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RcsB Is Required for Inducible Acid Resistance in Escherichia coli and Acts at *gadE*-Dependent and -Independent Promoters

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NOTES

RcsB Is Required for Inducible Acid Resistance in *Escherichia coli* and Acts at *gadE*-Dependent and -Independent Promoters▽

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RcsB interacts with GadE to mediate acid resistance in stationary-phase *Escherichia coli* K-12. We show here that RcsB is also required for inducible acid resistance in exponential phase and that it acts on promoters that are not GadE regulated. It is also required for acid resistance in *E. coli* O157:H7.

The glutamate-dependent acid resistance system AR2 of *Escherichia coli* is regulated by a regulatory network that responds to general stress, via the alternative sigma factor RpoS, and to low pH (8, 13, 17, 18, 20, 22, 25). AR2 requires several regulators, including the central regulator GadE, to integrate signals from the EvgAS and PhoPQ two-component systems (3, 10–13, 17). RcsB, a regulator with a wide range of roles in many enteric bacteria (5), is essential for survival during extreme acid challenge (pH 2.5 or below) during stationary phase and regulates transcription of some AR2 genes by forming a heterodimer with GadE (2, 4, 15, 16). RcsB also forms heterodimers with RcsA, TviA, and BglJ to regulate colanic acid synthesis, antigen VI expression, and sugar transport, respectively (26–28). Here we show that the inducible acid resistance of exponential-phase *E. coli* is also completely dependent on RcsB and that this resistance correlates with dependence of activation of the AR2 network on RcsB. We show that several AR2 genes that are not GadE regulated require RcsB and that RcsB must interact downstream of the sensor kinase EvgS but upstream of the first regulator, YdeO. These results suggest an additional role for RcsB in the activation of acid resistance and show that there is cross talk between the Rcs and EvgAS systems. We show that the role of RcsB extends to the pathogenic strain *E. coli* O157:H7 (Sakai).

Induced acid resistance is *rcsB* dependent. A Δ*rcsB* derivative of *E. coli* K-12 MG1655 was constructed as previously described (7). We determined the survival of this strain and the wild-type parent to extreme acid challenge in exponential phase, with and without induction by mild acidification (pH 5.7). Both strains grew at the same rate. Cells were grown, from overnight cultures diluted at least 500-fold, to an optical density at 600 nm (OD_{600}) of 0.2 in M9 with glucose (22.2 mM) and Casamino Acids (0.2% [wt/vol]), buffered with morpholinepropanesulfonic acid (MOPS) and morpholineethanesulfonic acid (MES) as described previously (M9supp) (1), and then incubated at pH 5.7 or pH 7 for 70 min before acid challenge at pH 2.4 for 2 h. We determined that under these conditions there are no detectable carryover effects from stationary phase on gene expression or cell survival. Cultures were always checked to ensure that the adjusted pH values remained constant for the entire experiment (data not shown). Survival was measured as previously described (1). Induction at pH 5.7 caused a significant increase in resistance in the wild-type strain (Fig. 1a). Survival of the Δ*rcsB* strain was below the level of detection in both induced and uninduced cultures (Fig. 1a). To complement the *rcsB* deletion, we constructed plasmid p*rcsB* by cloning *rcsB* under the control of its own promoter (−1144 to +674, relative to the RcsB translation start site) into the low-copy-number plasmid pZC230 (15, 24). Introduction of p*rcsB* into the Δ*rcsB* strain restored survival to wild-type levels, while the presence of the vector alone had no effect (Fig. 1a). These results show that RcsB is essential for inducible acid resistance in exponential-phase cells.

RcsB is required for the activation of GadE-dependent and -independent promoters. Several inducible acid resistance genes are under the control of the GadE-RcsB heterodimer (2, 4, 15, 16). However, genes outside the GadE regulon also contribute to resistance to extreme acid shock and are induced by mild pH shock (16, 19–21). Using a luciferase reporter system as previously described (1), we compared the induction kinetics of the *evgA*, *gadA*, *gadB*, *gadE*, *gadW*, *gadX*, *gadY*, *hdeA*, *hdeD*, *mgtA*, *safA*, and *ydeP* promoters in wild-type and *rcsB* knockout backgrounds. Table 1 shows the ratios of expression levels for all these promoters with and without acid induction. Note that the *safA* promoter drives expression of both *safA* (also called b1500) and *ydeO* (20); SafA activates the PhoPQ system in response to acid, and YdeO directly activates GadE expression as well as the expression of several other acid resistance genes (9–11, 13). *mgtA* was included as an example

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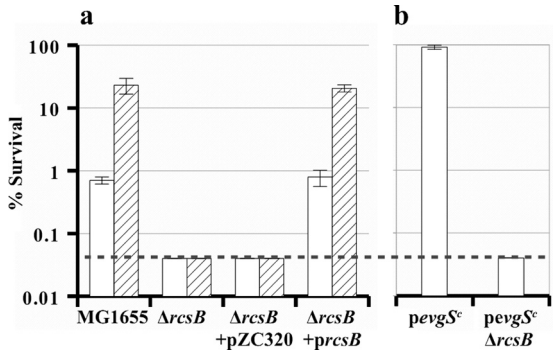

FIG. 1. Acid-inducible resistance is dependent on *rcsB*. Survival was calculated as the number of colonies after 2 h at pH 2.4 in M9supp as a percentage of total colonies at time zero. (a) Wild-type *E. coli* MG1655 and Δ*rcsB* strains after induction by acid shift (striped bars) or not induced by acid shift (white bars). (b) Uninduced MG1655 containing a plasmid expressing a constitutive EvgS (p*evgS*^{c}) compared to an uninduced Δ*rcsB* mutant containing the same plasmid. The broken line represents the limit of detectable survival, determined to be 0.04%. Here and in other figures, error bars represent the standard deviations of at least three independent replicates.

of a promoter that is directly regulated by PhoPQ and is hence also activated by acid (1). We have previously determined the GadE dependence of these promoters (1). The inducible activities of the GadE-dependent promoters *gadA*, *gadB*, *hdeD*, and *hdeA* were reduced at least 20-fold in the Δ*rcsB* strain (Table 1). The activity of the *gadE* promoter, which is partially dependent on itself for full activity (1, 23), was reduced nearly 100-fold in the Δ*rcsB* strain (Table 1). Thus, the effect of loss of RcsB on the regulation of GadE-dependent promoters may be due simply to loss of GadE expression, distinct from the reported situation in stationary phase (4). In addition, the GadE-independent promoters for *safA*, *slp*, *gadW*, *gadY*, *mgtA*, and *ydeP* showed significant reductions in acid-inducible activity in the Δ*rcsB* strain (Table 1). The *gadX* promoter, which is not activated by GadE or (unlike all the above promoters) by induction via EvgA but does require RpoS for induction by low pH (1), also showed significantly reduced acid-induced expression in the Δ*rcsB* strain (Table 1). The *evgAS* promoter, which is not acid regulated (1), was not affected in the Δ*rcsB* strain (Table 1), nor were two other non-acid-regulated promoters, the *csrA* and *acpP* promoters (data not shown). We confirmed that the induction kinetics of the *gadA*, *gadE*, *safA*, and *mgtA* promoters were restored to close to wild-type behavior in the presence of plasmid p*rcsB* (Fig. 2). Together, these results show that RcsB has additional GadE-independent roles in the regulation of both EvgAS-dependent and EvgAS-independent promoters.

RcsB regulates GadE-dependent and -independent promoters in stationary phase. We confirmed that the Δ*rcsB* strain grown to stationary phase in M9supp was sensitive to low pH and that this sensitivity could be complemented with plasmid p*rcsB* (Fig. 3a). No increase in acid resistance could be obtained in the stationary-phase Δ*rcsB* mutant by acid induction (data not shown). We again used the luciferase reporter system to measure promoter activity in stationary-phase cultures. Cells were grown for 18 h with shaking in M9supp at 37°C, then diluted 10-fold, and incubated for 10 min at 37°C with shaking to briefly aerate the cultures (required for optimal luciferase activity). The expression of the GadE-RcsB-regulated gene *gadA* was significantly reduced in the Δ*rcsB* background, as was expression from the GadE-dependent promoters of *hdeA* and *hdeB* (Table 1), consistent with earlier reports. Expression from the GadE-independent *gadX* and *gadW* promoters, and the partially GadE-dependent *gadE* promoter, was reduced in the Δ*rcsB* background in stationary phase (Table 1), although the effect on *gadW* activity was only 2-fold. The activities of the *safA* and *ydeP* promoters, which show RcsB- and EvgAS-dependent activation by low pH (1, 17), were below the limit of detection in both wild-type and Δ*rcsB* backgrounds. Consistent with this finding, no significant effect was seen on expression of the *mgtA* promoter, which is indirectly activated by SafA via PhoPQ (1). The loss of expression of the *gadE*, *gadA*, and *gadX* promoters in the Δ*rcsB* background was fully complemented by the p*rcsB* plasmid (Fig. 3b). Our results confirm that RcsB is required for acid resistance and activates GadE-regulated promoters in stationary phase and show that some GadE-independent promoters also require RcsB under these conditions. The results for *gadE* and *gadX* are different from those reported earlier (4), which may relate to different growth conditions or the effects of different strain backgrounds. Intriguingly, the *gadY* promoter, which requires *rcsB* in exponential phase, was independent of *rcsB* in stationary phase.

RcsB acts downstream of EvgS. Most of the promoters studied here are regulated by the EvgAS two-component system in response to low pH (1, 6, 13, 17, 19, 20), the expression of which is not affected by loss of RcsB (Table 1 and data not shown). Thus, the effects of RcsB on these promoters could be explained by it being required for the function of EvgA or EvgS. If so, the Δ*rcsB* phenotype would not be suppressed by constitutive mutations in EvgS, which causes increased acid resistance in exponential phase without induction by acidifica-

TABLE 1. Ratios of expression levels for promoters with and without acid induction

Promoter	Promoter dependency[a]		Relative activity[b]	
	GadE	EvgA	Exp[c]	Stat[d]
gadA	d	d	0.050^{*}	0.005^{*}
gadB	d	d	0.014^{**}	—
hdeA	d	d	0.002^{***}	0.027^{**}
hdeD	d	d	0.100^{*}	0.160^{*}
gadE	pd	d	0.013^{***}	0.086^{**}
gadW	i	d	0.208^{*}	0.534^{ns}
gadY	i	d	0.034^{**}	0.950^{ns}
slp	i	d	0.039^{***}	—
safA	i	d	0.030^{**}	ND
mgtA	i	d	0.166^{***}	0.915^{ns}
ydeP	i	d	0.002^{**}	ND
evgA	i	i	0.900^{ns}	—
gadX	i	i	0.283^{*}	0.256^{**}

[a] Promoter dependency was determined as described in reference 1 and is indicated as follows: d, dependent; i, independent; pd, partially dependent.

[b] *P* values of >0.95, >0.99, and >0.999 are indicated by one, two, or three asterisks, respectively; ns, not significant. ND, not determined as expression levels were too low to be accurately measured; —, not done.

[c] Promoter activity in the Δ*rcsB* strain relative to the wild type after 40 min of induction at pH 5.7 at an OD_{600} of 0.2.

[d] Promoter activity in the Δ*rcsB* strain relative to the wild type in stationary phase.

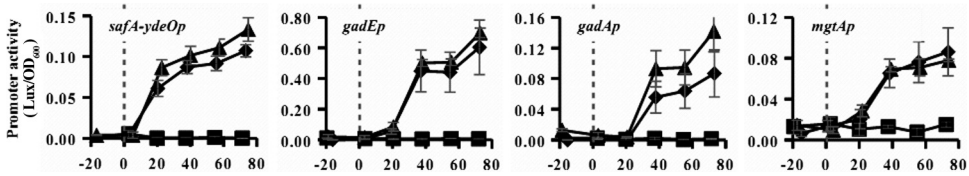

FIG. 2. Promoter activity after induction by acid shift to pH 5.7 during exponential phase in M9supp. The activity of each promoter in wild-type MG1655 (triangles) and the Δ*rcsB* mutant without (squares) and with (diamonds) the complementing plasmid p*rcsB* is shown. Time (in minutes) is shown on the *y* axis. Time zero represents the point of acidification to pH 5.7 (indicated by a vertical broken line). Lux, luciferase activity.

tion (13, 14). We therefore assayed the survival of wild-type and Δ*rcsB* cells containing the p*evgS*^{c} plasmid, which expresses a constitutive mutant of EvgS from the EvgA native promoter (M. D. Johnson, N. A. Burton, and P. A. Lund, unpublished data). Wild-type cells expressing the *evgS* mutant survived exposure to pH 2.4 without induction as well as the pH 5.7-induced wild-type strain. However, no survival of the Δ*rcsB* p*evgS*^{c} strain could be detected after 2 h at pH 2.4 (Fig. 1b), showing that RcsB must act downstream of EvgS. Expression of the first major regulator immediately downstream of EvgA, YdeO, is itself completely dependent on RcsB, making a direct effect of RcsB on EvgS or EvgA more likely. We hypothesize that RcsB is required either for the EvgS-dependent phosphorylation of EvgA or for the regulatory activity of EvgA itself. These possibilities are under investigation.

RcsB is required for acid resistance in *E. coli* O157:H7. To investigate whether the role in acid resistance of *rcsB* extends beyond *E. coli* K-12, an *rcsB* mutant was constructed in *E. coli* O157:H7 (Sakai). The survival of wild-type and mutated O157:H7 (Sakai) at pH 2.4 was assayed as described above. Wild-type *E. coli* O157:H7 (Sakai) survives much better during exponential phase than strain MG1655 does. However, this resistance was reduced to lower than the limit of detection in the absence of *rcsB* (Fig. 4a), and this sensitivity was also seen in stationary-phase cells (Fig. 4b). The resistant phenotype in both phases was restored by the presence of the p*rcsB* complementation plasmid.

Our results show that activation of the AR2 network in response to mild acid shock in exponential and stationary phases is completely dependent on RcsB and that this effect

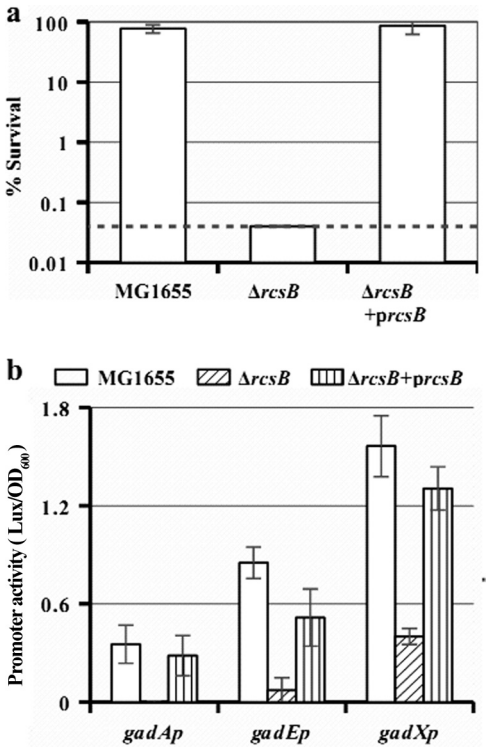

FIG. 3. Effect of deletion of *rcsB* in stationary phase. (a) Survival of cells in stationary phase without acid induction in M9supp. The horizontal broken line represents the limit of detection at 0.04%. (b) Stationary-phase promoter activity of the specified promoters, without acid induction, in *E. coli* MG1655, Δ*rcsB* mutant, and Δ*rcsB* mutant complemented with p*rcsB* plasmid.

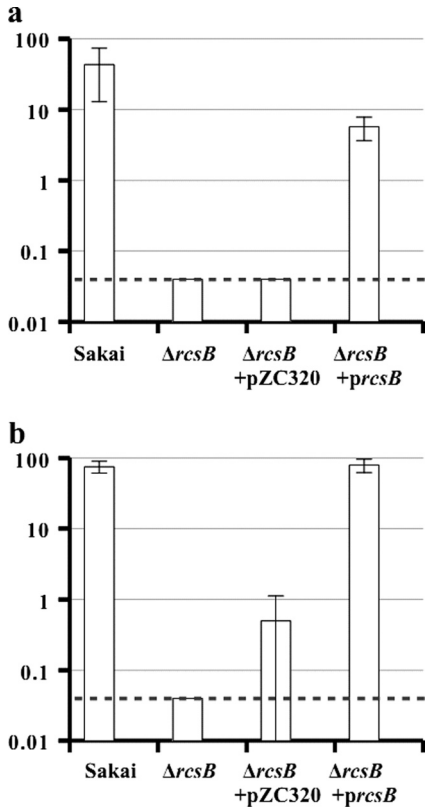

FIG. 4. Effect of deletion of *rcsB* on exponential- and stationary-phase survival of *E. coli* O157:H7 (Sakai). Survival was measured as described in the legend to Fig. 1 during exponential phase (a) and stationary phase (b). Percent survival is shown on the *y* axes.

is not suppressed by an EvgS constitutive mutant. Reporter analysis of the promoters involved in the AR2 network revealed that these promoters are completely dependent on RcsB whether or not they are regulated by GadE, so the GadE-RcsB heterodimer model does not explain all the effects of RcsB on acid resistance. We speculate that RcsB is required either for the phosphorylation of EvgA by EvgS or for the binding of EvgA to promoter elements. Given the fact that RcsB often forms active heterodimers, it is tempting to speculate that it may do so with phosphorylated EvgA.

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