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The RcsCB His-Asp Phosphorelay System Is Essential To Overcome Chlorpromazine-Induced Stress in *Escherichia coli*

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The RcsCB His-Asp phosphorelay system regulates the expression of several genes of *Escherichia coli*, but the molecular nature of the inducing signal is still unknown. We show here that treatment of an exponentially growing culture of *E. coli* with the cationic amphipathic compound chlorpromazine (CPZ) stimulates expression of a set of genes positively regulated by the RcsCB system. This induction is abolished in *rcsB* or *rcsC* mutant strains. In addition, treatment with CPZ inhibits growth. The wild-type strain is able to recover from this inhibition and resume growth after a period of adaptation. In contrast, strains deficient in the RcsCB His-Asp phosphorelay system are hypersensitive to CPZ. These results suggest that cells must express specific RcsCB-regulated genes in order to cope with the CPZ-induced stress. This is the first report of the essential role of the RcsCB system in a stress situation. These results also strengthen the notion that alterations of the cell envelope induce a signal recognized by the RcsC sensor.

The *Escherichia coli rcsC* and *rcsB* genes encode a histidine-aspartate phosphorelay system (11). *rcsB* codes for the response regulator which, by analogy with other phosphorelay systems, is probably activated through the transfer of a phosphate group from either its cognate sensor, RcsC (20), or from another protein, RcsF (9). YojN, a histidine-containing phosphotransmitter (Hpt) protein, is used as a phosphorelay between RcsC and RcsB (22).

The RcsCB system, initially discovered in *E. coli*, is conserved in many enterobacterial species, as well as in some plant pathogens (1, 22, 26, 27). In *E. coli*, targets regulated by the RcsCB system include the exopolysaccharide synthesis genes *cps* (20), cell division genes (4), the osmoregulated gene *osmC* (8), and genes involved in motility and chemotaxis (22; our unpublished data). Although the *rcsC* and *rcsB* genes are not essential, their conservation in many bacterial species and the variety of functions under their control suggest that they have an important role in the physiology of the cell.

The *rcsC*/*rcsB* system is activated by environmental conditions such as desiccation and osmotic shock (16, 19), by overproduction of the chaperone DnaJ-like transmembrane protein DjlA (5, 13), and by several mutations affecting the composition of the envelope (6, 11). All these activating conditions have a common consequence: they alter the envelope composition and/or topology. This observation has led to the suggestion that perturbations in the cell envelope might be the inducing signal recognized by the RcsC sensor (11).

This work shows that the RcsCB system is essential for the cell's ability to overcome stress induced by chlorpromazine (CPZ) and supports previous suggestions that RcsC senses alterations of the cell envelope.

The RcsCB regulation pathway is activated by CPZ. CPZ is a cationic amphipathic molecule that, by insertion into the inner leaflet of the membrane lipid bilayer, induces modifications of the membrane topology (17, 25). This property of CPZ was used to test the hypothesis that the RcsC sensor is activated by alterations of the envelope integrity by measuring the expression of several RcsCB-regulated genes in cultures treated with CPZ. Amphipathic compounds alter the curvature stress in membranes, and osmotic stress is believed to exert a similar effect (25). Therefore, for optimal conditions, all our experiments were performed after growing the bacteria in LB0N (Luria-Bertani broth containing no NaCl), a low-osmolarity medium. The RcsCB-regulated genes were tested as transcriptional fusions in single copy on the chromosome of *E. coli* strain CF6343 (8), a Δ*lacZ* derivative of the wild-type strain MG1655. These included the promoters of the *cps* capsule synthesis genes (*cps*::*lacZ* [8]), the cell division genes *ftsAZ* ($ftsA_{1p}$::*lacZ* [4]), and $osmCp_1$, one of the two promoters of the gene *osmC* ($osmCp_1$::*lacZ* [8]). The *cps* and $osmCp_1$ promoters are both osmoregulated. Osmotic induction of *cps* is RcsB dependent and stimulated by RcsA (19). In contrast, osmotic induction of $osmCp_1$ does not depend on RcsB, RcsC, or RcsA. Its activity is mediated by another transcriptional activator, NhaR (8, 23). In minimal medium containing glucose, transcription from $osmCp_1$ is repressed by the H-NS and Lrp proteins (2). However, none of these proteins affects significantly the expression of $osmCp_1$ during growth in rich medium (23; our unpublished data). No regulator other than RcsB is known to regulate *ftsA1p*. For a control for our experiments, we also measured the expression of a *lacZ* fusion with *osmEp*, another stress-inducible promoter whose expression is independent of RcsB (7; our unpublished data).

The β-galactosidase activity produced from an *osmEp-lacZ* fusion [*E. coli* strain CLG530; F^- *araD139* Δ(*argF-lac*)*U169* *deoC1 flbB5301 rpsL150 relA1 ptsF25 rbsR* Φ(*osmEp-lacZ*) (7)] was not affected after 1 h of treatment with CPZ (100 μM), demonstrating that the drug did not lead to nonspecific induc-

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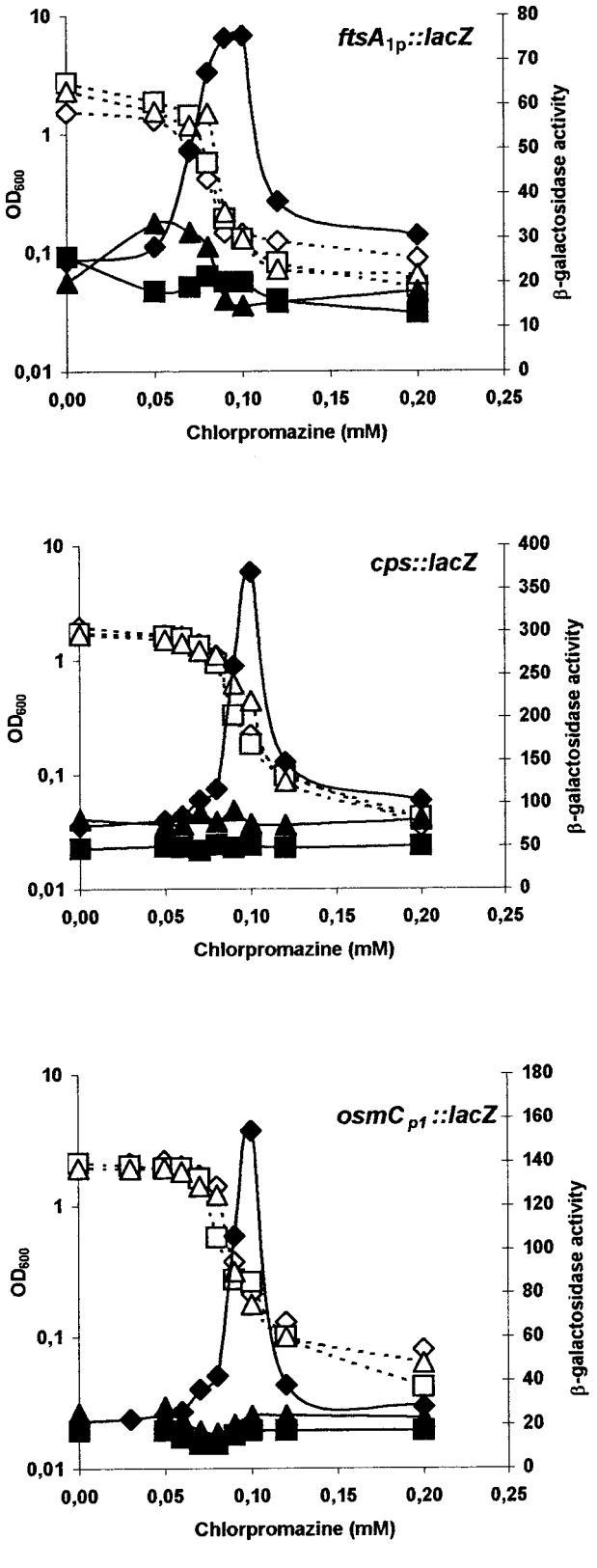

FIG. 1. CPZ induces transcription of RcsCB-dependent genes. Cultures of strains carrying the transcriptional fusions tested (*ftsA1p*::*lacZ* fusion, *cps*::*lacZ* fusion, and *omsCp*$_1$::*lacZ* fusion) grown overnight in LB0N were diluted 1,000-fold in the same medium and grown to an OD_{600} of ~0.2 at 37°C. Different amounts of CPZ were added at this time. After 2 h, OD_{600} was measured and β-galactosidase activity was assayed by the method of Miller (14) and expressed in Miller units. The OD_{600} (open symbols) and β-galactosidase specific activity (closed symbols) for the wild-type (diamond), *rcsB* (square), and *rcsC* (triangle) strains are shown. The *E. coli* strains containing the transcriptional fusions *cps*::*lacZ* [SK1291; F$^-$ λ$^-$ *rph-1* Δ*lacIZ*(*Mlu*I) Φ(*cps-lacZ*)] and *omsCp*$_1$::*lacZ* [CLG686; F$^-$ λ$^-$ *rph-1* Δ*lacIZ*(*Mlu*I) Φ(*omsCp*$_1$ *-lacZ*)] have been described previously (8). The *ftsA1p*::*lacZ* fusion strain [SK1146, F$^-$ *rph-1* Δ*lacIZ*(*Mlu*I) Φ(*ftsA1p-lacZ*)] was constructed by lysogenization of CF6343 with phage λFAB4 (4) by the method of Simons et al. (18). The *rcsB*::*tet* and *rcsC*::*tet* alleles were transduced with phage P1 from the donor strains MZ60 and MZ63, respectively (28).

tion of transcription or introduce artifacts in the measurements of β-galactosidase activity. In contrast, the patterns of expression of the three RcsB-dependent *lacZ* fusions tested were affected by CPZ in a similar fashion (Fig. 1). In the wild type, their expression was increased by treatment with CPZ in a dose-dependent manner. Expression peaked at an CPZ concentration of 100 μM. Higher concentrations of the drug did not result in induction. However, cells treated with CPZ at concentrations above 60 μM exhibited a severe growth defect (Fig. 1). We observed that cells treated with concentrations of CPZ higher than 100 μM had lost the ability to yield colonies on rich medium without drug, implying that the absence of induction was probably a consequence of a lethal effect of CPZ. In contrast to the wild type, an isogenic *rcsB* mutant strain exhibited no induction of the tested fusions at any concentration of CPZ (Fig. 1). In addition, *rcsC* mutants, in which the sensor of the two-component system was missing, did not exhibit induction either. These results therefore suggested that the stimulation of the expression of the *cps*, *ftsAZ,* and *osmC* genes by CPZ was due to the activation of the RcsCB pathway. Similar results (not shown) were obtained with tetracaine, another cationic amphipathic compound leading to similar alterations of the membrane. CPZ is also known to induce a relaxation of DNA negative supercoiling (15). We then grew strain CLG686, carrying an *osmCp*$_1$*-lac* fusion, in LB0N and treated part of the culture with novobiocin (20 μg·ml^{-1}), an inhibitor of DNA gyrase known to reduce supercoiling density at this sublethal concentration (7). At time zero of this experiment, CLG686 exhibited a β-galactosidase activity of 14 Miller units. After 1 h, the treated and untreated cultures exhibited β-galactosidase activity of 18 and 24 Miller units, respectively. After 2 h, they exhibited activity of 22 and 49 Miller units. This twofold stimulation of *osmCp*$_1$ was independent of RcsB (data not shown), and it was not sufficient to account for the eightfold induction following treatment with CPZ (Fig. 1).

The effect of CPZ on the curvature stress in the membrane supports the hypothesis that the RcsC sensor is activated by alterations of the cell envelope. However, we cannot rule out the possibility that CPZ activates RcsC through other mechanisms. For instance, it is known to affect regulators of K^+ transport and could thereby alter the turgor pressure (21). It is also possible that the growth arrest following treatment with CPZ per se is inducing the Rcs pathway. However, this is unlikely, because the *osmCp*$_1$ promoter is not induced when growth stops upon entry into stationary phase (2).

The RcsCB system is essential to overcome CPZ-mediated

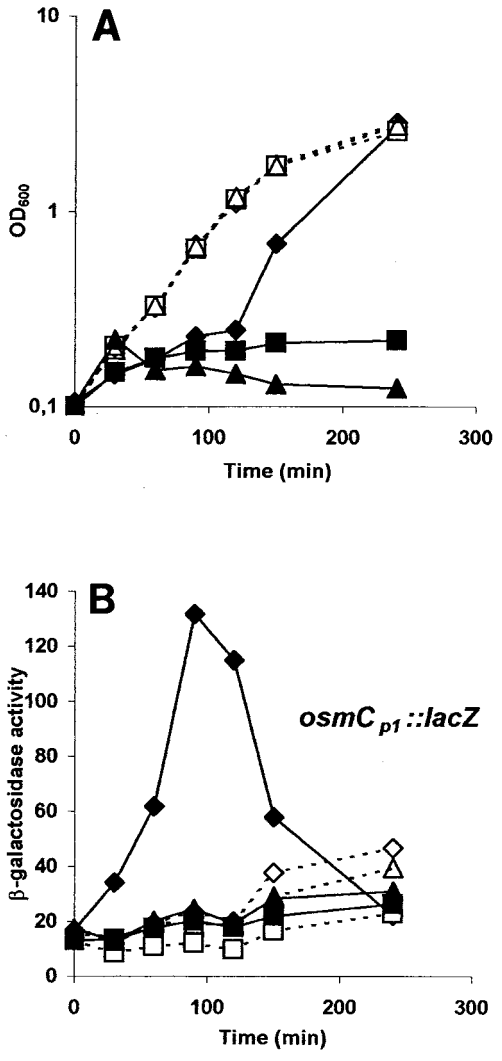

FIG. 2. The RcsCB system is necessary to overcome inhibition of growth by CPZ. Cultures of strains CLG686 [Φ(*osmCp*$_1$-*lacZ*) *rcsB*$^+$ *rcsC*$^+$; diamond), CLG698 (CLG686 *rcsB*::*tet*; square), and CLG768 (CLG686 *rcsC*::*tet*; circle) in LB0N broth grown overnight were diluted 1,000-fold in the same medium and grown to an OD_{600} of ~0.1 at 37°C. At time zero, CPZ was added to a final concentration of 100 μM, and the OD_{600} (A) and the β-galactosidase specific activity (B) were determined at the indicated times. β-Galactosidase activity is expressed in Miller units. Cultures were treated with CPZ (closed symbols) or were not treated (controls) (open symbols).

growth inhibition. The growth of cells treated with CPZ was dramatically affected at concentrations above 0.06 mM (Fig. 1). To further characterize this effect, growth was monitored over time after the addition of CPZ to a final concentration of 0.1 mM to exponentially growing cultures. As shown in Fig. 2A, the growth curves of the untreated cultures of the wild-type, *rcsB,* and *rcsC* strains were identical. After addition of the drug, growth of the three strains immediately slowed and almost stopped after 1 h. Interestingly, 2 h after treatment, the growth of the wild-type strain abruptly resumed and reached a plateau comparable to that of the untreated culture. In contrast, the *rcsB* or *rcsC* mutant strains were not able to resume growth, even after 4 h. This result therefore indicated that the RcsCB system was required to overcome the toxic effect of CPZ. At present, the inducing signal sensed by RcsC remains unknown, and CPZ is unlikely to be encountered by *E. coli* in its natural environment. However, to our knowledge, this is the first report of an essential role of the RcsCB system in cells coping with stress conditions. This observation suggests a justification for conservation of the *rcsC* and *rcsB* genes in several bacterial species, including pathogenic *E. coli* strains (our unpublished results).

The analysis of the effect of CPZ on cell growth was performed with strains containing the *omsCp*$_1$::*lacZ* fusion (8). It was thus possible to monitor both growth (optical density at 600 nm [OD_{600}]) and expression of the fusion over time upon addition of the drug in the same experiment. As shown in Fig. 2B, in the wild-type strain, the expression of the *omsCp*$_1$::*lacZ* fusion increased immediately after addition of the drug, leading to a maximum β-galactosidase activity of 130 Miller units after 100 min. Then, the induction ceased, and the β-galactosidase activity fell back to the basal level. As expected, no such induction was observed in the *rcsB* or *rcsC* strains. Interestingly, the maximum value was reached just a few minutes before the wild-type strain resumed growth. This observation suggested that some cellular functions under RcsBC control are needed at a certain level to overcome the stress generated by the addition of CPZ. For instance, synthesis of the capsule induced by the RcsCB system might contribute to the ability of the cells to resume growth by preventing or limiting the accessibility of the drug to the membrane. We then tested the sensitivity of a derivative of MG1655 carrying a *cps-3*::Tn*10* mutation (24). This strain exhibited a behavior very similar to that of the *rcsB* derivative of MG1655 (data not shown). However, the interpretation of this experiment is uncertain. Indeed, strains carrying insertions inactivating some of the *cps* genes cannot accommodate an *rcsB*$^+$ plasmid, presumably because they accumulate deleterious capsular polysaccharide precursors (3). Here, the induction of the Rcs pathway by CPZ may enhance the toxic effect of the drug in a *cps-3*::Tn*10* mutant strain.

We also tested the effect of a Δ*osmC*::*kan* mutation that completely abolishes the expression of *osmC* (12). This mutation made cells sensitive to CPZ, suggesting that OsmC participates in the adaptation to the CPZ stress. A complete inhibition of growth was observed at a lower concentration of CPZ than with the *rcsB* mutant (data not shown). This increased sensitivity is probably due to the complete lack of OsmC in the Δ*osmC*::*kan* null mutant, whereas the *rcsB* mutant contains residual amounts of OsmC expressed mainly from the second *osmC* promoter, *omsCp*$_2$, which is σ^S dependent and RcsB independent (8, 10).

Together, these data suggested that the sensitivity of *rcsB* mutants to CPZ was multifactorial due to reduced expression of several targets of this regulator. The identification of RcsCB-regulated genes is probably incomplete at present, and other members of this regulon may also contribute to the adaptive response to CPZ. Although this study has shown the biological importance of the RcsCB His-Asp phosphorelay system, understanding its real contribution to cell physiology will require the identification of all the targets controlled by this system and of the stimuli leading to its activation in natural environments.

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