The Two-Component Response Regulator RcsB Regulates Type 1 Piliation in Escherichia coli

William R. Schwan, Satoshi Shibata, Shin-Ichi Aizawa, and Alan J. Wolfe

Type 1 pili are filamentous proteinaceous appendages produced by many members of the family Enterobacteriaceae that play a major role in biofilm development and pathogenesis during the course of human infections (26). Escherichia coli cells can switch from a completely piliated state to a completely nonpiliated state (7). This ability depends on a process called phase variation (Fig. 1), in which a 314-bp invertible DNA element switches between two interconvertible orientations (1). When this element is in the “phase-on” orientation, the fimA promoter (fimAp) faces the operon and thus can drive its transcription. Since the fim operon includes genes for structural components and the machinery required for pilus assembly, cells in the “phase-on” orientation elaborate numerous type 1 pili. When the element is oriented in the “phase-off” state, the promoter faces the opposite direction, the promoter cannot drive fimA transcription, and the cells lack type 1 pili altogether. The invertible nature of the fimAp element depends upon two site-specific recombinases, FimB and FimE. Whereas FimE recombinase activity favors switching from “phase-on” to “phase-off,” FimB facilitates switching in both directions (16, 22, 23).

We previously reported that the fim operon is regulated by acetyl phosphate (35), a central metabolite that functions as a global signal (12, 34) by donating its phosphoryl group to a subset of response regulators (RRs) of the two-component signal transduction (2CST) pathways (8, 15a, 19). The most fundamental of 2CST pathways consists of an RR, which autophosphorylates a conserved aspartyl residue (6, 29, 33). For a subset of RRs, the central metabolite acetyl phosphate serves as an alternative phosphoryl donor (reviewed in reference 34). As its name implies, the RR is typically associated with a response domain, often one that permits binding to DNA. Thus, many RRs function as transcription factors (6, 29, 33).

The phosphorelay, a more complex version of the 2CST pathway, contains two additional domains. As in the fundamental 2CST pathway, ATP donates a phosphoryl group to the SK, which then donates it to an RR. In the phosphorelay, a histidine phosphotransferase transfers the phosphoryl group from the first RR to a second one (reviewed in references 2, 14, and 24). The core of the Rcs phosphorelay is composed of three proteins: RcsC (a hybrid SK-RR), RcsD (a histidine phosphotransferase also known as YojN), and RcsB (the terminal RR) (reviewed in references 10 and 20). RcsB can bind DNA either as a homodimer (3) or as a heterodimer in association with the accessory protein RcsA (31, 32). The stability of RcsA is controlled by the proteases Lon (30) and ClpYQ (18). Another accessory protein, the outer membrane lipoprotein RcsF, serves to activate the kinase activity of RcsC (20).

The Rcs phosphorelay is estimated to regulate some 5% of the Escherichia coli genome (reviewed in references 21 and 25). Most of these genes encode functions associated with the cell envelope. For instance, the Rcs phosphorelay activates the genes required for the biosynthesis of colanic acid, an extracellular polysaccharide required for biofilm development (13). It also activates the expression of several multiple-stress effectors that localize to the periplasm (3, 5), while repressing genes required for the biogenesis of flagella (11).

While studying the impact exerted by acetyl phosphate upon the network of 2CST pathways (12), we obtained electron microscopic evidence that led us to hypothesize that RcsB functions as a positive regulator of type 1 pili. Here, we report attempts to test that hypothesis.

We grew cells at 37°C in tryptone broth (1% [wt/vol] tryptone, 0.5% [wt/vol] NaCl), harvested them during mid-exponential growth and shortly after entry into stationary phase, and monitored piliation by transmission electron microscopy as described previously (12). At both stages of growth, about 60% of wild-type (WT) cells (strain AJW678) (17) elaborated...
pili (Fig. 2A and C); isogenic rcsC mutant cells (strain AJW2143) (12) displayed similar behavior (Fig. 2C). In contrast, most isogenic rcsB mutant cells (strain AJW2143) (12) were nonpiliated (Fig. 2B). About 40% of the rcsB mutant cells elaborated pili after entry into stationary phase, while only 20% displayed pili during exponential growth (Fig. 2C).

The observation that the percentage of piliated cells was affected rather than the number of type 1 pili per cell or their length led us to hypothesize that RcsB influences the orientation of the 314-bp fimAp invertible element. To test this hypothesis, we initially performed multiplex PCR amplifications on chromosomal DNA extracted from the rcsB mutant, the rcsC mutant, or their WT parent, using oligonucleotide primers specific for the “phase-on” and “phase-off” orientations of the invertible element (28) or, as a control, the E. coli ftsZ gene (27).

We previously reported that pH values and salt relevant to murine urine exerted substantial effects on phase variation (27). Therefore, we grew cells at 37°C to mid-exponential phase in LB (1% [wt/vol] peptone 140, 0.5% [wt/vol] yeast extracts, 1% [wt/vol] glycerol, 0.1 M sodium phosphate, 0.5% [wt/vol] NaCl) at either neutral pH (7.0) or acidic pH (5.5) and in either the presence of additional NaCl (final concentration, 490 mM [referred to as high salt]) or its absence (final concentration, 90 mM [referred to as low salt]).

Overall, the PCR results followed those of our previous study (27): more WT cells grown at neutral pH (Fig. 3A, lanes 1 and 4, and B) than cells grown at acidic pH (Fig. 3A, lanes 7 and 10, and B) positioned their invertible fimAp element in the “phase-on” orientation. This analysis also showed that rcsB mutant cells grown under neutral-pH, low-salt conditions (Fig. 3A, lane 2) positioned their invertible element more in the “phase-off” orientation than did cells of either their WT parent (lane 1) or the isogenic rcsC mutant (lane 3) grown under the same conditions. Under neutral pH, high-salt conditions, the rcsC mutant (lane 6) appeared to position the fimAp promoter element less in the “phase-on” orientation than did either its WT parent (lane 4) or the rcsB mutant (lane 5). At acidic pH, regardless of salt, all strains produced similar “phase-off” and “phase-on” distributions (lanes 7 to 12).

To confirm the rcsB finding, we complemented the rcsB mutant with a plasmid containing a His-tagged WT rcsB gene (pHRcsB) (5), harvested cells during mid-exponential growth, and compared the complemented strain to the WT strain and the rcsB mutant parent (Fig. 3C and D). At the neutral-pH, low-salt condition, the complemented rcsB mutant (lane 3) oriented its invertible element more like the WT strain (lane 1) than the rcsB mutant (lane 2). Similar results were obtained when cells were harvested following entry into stationary phase (data not shown).

Taken together, these results support the hypothesis that RcsB regulates piliation under neutral-pH, low-salt conditions by influencing the orientation of the fimAp invertible element. These results also suggest that RcsC may influence the positioning of the invertible element under high-salt growth conditions. Finally, they show that neither RcsB nor RcsC exerts much influence at acidic pH and that some other factor must be involved.

To determine how RcsB may regulate the orientation of the invertible element, we asked whether RcsB and/or RcsC influences the transcription of fimB, fimE, or both recombinase genes. The rcsB mutant, the rcsC mutant, and their WT parent were transformed with single-copy plasmids pJB5A and pJLE4-3, which express the transcriptional fusions fimB-lacZYA and fimE-lacZYA, respectively (27). Because pH and salt have been shown to influence the transcriptional states of both fimB and fimE (27), we performed these reporter studies under the growth conditions used for the PCR analyses.

When cells were grown at acidic pH in the presence of either the low or high salt, transcription of either fimB or fimE was largely unaffected by the status of the Rcs phosphorelay (Table
(strain AJW678), an rcsB mutant (strain AJW2143), and an rcsC mutant (strain AJW2144). Cells were harvested during mid-exponential phase following aerobic growth with 250-rpm agitation at 37°C in pH 7.0 LB medium with either no added NaCl (low salt) or 400 mM added NaCl (+, high salt) or in pH 5.5 LB medium with either no added NaCl (low salt) or 400 mM NaCl (high salt). Multiplex PCRs were set up with INV and FIMA primers to amplify “phase-off”-oriented DNA (ON; 450-bp product) (28), FIME and INV primers to amplify “phase-off”-oriented DNA (OFF; 750-bp product) (28), and EcFtsZ1 and 2 primers to amplify the ftsZ gene (302-bp product) (27). Each multiplex was run at least three separate times. The lanes were loaded as follows: lane 1, AJW678 (pH 7.0, low salt); lane 2, AJW2143 (pH 7.0, low salt); lane 3, AJW2144 (pH 7.0, low salt); lane 4, AJW678 (pH 7.0, high salt); lane 5, AJW2143 (pH 7.0, high salt); lane 6, AJW2144 (pH 7.0, high salt); lane 7, AJW678 (pH 5.5, low salt); lane 8, AJW2143 (pH 5.5, low salt); lane 9, AJW2144 (pH 5.5, low salt); lane 10, AJW678 (pH 5.5, high salt); lane 11, AJW2143 (pH 5.5, high salt); and lane 12, AJW2144 (pH 5.5, high salt). (B) Quantification of the data from panel A. Using ImageQuant software (Molecular Dynamics), the number of pixels for each band was quantified. For each lane, the intensities of the OFF and ON states were corrected to the intensity of the ftsZ band. The corrected values for both states were plotted as log10 numbers, the WT strain for both the OFF and ON states were standardized to the respective WT band (lane 1). Since the resultant values were plotted as log10 numbers, the WT strain for both the OFF and ON states had a value of zero, while increased and decreased PCR products resulted in a higher or lower value, respectively. Solid bars, strain AJW678 (WT); open bars, strain AJW2143 (rcsB mutant); hatched bars, strain AJW2144 (rcsC mutant).

1). This is consistent with the lack of any significant effect upon the populations of invertible elements (Fig. 3). In contrast, when cells were grown at neutral pH, the states of certain Rcs phosphorelay components influenced transcription. Furthermore, the critical Rcs component and the promoter affected depended upon the salt. For example, at the low salt, rcsB mutant cells transcribed fimB at significantly reduced levels relative to their WT parent and the rcsC mutant (Table 1). This effect appears to be specific because expression of the His-tagged WT rcsB allele from a compatible plasmid (pHRcsB) restored transcription to WT levels. In contrast, growth in high-salt medium resulted in a distinctly different pattern. Under this condition, relative to WT cells, both the rcsB and rcsC mutants exhibited elevated fimE transcription. Here, both the RcsB and RcsC effects appeared to be specific because expression from a compatible plasmid either with the His-tagged WT rcsB allele in the rcsB mutant (Table 1) or with the WT rcsC allele (pSG980) (9) in the rcsC mutant restored fimE transcription to WT levels (data not shown). In contrast, the vector controls had no effect (data not shown).

The PCR analysis (Fig. 3) and the fim-lacZ fusion data (Table 1) supported the hypothesis that RcsB can influence the orientation of the fimAp invertible element by controlling the transcription of the recombinase genes fimB and fimE. This RcsB-dependent behavior occurred only at neutral pH and depended upon RcsC only in the presence of the high salt. On the basis of these observations, we predicted that type 1 pilus expression would be affected in a condition-dependent manner by mutations in rcsB and rcsC. To test this prediction, enzyme immunoassays were performed according to the procedure of Hultgren et al. (15). As predicted, at neutral pH with the low salt, the rcsB mutant strain displayed significantly reduced type 1 pilus expression relative either to its WT parent or to the rcsC mutant (Fig. 4). At neutral pH with the high salt, however, both the rcsB and rcsC mutants displayed lower levels of type 1 pilus expression.

### Table 1. Effects of pH and salt on fimB-lacZ and fimE-lacZ fusions in WT E. coli compared to effects on isogenic rcsB and rcsC mutants

<table>
<thead>
<tr>
<th>Fusion and strain</th>
<th>Expression level (Miller units) under indicated growth condition*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 7.0</td>
</tr>
<tr>
<td>fimB-lacZ fusion</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>345 ± 30</td>
</tr>
<tr>
<td>rcsB</td>
<td>201 ± 19†</td>
</tr>
<tr>
<td>rcsB/pRcsB</td>
<td>329 ± 21</td>
</tr>
<tr>
<td>rcsC</td>
<td>305 ± 34</td>
</tr>
<tr>
<td>fimE-lacZ fusion</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>335 ± 26</td>
</tr>
<tr>
<td>rcsB</td>
<td>332 ± 34</td>
</tr>
<tr>
<td>rcsB/pRcsB</td>
<td>339 ± 32</td>
</tr>
<tr>
<td>rcsC</td>
<td>365 ± 21</td>
</tr>
</tbody>
</table>

* Values indicate fimB and fimE promoter expression in terms of β-galactosidase activity and are means ± standard deviations from at least three independent experiments. Cells were grown in LB at pH 7.0 or pH 5.5 with the low or high (+) salt and harvested during mid-exponential phase.

† Bold denotes that the differential expression is significant (P < 0.05) as determined by Student's t test.

rcsB/pRcsB is the rcsB mutant transformed with pHRcsB, which encodes a His-tagged WT rcsB allele under the control of the lac promoter. Induction, however, was unnecessary.
At neutral pH, therefore, it appears that RscB helps mediate the inversion of the fimAp element either by increasing fimB transcription (low salt) or by decreasing fimE transcription (high salt). These results are consistent with our observation that significantly fewer rcsB mutant cells than cells of their WT parent expressed pili. Whether RcsB acts directly upon fim transcription awaits further experimentation; however, inspection of the sequence upstream of the fimB and fimE open reading frames reveals several sequences with some similarity to RcsB and RcsAB boxes (31, 32). Although RcsC appeared to have no discernible effect under neutral-pH, low-salt growth conditions, it appeared to influence the orientation of the invertible element under neutral-pH, high-salt conditions, presumably by decreasing fimE transcription. These results argue for the hypothesis that RscB receives its phosphoryl groups from RcsC (its cognate SK) under neutral-pH, high-salt conditions, but from an alternative donor (e.g., acetyl phosphate or a noncognate SK) under neutral-pH, low-salt conditions.

Our previous work showed that acidic growth conditions reduce type 1 pilus expression, while implicating the EnvZ/OmpR 2CS pathway as a fim inhibitor at least in the presence of the high salt (27). The current study supports the former conclusion and shows that it occurs in an RcsB-independent manner. Whether fim regulation under acidic growth conditions involves the EnvZ/OmpR pathway or some other acid tolerance gene product is currently under examination.

In summary, we propose that the fim locus is part of the RcsB regulon and that this global regulator inversely affects the transcription of fimB and fimE. Furthermore, this inverse regulation increases the probability of the “phase-on” orientation of the fimAp element and consequently the elaboration of type 1 pili. On the basis of the current study and our previous report (12), we can conclude that RcsB enhances the production of type 1 pili as well as the synthesis of the capsule, while inhibiting the biogenesis of flagella. Since these surface organelles play critical and/or essential roles in biofilm development and urinary tract infections, RcsB should now be considered a coordinator of these processes.

We thank Sylvia Reimann for strain construction.

This work was supported by NIH grant GM066130, awarded to A.J.W., grant RAI065432A, awarded to W.R.S., and a grant from the CREST project of JST to S.-I.A.

REFERENCES


