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Eliminating a Set of Four Penicillin Binding Proteins Triggers the Rcs Phosphorelay and Cpx Stress Responses in *Escherichia coli*

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Penicillin binding proteins (PBPs) are responsible for synthesizing and modifying the bacterial cell wall, and in *Escherichia coli* the loss of several nonessential low-molecular-weight PBPs gives rise to abnormalities in cell shape and division. To determine whether these proteins help connect the flagellar basal body to the peptidoglycan wall, we surveyed a set of PBP mutants and found that motility in an agar migration assay was compromised by the simultaneous absence of four enzymes: PBP4, PBP5, PBP7, and AmpH. A wild-type copy of any one of these restored migration, and complementation depended on the integrity of the PBP active-site serine. However, the migration defect was caused by the absence of flagella instead of improper flagellar assembly. Migration was restored if the *flhDC* genes were overexpressed or if the *rcsB* or *cpxR* genes were deleted. Thus, migration was inhibited because the Rcs and Cpx stress response systems were induced in the absence of these four specific PBPs. Furthermore, in this situation Rcs induction depended on the presence of CpxR. The results imply that small changes in peptidoglycan structure are sufficient to activate these stress responses, suggesting that a specific cell wall fragment may be the signal being sensed. The fact that four PBPs must be inactivated may explain why large perturbations to the envelope are required to induce stress responses.

The peptidoglycan wall is vital to the bacterial cell. Situated between the inner and outer membranes of Gram-negative bacteria, this covalently linked scaffold of glycan chains and short peptides maintains cell shape and resists osmotic lysis (1). Peptidoglycan is synthesized and modified by a set of periplasmic penicillin binding proteins (PBPs), including the high-molecular-weight transglycosylase-transpeptidase PBPs, which polymerize and cross-link the glycan chains, and the low-molecular-weight (LMW) PBPs, which modify existing chains (2). Among the latter, the LMW PBPs 4 and 7 and AmpH are primarily endopeptidases that cleave peptide side chains and disconnect the glycan polymers (2), and PBPs 5 and 6 and DacD are D,D-carboxypeptidases that remove the terminal D-alanine from pentapeptide side chains (2). In *Escherichia coli*, the loss of several of these nonessential LMW PBPs affects the symmetry of cell division, slows daughter cell separation, and gives rise to abnormally shaped cells (3–5), but other than this, few physiological effects of the LMW PBPs are known. These proteins may modify peptidoglycan to accommodate cell growth or to allow large macromolecular structures, such as secretion systems or flagella, to assemble onto or across the cell wall (1, 6).

Because the wall is so important, it is not surprising that bacteria monitor its integrity and stability. In *E. coli*, the RcsBCD, CpxAR, BaeSR, and EnvZ-OmpR signal transduction systems are generally believed to sense and respond to stresses that damage or alter the cell envelope (7–11). For example, the Rcs multicomponent phosphorelay responds to stimuli such as osmotic shock and β-lactam exposure (10, 11) and modulates gene expression when one or more signals are sensed by the outer membrane lipoprotein RcsF or by the inner membrane sensor kinase RcsC (11–13). Upon activation, RcsC autophosphorylates a conserved histidine residue in its cytoplasmic domain; the phosphate moves to a neighboring aspartate, then to a histidine residue on the inner membrane protein RcsD, and finally to an aspartate residue on RcsB, the cytoplasmic response regulator (13, 14). A homodimer of phosphorylated RcsB or an RcsB-RcsA heterodimer modulates the expression of target genes, including those that encode the colanic acid synthesis pathway, the genes *rprA*, *osmC*, and *ftsZ*, and about 180 other genes (10). The Rcs phosphorelay also negatively regulates transcription of *flhDC*, the master regulator of flagella synthesis, thereby turning off motility when cells come under stress (15, 16). The CpxAR two-component system consists of the sensor kinase CpxA, the response regulator CpxR, and the periplasmic inhibitor CpxP (17, 18). This response is activated by surface attachment, misfolded pilus subunits, and changes in pH and osmolarity, among other stresses (18), and regulates the expression of bundle forming pili in enteropathogenic *E. coli* (19), the production of P pili in uropathogenic *E. coli* (20), and the attachment, invasion, and intracellular growth of *Salmonella enterica* serovar Typhimurium (21). Although the phosphotransfer pathways in these systems are well characterized, the molecular identities of the signals and the mechanisms by which they are sensed remain unknown.

Here, we hypothesized that the LMW PBPs might play a role in anchoring the flagellar basal body to the peptidoglycan cell wall, which is required for *E. coli* to be fully motile (22, 23). In fact, we found that the simultaneous removal of four specific PBPs (PBPs 4, 5, and 7 and AmpH) did inhibit bacterial motility, but this effect was not due to mis-assembly of flagella. Instead, these mutations induced the Rcs and Cpx stress responses, and the Rcs response inhibits motility by downregulating transcription of flagellar genes. The enzymatic activity of any one of these four PBPs was

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TABLE 1 *E. coli* strains and plasmids

Strain or plasmid	Description[a]	Source or reference
E. coli strains		
CS109	W1485 *rph-1 katF* (*rpoS*)	3
CS204-1	CS109 *dacA*::*res pbpG*::*res*	3
CS206-3	CS109 *ampH*::*res pbpG*::*res*	3
CS315-1	CS109 *dacA*::*res pbpG*::*res dacB*::*res*	3
CS317-3	CS109 *ampH*::*res pbpG*::*res dacB*::*res*	3
CS326-3	CS109 *ampH*::*res dacA*::*res dacB*::*res*	3
CS345-3	CS109 *ampH*::*res dacA*::*res pbpG*::*res*	3
CS448-3	CS109 *dacB*::*res dacA*::*res pbpG*::*res ampH*::*res*	3
KE2	CS109 *ampG*::kan	This study
KE8	CS109 *rcsB*::tet	This study
KE9	CS448-3 *rcsB*::tet	This study
KE11	CS109 *rcsF*::cam	This study
KE12	CS448-3 *rcsF*::cam	This study
KE14	CS109 *rcsC52*::tet	This study
KE15	CS448-3 *rcsC52*::tet	This study
KE18	CS109 *envZ*::kan	This study
KE19	CS109 *ompR*::kan	This study
KE20	CS448-3 *envZ*::kan	This study
KE21	CS448-3 *ompR*::kan	This study
KE36	CS109 *cpxR*::kan	This study
KE37	CS448-3 *cpxR*::kan	This study
KE39	CS448-3 *ampG*::kan	This study
KE41	CS109 *fliC*::kan	This study
KE42	CS448-3 *fliC*::kan	This study
SKCS49-1	CS109 *flhA*::kan	This study
Plasmids		
pCP20	*c*I857 *repA*(Ts) P_R::*flp*, Amp^r Cam^r	25
pDKR1	*colE1* P_{rprA}::*sfgfp*; Kan^r	52
pFlhDC	P_{BAD}-*flhDC*; Cam^r	R. Harshey
pJW1	pNLP10 with P_{cpxP}::*luxCDABE*; Kan^r	33
pKD46	Lambda Red$^+$ (Amp^r pSC101 oriTS)	25
pLCM1	P_{lac}-*ampH lacI*^{q}; Kan^r	53
pLP8	P_{lac} *lacI*^{q}, Kan^r, pMLB1113 background	54
pLP405	P_{BAD}-*dacB*(*S62A*); Kan^r	P. Potluri
pLP514	P_{lac}-*dacA*(*S44G*) *lacI*^{q}; Kan^r	54
pLP515	P_{lac}-*dacA lacI*^{q}; Kan^r	54
pLP706	P_{BAD}-*pbpG*(*S67A*); Kan^r	P. Potluri
pLP18	P_{BAD}, Kan^r	55
pLPKC403	P_{BAD}-*dacB*, Kan^r	55
pLPKC704	P_{BAD}-*pbpG*, Kan^r	55
pMEL1	P_{lac}-*ampH*(*S87A*) *lacI*^{q}; Kan^r	53
pVS182	*flhD*::*lacZ* in pRS551; Amp^r	56

[a] Cam^r, chloramphenicol resistance; Amp^r, ampicillin resistance; Kan^r, kanamycin resistance.

sufficient to rescue motility, strongly suggesting that these two stress responses respond to minor, possibly specific changes in peptidoglycan structure.

MATERIALS AND METHODS

Bacteria, plasmids, growth conditions, and migration assays. *E. coli* strains and plasmids are listed in Table 1. The parental strain was *E. coli* CS109 (W1485F$^-$ *rpoS rph-1 acnA*) (24). Deletions marked with antibiotic resistance cassettes flanked by *res* sites were introduced by P1 transduction and cured by using the RP4 ParA resolvase (3). Deletions marked with antibiotic resistance cassettes flanked by FRT sites were introduced by P1 transduction and cured by using the FLP helper plasmid pCP20 (25). Bacteria were grown at 30 or 37°C in Luria-Bertani (LB) broth or agar (Difco) or at 30°C in tryptone broth (1% tryptone plus 0.25% NaCl) (Difco) or agar (tryptone broth plus 0.26% agar). When needed, kanamycin (50 μg/ml), chloramphenicol (20 μg/ml), or arabinose (0.05%) was added. For migration assays, bacteria were grown in LB broth overnight at 30°C, and 1.5 μl of this culture was spotted onto the surface of a migration plate (tryptone agar) and incubated at 30°C for 10 or 24 h.

Enzyme assays and muropeptide analysis. The β-galactosidase activity was assayed by growing bacteria in LB broth overnight at 30°C, diluting the culture 1:100 into 1% tryptone, and growing the cells until the optical density at 600 nm (OD_{600}) of the culture reached 0.5 to 0.6. The enzyme activity was determined according to the procedure of Miller (26). The amounts of green fluorescent protein (GFP) and luciferase were determined by growing bacteria in LB broth as described above until the OD_{600} reached 0.5 to 0.6 and then transferring 100 μl (in triplicate) to wells of a 96-well microplate (Costar, catalog no. 3603; Corning, Inc.). Activity was expressed as the ratio of GFP fluorescence, 485 nm (excitation) and 528 nm (emission), or luminescence counts per second (cps) to OD_{630}. For assays of cells from migration agar, a plug of cells and agar from 10- and 24-h migration plates was weighed and then sheared by passage through a 200-μl pipette tip. A 150-μl portion of 1% tryptone was added to the mixture, 100 μl of the sample was transferred in triplicate to a 96-well microplate, and the GFP fluorescence was measured as described above. The CFU were determined to ensure that equal numbers of cells were assayed.

Peptidoglycan was prepared and analyzed as described previously (27). Strains were grown overnight in LB medium at 30°C, diluted 1:100 into 150 ml of 1% tryptone, and grown at 30°C to an OD_{600} of 0.5 to 0.6. The cells were pelleted at 7,400 × *g* for 10 min at 4°C (Thermo Scientific Sorvall Legend XTR Centrifuge, F15 Fiberlite rotor). The cells were resuspended in 3 ml of LB medium and added dropwise to 6 ml of boiling 6% sodium dodecyl sulfate (SDS) in glass culture tubes. The samples were boiled and stirred for 1 h, then stirred at room temperature overnight. To isolate the peptidoglycan, the samples were boiled and stirred for an additional hour and pelleted by centrifugation at 65,000 rpm for 10 min at 30°C in an ultracentrifuge (Beckman-Optima TLX, TLA110 rotor). The pellet was resuspended in 3 ml of distilled H_2O to wash the peptidoglycan. The wash step was repeated three to four times, and the pellet was resuspended in 1.5 ml of 10 mM Tris-HCl (pH 7.0), after which α-amylase was added to a final concentration of 100 μg/ml, and the sample was incubated at 37°C for 2 h. The samples were incubated with Pronase (100 μg/ml) at 60°C for 2 h and then boiled for 30 min in 1% SDS to destroy the Pronase activity. The samples were pelleted, washed three to four times with distilled H_2O to remove the SDS, resuspended in 400 μl of $NaPO_4$ (50 mM) buffer containing *N*-acetylmuramidase SG (catalog no. A0534; US Biologicals) (40 μg/ml), and incubated overnight at 37°C. The samples were then boiled for 5 min to destroy the muramidase activity and centrifuged at top speed for 10 min in a table-top microcentrifuge. The supernatant was transferred to a clean tube and vacuum dried for 2.5 h.

Western blotting. Blots of whole cells were prepared as described previously (28). Cultures were grown overnight at 30°C in LB medium, diluted 1:100 into tryptone broth, and grown to an OD_{600} of 0.5 to 0.6, and 500 μl of the cells was pelleted by centrifugation and resuspended in 200 μl of double-distilled H_2O. Next, 5 μl of this suspension was spotted onto a polyvinylidene difluoride (PVDF) membrane prewetted with methanol and allowed to absorb overnight at room temperature. Primary anti-FliC antibody (catalogue no. ab93713; Abcam, Cambridge, MA) was used at a 1:10,000 dilution. Secondary α-rabbit-HRP was used at a 1:25,000 dilution. Standard procedures were followed for Western blotting (29).

Flagellum staining. Flagella were stained as described previously (30), with some modifications. All wash steps were carried out with extreme care, allowing up to 10 min for each wash. All centrifugation steps were 2,000 × *g* for 10 min in an Eppendorf 5415D tabletop centrifuge. Overnight cultures were grown in LB medium at 30°C, diluted 1:100 into 1% tryptone broth, and incubated until the OD_{600} reached 0.5 to 0.6. Motility was confirmed by microscopy before proceeding. The cell pellet was

washed three times in equal volumes of phosphate buffer (pH 7.0) at room temperature, washed once in equal volumes of phosphate buffer (pH 7.5), and resuspended in 100 μl of Alexa Fluor 488 carboxylic acid succinimidyl ester (AF488, catalogue no. A20000; Invitrogen, Carlsbad, CA), which had been dissolved in phosphate buffer (pH 7.5). Then, 20 μl of 1 M $NaHCO_3$ was added, and the dye-cell suspension was incubated at room temperature in the dark for 90 min, with gentle mixing on a rotator (Denville Scientific, South Plainfield, NJ). The stain was removed by centrifugation, and the cells were washed twice in 500 μl of phosphate buffer (pH 7.0) containing 0.002% Tween 20. This wash was carried out by gently swirling the liquid back and forth over the pellet. The pellet was resuspended in 500 μl of phosphate buffer (pH 7.0), containing 0.002% Tween 20, spotted onto a clean glass slide, and imaged using a wide-field epifluorescent Zeiss Axio Imager.Z1 microscope fitted with a 100× differential interference contrast objective (1.45 NA). Images were acquired with a Zeiss Axiocam MRm cold charge-coupled device camera, using appropriate filter cubes for fluorescence image acquisition.

RESULTS

Loss of four specific LMW PBPs inhibits motility. We hypothesized that one or more of the LMW PBPs helps attach the flagellar machinery to the peptidoglycan cell wall. To test this, we screened 60 *E. coli* PBP mutants for defects in migration, expecting to find that nonmigratory strains had defects in flagellar assembly. Bacteria were inoculated into migration agar and incubated at 30°C for 10 h, and the diameters of their migration rings were measured. Of the 60, 8 did not migrate (Table 2), producing no ring extending beyond the original inoculum. All mutants lacking three or fewer PBPs migrated, as did all but one of those tested that lacked four (Table 2). The exception in this latter group was strain CS448-3 (Fig. 1A, row 2), from which the genes encoding PBP4, PBP5, PBP7, and AmpH had been deleted. Furthermore, from among strains lacking five to seven LMW PBPs, the only nonmigratory mutants were those from which these four PBPs were missing (Table 2). Tellingly, even strains lacking six LMW PBPs migrated as long as at least one of these four PBPs was intact (Table 2, strains with "CS6xx" prefixes). In no case did strains have growth defects that could account for these differences in migration (not shown). The absence of all four of these PBPs was necessary for the loss of motility, because mutants lacking any three of these four PBPs migrated (Fig. 1A, rows 3 to 6). As further verification, we complemented strain CS448-3 with plasmids carrying wild-type versions of each of these genes. Indeed, the presence of any one of these PBPs restored motility (Fig. 1B, rows 2, 4, 6, and 8). Finally, we determined whether loss of migration was caused by the absence of the proteins themselves or by the absence of their enzymatic activities. CS448-3 did not migrate when complemented with inactive versions of PBP 4, 5, or 7 or AmpH (Fig. 1B, rows 3, 5, 7, and 9) in which the active-site serine had been replaced with alanine or glycine. Thus, the enzymatic activity of these PBPs was responsible for the phenotype, not the physical presence or absence of the proteins. In sum, the results show that removing this one particular combination of four LMW PBPs was necessary and sufficient for inhibiting *E. coli* migration.

PBP loss antagonizes flagellum assembly. Several scenarios could account for the inhibition of migration in these mutants: flagella might not be produced at all, the basal body might be so weakly anchored to peptidoglycan that the flagella become detached, or the flagella could be attached but not rotate properly. As a first step in distinguishing among these possibilities, we stained flagella with AF488 to determine whether flagella were present.

TABLE 2 Migration of selected PBP mutants

Strain	PBP(s) deleted[a]	Migration[b]
BMCS20K-1	1b	+
BMCS04-1K	1a	+
CS9-19	7	+
CS12-7	5	+
CS13-2	1a	+
CS14-2	C	+
CS15-3	H	+
CS16-1	1b	+
CS17-1	6	+
CS206-3	7, H	+
CS211-2	5, 6	+
CS212-3	6, H	+
CS221-3	4, H	+
CS224-2	1b, 5	+
CS225-1	1b, 6	+
CS226-3	1b, H	+
CS230-1K	1a, H	+
CS236-1K	4, dacD	+
CS315-1	4, 5, 7	+
CS317-3	4, 7, H	+
CS320-1	4, C, H	+
CS322-1	4, 5, 6	+
CS323-3	4, 6, H	+
CS326-3	4, 5, H	+
CS331-1	5, 6, 7	+
CS332-3	6, 7, H	+
CS336-3	5, 6, H	+
CS345-3	5, 7, H	+
CS349-1	5, C, H	+
CS389-1K	4, H, dacD	+
CS440-3	4, 6, 7, H	+
CS441-1	5, 6, C, H	+
CS442-3	4, 5, 6, H	+
CS443-3	5, 6, 7, H	+
CS444-1	5, 6, C, H	+
CS445-1	4, 5, 6, C	+
CS446-1	4, 5, 6, 7	+
CS447-1	4, 6, 7, C	+
CS448-3	4, 5, 7, H	−
CS449-2	4, 5, 7, C	+
CS450-1	5, 6, 7, C	+
CS533-1	4, 5, 6, 7, C	+
CS534-1	4, 5, 7, C, H	−
CS535-1	4, 6, 7, C, H	+
CS536-1	5, 6, 7, C, H	+
CS539-1K	4, 5, 6, C, H	+
CS607-4	1b, 4, 5, 6, 7, H	−
CS608-1	1b, 4, 5, 6, 7, C	+
CS609-1	1b, 4, 5, 7, C, H	−
CS610-1	1b, 4, 6, 7, C, H	+
CS611-1	1b, 5, 6, 7, C, H	+
CS612-1	4, 5, 6, 7, C, H	−
CS613-1	1b, 4, 5, 6, C, H	+
CS615-1K	1a, 4, 5, 6, 7, C	+
CS616-1K	1a, 4, 5, 7, C, H	−
CS617-1K	1a, 4, 6, 7, C, H	+
CS618-1K	1a, 5, 6, 7, C, H	+
CS619-1K	1a, 4, 5, 6, C, H	+
CS702-1	1b, 4, 5, 6, 7, C, H	−
CS703-1K	1a, 4, 5, 6, 7, C, H	−

[a] "C" and "H" refer to AmpC and AmpH, respectively.
[b] "+" indicates the strain was as motile as the wild type after 10 h, and "−" indicates the strain was not motile after 10 h.

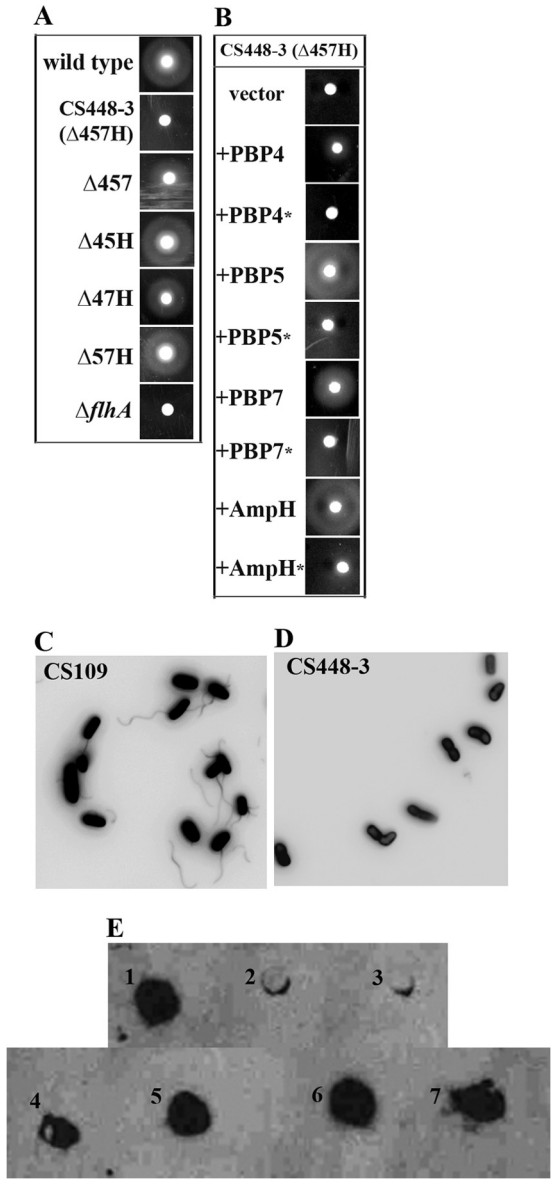

FIG 1 Loss of four specific PBPs inhibits migration by inhibiting flagellum production. The migration of each strain was assayed over 10 h on migration agar. (A) *E. coli* strain CS448-3, lacking PBPs 4, 5, and 7 and AmpH (Δ457H, row 2), is nonmotile after 10 h. Strains lacking any three of these PBPs remained motile: Δ457 (CS315-1), Δ45H (CS326-3), Δ47H (CS317-3), and Δ57H (CS345-3). A strain unable to make flagella, an Δ*flhA* mutant strain (SKCS49-1), served as a negative control. (B) Complementation with any one wild-type PBP gene restores the ability to migrate to *E. coli* CS448-3. CS448-3 was transformed with the following plasmids (the wild-type PBP that was expressed is noted in parentheses): pLPKC403 (PBP4, row 2), pLP515 (PBP5, row 4), pLPKC704 (PBP7, row 6), and pLCM1 (AmpH, row 8). No complementation occurred when PBPs with mutated active sites were expressed: pLP405 (PBP4*, row 3), pLP514 (PBP5*, row 5), pLP706 (PBP7*, row 7), and pMEL1 (AmpH*, row 9). CS448-3 transformed with plasmid vectors was nonmotile: CS448-3 pLP18 (top row) and CS448-3 pLP8 (data not shown). The parental strain containing plasmid vectors was motile (data not shown). (C and D) PBP loss inhibits flagellum production. *E. coli* CS109 (C) and CS448-3 (D) were stained with Alexa Fluor 488. Wild-type cells displayed at least one flagellum per cell (C), whereas CS448-3 lacked flagella (D). (E) Whole cells were spotted onto a PVDF membrane prewetted in methanol, and the FliC flagellar protein was detected by Western blotting. E1, CS109; E2, CS448-3; E3, CS109Δ*flhA* (SKCS49-1); E4, CS109Δ*rcsB* (KE8); E5, CS448-3Δ*rcsB* (KE9); E6, CS109/pFlhDC; E7, CS448-3/pFlhDC.

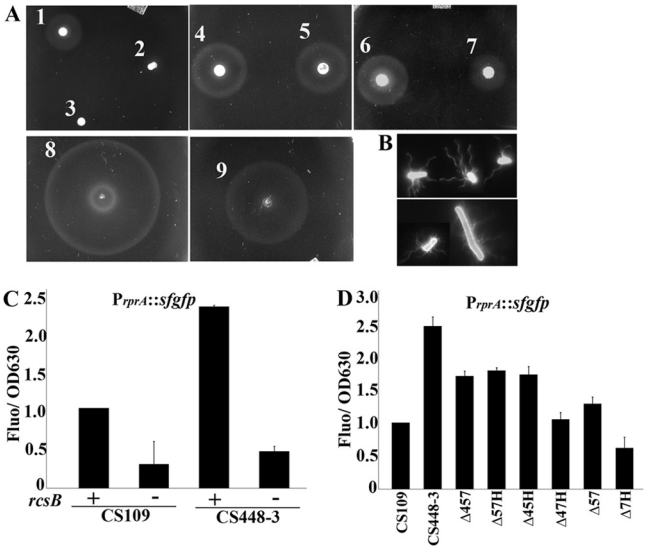

FIG 2 PBP loss activates the Rcs phosphorelay. (A) Migration assays. A1, *E. coli* CS109; A2, CS448-3; A3, CS109 Δ*flhA* (SKCS49-1); A4, CS109 Δ*rcsB* (KE8); A5, CS448-3 Δ*rcsB* (KE9); A6, CS109 Δ*rcsC* (KE17); A7, CS448-3 Δ*rcsC* (KE15); A8, CS109/pFlhDC; A9, CS448-3/pFlhDC. The *flhDC* genes were expressed from plasmid pFlhDC by supplementing the plates with arabinose (0.05%). (B) Overexpressing *flhDC* produces hyperflagellated cells. Cells were stained with Alexa Fluor 488. CS109/pFlhDC (upper panel) and CS448-3/pFlhDC (lower panel) exhibited many more flagella than did CS109 (Fig. 1D). Some cells appear enlarged because of the intensity of the fluorescence. (C) The absence of four PBPs increases the relative amount of P_{rprA}::*sfgfp* expression in an RcsB-dependent manner in *E. coli* CS448-3. "+", Wild-type *rcsB* present; "−", *rcsB* is deleted. (D) Rcs reporter expression in double and triple PBP mutants: Δ457 (CS315-1), Δ57H (CS345-3), Δ45H (CS326-3), Δ47H (CS317-3), Δ57 (CS204-1), Δ7H (CS206-3). The strains in panels C and D contain plasmid pDKR1, which carries the P_{rprA}::*sfgfp* reporter gene. In panels C and D, data from three experiments were normalized relative to CS109.

The parent, *E. coli* CS109, had flagella (Fig. 1C), but the PBP mutant CS448-3 did not (Fig. 1D). To verify that flagella were not sheared off during the centrifugation steps, cells were spotted directly onto a PVDF membrane and probed with anti-FliC antibody. The parental strain produced cell surface FliC (Fig. 1, E1), but CS448-3 did not (Fig. 1, E2). Furthermore, flagellar proteins were not simply trapped inside CS448-3 cells, because Western blots of whole-cell lysates revealed that CS448-3 did not contain FliC (data not shown). We concluded that strain CS448-3 did not migrate simply because it did not make flagellar proteins.

PBP loss activates the Rcs phosphorelay. The simplest explanation for the lack of flagella was that transcription or translation of these genes was inhibited in CS448-3. The Rcs phosphorelay negatively regulates motility by inhibiting transcription of *flhDC*, the master transcriptional activator of flagella genes (15). Indeed, expression of a *flhD*::*lacZ* reporter gene was decreased 4-fold in CS448-3 relative to the wild type (not shown). To determine whether the Rcs phosphorelay inhibited motility and thus migration, we removed the response regulator RcsB from CS448-3 (Fig. 2, A5) and found that this mutant was as capable of migration as the wild type at 10 h (Fig. 2, A1). Removing RcsC, the phosphorelay sensor histidine kinase, also restored the ability to migrate to CS448-3 at 10 h (Fig. 2, A7). If the Rcs system was inhibiting the transcription of *flhDC* in CS448-3, then overexpressing *flhDC* should restore migration. In fact, ectopic expression of *flhDC* in

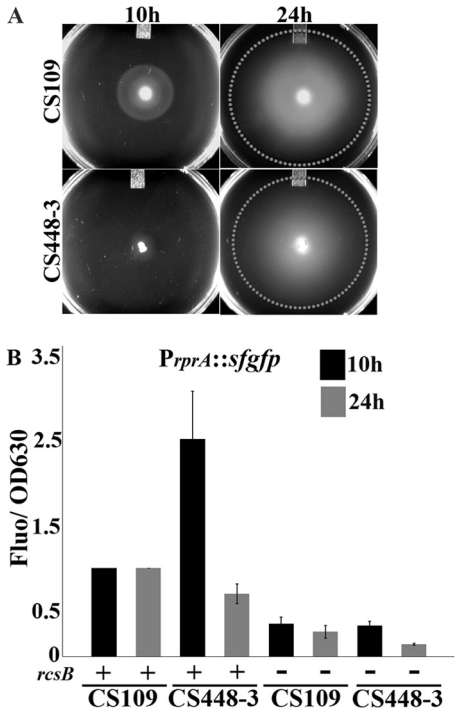

FIG 3 CS448-3 exhibits wild-type migration and an attenuated Rcs response after 24 h of incubation. (A) Strains were assayed for migration at 10 and 24 h. At 10 h, CS109 migrates (top left), but CS448-3 does not migrate (bottom left). CS448-3 recovers wild-type migration after 24 h (bottom right, white dotted circle indicates migration ring). (B) Cells were removed from the migration plate and assayed for P_{rprA}::*sfgfp* expression (black bars, 10 h; gray bars, 24 h). Each strain contained plasmid pDKR1, which contains the P_{rprA}::*sfgfp* reporter. Reporter expression in CS448-3 pDKR1 returns to wild-type levels at 24 h. "+", Wild type *rcsB* is present; "−", *rcsB* is deleted. The data from three experiments were normalized relative to CS109.

the parent strain produced a much enlarged migration ring (Fig. 2, A8), and ectopic expression of *flhDC* in CS448-3 also restored the ability to migrate (Fig. 2, A9). This latter ring was greater than that exhibited by the parental strain without an ectopic source of FlhDC (Fig. 2, A1). At the cellular level, removing RcsB (Fig. 1, E5) or overexpressing *flhDC* (Fig. 1, E7) restored cell surface flagellin to CS448-3, and these cells were also hyperflagellated (Fig. 2B, lower panel), which is the likely explanation for the abnormally large migration rings in these strains (15). Finally, transcription of the *rprA* gene is positively regulated by RcsB and is a surrogate for activation of the Rcs pathway (31). Expression of a P_{rprA}::*sfgfp* reporter gene was significantly greater in CS448-3 than in the parental strain, and deleting RcsB diminished reporter expression (Fig. 2C). Reporter expression in CS315 and additional PBP mutants was intermediate between wild type and CS448-3 (Fig. 2D), suggesting that Rcs was moderately activated in these strains, although migration was not affected (Fig. 1A). Overall, the results established that CS448-3 was nonmigratory at 10 h because the Rcs phosphorelay was activated in this mutant and inhibited the expression of flagellum genes.

CS448-3 recovers motility after prolonged incubation. Curiously, though strain CS448-3 did not migrate at 10 h, it exhibited near-wild-type migration after 24 h of incubation (Fig. 3A, bottom right). This delay was not due to poor or slow growth because CS448-3 did not show significant growth defects (not shown). Because the loss of these PBPs produces aberrantly shaped cells (3), it was possible that migration was hindered or slowed because of a nonoptimum shape. However, this was not the case because CS448-3 remained misshapen even after motility was restored at 24 h (data not shown). Also, the loss of RcsB, although it restored the ability to migrate to CS448-3, did not improve the shape of this mutant (not shown). Furthermore, mutants lacking other combinations of PBPs 4, 5, and 7 or AmpH migrated (Fig. 1A, rows 3 to 6) but were aberrantly shaped (not shown). These results indicated that neither growth nor morphology accounted for the delay in migration. Another potential explanation for migration recovery at 24 h was that CS448-3 had accumulated compensatory suppressor mutations during this time. If so, then cells isolated from the extremities of the migration ring should be motile at 10 h in subsequent assays. However, when such cells were transferred to LB broth, grown overnight at 30°C and inoculated onto a migration plate, none migrated after 10 h (not shown), indicating that migration at 24 h was not caused by the accumulation of suppressor mutants.

Finally, CS448-3 might have regained motility and thus the ability to migrate if Rcs phosphorelay activity was attenuated after 24 h. To test this, the parent strain and CS448-3 carrying a P_{rprA}::*sfgfp* reporter gene (on plasmid pDKR1) were incubated on migration plates, agar plugs containing cells from the inoculation site extending to the periphery of the migration ring were collected, and the amount of sfGFP in these cells at 10 and 24 h was determined. There was some expression of the P_{rprA}::*sfgfp* gene in the parental strain CS109 (Fig. 3B), which was probably nonspecific background caused by leaky transcriptional control. This level was the same in CS109 at both 10 and 24 h (Fig. 3B). In contrast, P_{rprA}::*sfgfp* expression was elevated significantly in CS448-3 at 10 h (Fig. 3B, black bar). However, after 24 h, P_{rprA}::*sfgfp* expression in CS448-3 returned to wild-type levels (Fig. 3B, gray bar). Therefore, it appears that Rcs activity in CS448-3 was attenuated after prolonged incubation, which would account for the restored motility. Such a scenario further corroborates the conclusion that in this mutant the Rcs response delays motility and thus the ability to migrate.

The Cpx stress response is activated in CS448-3. Signal transduction systems other than the Rcs pathway also affect flagellar motility. In the EnvZ-OmpR two-component system, OmpR, like RcsAB, negatively regulates *flhDC* transcription (32). To determine whether the EnvZ-OmpR response was activated in CS448-3, we removed either EnvZ, the sensor kinase, or OmpR, the response regulator. The loss of neither restored the ability to migrate to CS448-3 at 10 h (not shown). Similarly, the *mdtABCD*::*luxCDABE* reporter gene, which is induced by the BaeSR envelope stress response, was not expressed in CS448-3 (not shown), indicating that the BaeSR system was not active under these conditions. In contrast, inactivation of the Cpx two-component system in CS448-3 restored its ability to migrate (Fig. 4B5). This system affects motility in *E. coli* via CpxR-mediated transcriptional repression of the *motAB* genes (33). Also, expression of the P_{cpxP}::*luxCDABE* reporter, indicating Cpx activation, increased in CS448-3 relative to the wild type (Fig. 4C). Strains lacking different combinations of PBP 4, 5, or 7 or AmpH expressed the reporter at levels that fell in between those of wild type and CS448-3 (Fig. 4D). This indicated that Cpx was moderately activated in these mutants, although migration was not affected. Thus, the Cpx stress response was induced in CS448-3, as well as the Rcs

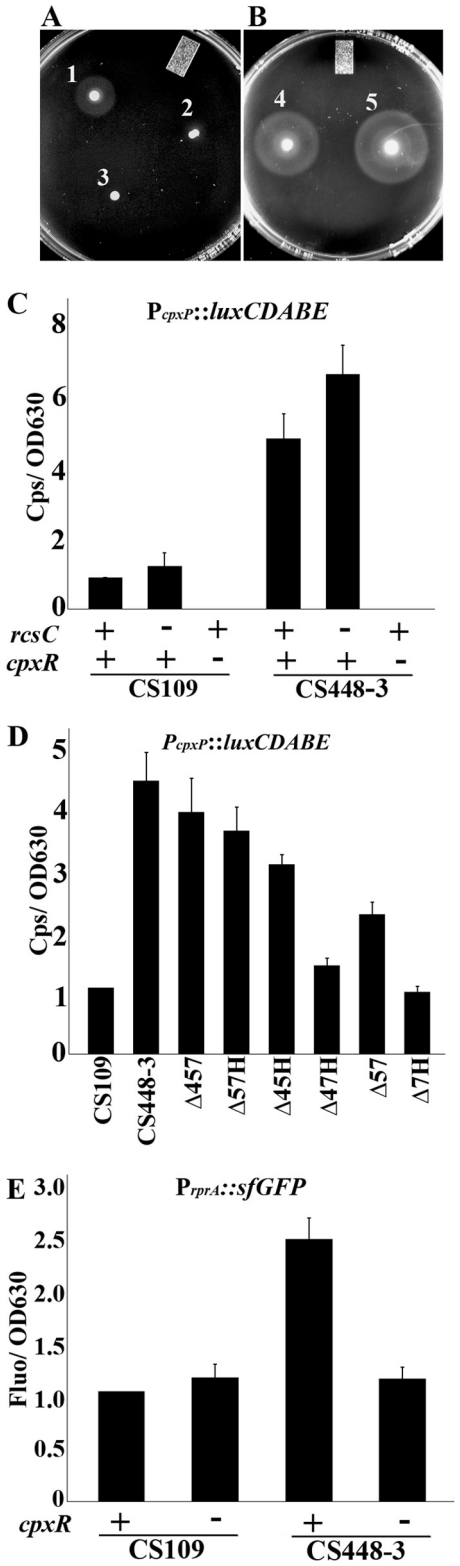

FIG 4 The Cpx stress response is activated in *E. coli* CS448-3. (A) Migration assays: A1, CS109; A2, CS448-3; A3, CS109 Δ*flhA* (SKCS49-1). (B) Deleting *cpxR* restores the ability to migrate to CS448-3: B4, CS109 Δ*cpxR* (KE36); B5, CS448 Δ*cpxR* (KE37). (C) Cpx reporter activity is increased in CS448-3 and is independent of the Rcs phosphorelay. (D) Cpx reporter activity in double and triple PBP mutants. Strains are the same as in Fig. 2D. (E) Rcs reporter activity depends on CpxR. The experimental details are the same as in the legend to Fig. 2. The strains in panels C and D contain plasmid pJW1, which carries the P_{cpxP}::*luxCDABE* reporter. The strains in panel E contain plasmid pDKR1, which carries the P_{rprA}::*sfgfp* reporter. "+", *rcsC* or *cpxR* are present; "−", the gene is deleted. In panels C, D, and E, the data from three experiments were normalized relative to CS109.

response. However, P_{cpxP}::*luxCDABE* expression remained high in CS448-3 mutants lacking *rcsB* (not shown) and *rcsC* (Fig. 4C), indicating that activation of the Cpx pathway was independent of the ability to mount an Rcs response. Surprisingly, however, P_{rprA}::*sfGFP* expression returned to a basal level when *cpxR* was deleted from *E. coli* CS448-3 (Fig. 4E), indicating that under these conditions Rcs induction depended on the presence of CpxR. The simultaneous activity of both of these pathways was required to fully inhibit motility and thus migration in CS448-3, because removing either RcsB (Fig. 2A5) or CpxR (Fig. 4B5) restored migration to CS448-3 at 10 h. The results suggest that the two responses must function cooperatively to antagonize motility.

Peptidoglycan composition of PBP mutants. Because the loss of PBPs 4, 5, and 7 and AmpH induced the Rcs and Cpx systems, we hypothesized that the signal(s) triggering these responses must be a type of modified peptidoglycan. To determine whether CS448-3 contained unique peptidoglycan modifications, sacculi from CS448-3 and mutants deleted for PBP4, PBP5, PBP7, or AmpH were isolated and analyzed by HPLC. Sacculi from CS448-3 contained high levels of monomer (M5) and dimer pentapeptides (D45), as well as having a greater number of crosslinked muropeptides and lower amounts of lipoprotein-containing muropeptides (Table 3).

The relevant signal could be the increase of a peptidoglycan component that activates the Rcs or Cpx systems. Alternately, the presence of a particular muropeptide in wild-type cells might inhibit these stress responses, in which case a decrease in the concentration of this inhibitor would activate these systems. With this in mind, we compared the abundance of specific muropeptides with Rcs and Cpx reporter expression in the respective strains. If the accumulation of a specific muropeptide activated the Rcs and Cpx pathways, then that muropeptide should be abundant in strains with high Rcs or Cpx reporter expression and low in strains with low reporter expression. The patterns of Rcs and Cpx induction were similar (Fig. 2D and Fig. 4D). In both cases, reporter genes were expressed in increasing amounts according to the following relationship: CS317-3 < CS326-3 ≤ CS345-3, CS315-1 < CS448-3 (Fig. 2D and Fig. 4D). A candidate inducer should increase accordingly, and the abundance of the following muropeptides followed this trend: M5, M4G, D45, D44G, T445, and T445N (Table 3). Note that the overall frequency of pentapeptide- and pentaglycine-containing muropeptides increased in concert with this predicted pattern (Table 3). If the signal is a negative regulator, then the candidate inhibitory muropeptide should decrease in these strains as follows: CS317-3 > CS326-3 ≥ CS345-3, CS315-1 > CS448-3. Muropeptides that followed this trend included: M3, M3G, D43, D44NN, and those with attached lipoprotein (Table 3).

Finally, we examined the possibility that the peptidoglycan signaling compound might be transported to the cytoplasm by the muropeptide recycling pathway (34). If so, then this could account for the attenuated Rcs response observed after 24 h (Fig. 3B). However, deleting the gene encoding the inner membrane permease AmpG did not prevent Rcs induction in strain CS448-3,

TABLE 3 Peptidoglycan composition of *E. coli* PBP mutants

Muropeptide	Avg value (mol%) for various *E. coli* strains (PBPs deleted)[a]					
	CS109 (wt)	CS315-1 (4, 5, 7)	CS317-3 (4, 7, AmpH)	CS326-3 (4, 5, AmpH)	CS345-3 (5, 7, AmpH)	CS448-3 (4, 5, 7, AmpH)
M3	9.73	5.58	7.30	7.50	5.33	3.11
M3G	2.22	1.15	1.56	1.38	1.20	0.46
M4	42.32	30.91	33.67	38.48	34.44	18.01
M4G	0.00	1.89	0.03	0.44	1.64	2.57
M2	3.60	2.21	4.03	2.39	2.41	2.57
M5	0.00	13.43	0.34	7.91	9.23	25.13
M3L	10.01	6.13	7.79	6.54	4.83	1.56
D33D	0.22	0.00	0.08	0.06	0.03	0.00
D34D	1.30	0.43	0.89	1.03	0.89	0.25
D43	4.19	1.38	4.07	1.86	1.89	1.20
D44G	0.00	2.70	0.45	0.62	2.68	3.52
D44	18.76	14.64	29.44	19.27	20.19	14.44
D45	0.21	13.13	0.47	7.00	8.31	19.66
T443	0.25	0.09	0.13	0.00	0.05	0.06
T444	1.51	1.15	2.21	1.17	1.60	1.30
D43L	2.97	2.21	4.12	2.15	2.55	1.60
T445	0.00	0.83	0.00	0.34	0.49	1.10
D43N	0.37	0.22	0.59	0.21	0.24	0.21
D44N	1.16	0.64	1.30	0.65	0.93	0.67
D45N	0.09	0.69	0.00	0.49	0.47	1.38
T444N	0.34	0.15	0.42	0.22	0.22	0.18
T445N	0.05	0.18	0.00	0.11	0.09	0.32
D44NN	0.36	0.23	0.37	0.18	0.18	0.16
Monomers	68.19	61.30	55.24	64.62	58.98	53.40
Dimers	29.67	36.31	42.01	33.55	38.58	43.40
Trimers	2.14	2.39	2.76	1.85	2.44	3.20
Lipoprotein	13.04	8.323	11.90	8.69	7.38	3.41
Anhydro	3.02	2.39	3.70	2.09	2.45	3.15
Dap-dap	1.58	0.49	1.21	1.15	1.16	0.33
Pentapeptide	0.33	28.25	0.81	15.84	18.58	47.83
Penta-glycine	0	4.59	0.48	1.05	4.33	6.09
Cross-linkage	33.98	41.08	47.50	37.25	43.45	49.78
Avg length	33.68	41.95	27.10	48.60	40.85	32.68

[a] Values (given as the mole percentage) are averages of four (CS109, CS315-1, and CS448-3) or three (CS317-3, CS326-3, and CS345-3) HPLC analyses of peptidoglycan composition. The PBP status (i.e., wild type [wt] or PBPs deleted) is indicated in parentheses after each strain.

nor did it prevent the resumption of migration at 24 h (not shown). Thus, the peptidoglycan signal did not have to enter the cytoplasm via the recycling pathway, nor was Rcs attenuated because the inducing signal was depleted in this way.

DISCUSSION

Envelope stress responses are activated by peptidoglycan modification. *E. coli* encodes multiple envelope stress responses, but the specific signals that trigger these different responses have not been described. Thus far, the inducing treatments that have been identified affect the envelope in multiple ways. For example, lysozyme (35) and EDTA (36) significantly damage the peptidoglycan and outer membrane, respectively. Exposure to alcohols (37), acid stress (38), and changes in osmolarity (39, 40) likewise probably damage numerous envelope proteins or seriously alter overall membrane integrity. These gross effects make it nearly impossible to identify specific molecular stimuli that activate any one envelope stress response. Here, we demonstrate that the loss of four LMW PBPs (PBP4, PBP5, PBP7, and AmpH) activates the Rcs and Cpx envelope stress responses. Importantly, the pathways are activated by the loss of PBP enzyme activity, not because the proteins themselves are absent, strongly suggesting that a peptidoglycan compound is the activating signal. The results imply that the Rcs and Cpx pathways detect changes among peptidoglycan species in the cell wall.

Rcs and Cpx sensor components in the periplasm and on the periplasmic side of the cytoplasmic membrane are conveniently located to respond to such a cell wall-associated stimulus, and the responses could be activated either because an inducer accumulates or because an inhibitory compound disappears. Thus, PBPs 4, 5, and 7 and AmpH may normally degrade or modify an activator (which would then accumulate in their absence), or they may synthesize an inhibitor (which would then disappear in their absence). Whatever its identity and mode of action, the activating compound may be sensed directly by Rcs or Cpx receptors or may induce these stress responses indirectly via a downstream event (e.g., alterations in periplasmic protein folding or changes in another envelope characteristic). In any case, the critical concentration of the signal(s) will depend on the activities of PBPs 4, 5, and 7 and AmpH.

Although we cannot yet identify the specific peptidoglycan species responsible, we can draw some conclusions about its nature. If the signal is an activator, then the fact that PBP5 must be inactivated indicates the compound probably contains a pentapeptide. PBP4 and AmpH also exhibit low levels of D,D-carboxypeptidase activity, and their loss may also contribute to the accumulation of such a pentapeptide. However, because PBPs 4 and 7 and AmpH are primarily endopeptidases (2), the fact that the stress responses are induced in their absence implies that the activator probably includes a cross-linked muropeptide. In fact, *E. coli* CS448-3 contains much higher amounts of pentapeptide and cross-linked muropeptides (Table 3). More specifically, the concentrations of two monomers (M5 and M4G), two dimers (D45 and D44G), and two trimers (T445 and T445N) rise in parallel with the degree to which the Rcs and Cpx responses become activated, making these good candidates for behaving as the activator. Interestingly, two of these muropeptides contain glycine as the terminal amino acid in the peptide side chains, making it possible that Rcs and Cpx sense these termini, which are not abundant under normal circumstances. On the other hand, if the signal involves the disappearance of an inhibitor, then by inverting the above considerations the inhibitory compound would most likely contain a tetrapeptide or tripeptide and would either be a monomer or dimer that appears frequently in wild-type *E. coli* but not in CS448-3. Finally, the proximal inducing signal might not be a specific peptidoglycan species but rather some downstream alteration that affects the cell envelope or its properties. At the moment, the most obvious possibility along this line is that *E. coli* CS448-3 has many fewer muropeptides that are covalently connected to Braun's lipoprotein (Table 3), and this alteration in envelope structure might destabilize the outer membrane (although there is no evidence of this as yet).

Although a small change in peptidoglycan structure may trigger the Rcs and Cpx responses, other types of envelope damage may induce these responses by independent routes or signals. Alternately, all stressors might eventually generate a common signal. For example, the Cpx response can be induced by overexpressing the outer membrane lipoprotein NlpE (41), which has no obvious relationship to peptidoglycan synthesis. However, it is possible that accumulation of periplasmic NlpE could affect the synthesis and composition of peptidoglycan, which would then be the proximal signal. Conversely, and even more theoretically, extensive peptidoglycan modification might affect the folding of periplasmic proteins, which might themselves act as the proximal stress-inducing signal. In either case, the involvement of a specific set of LMW PBPs offers a new avenue to explore these questions.

LMW PBPs perform different physiological roles. It is a mystery why *E. coli* and other bacteria contain so many PBPs with similar or overlapping enzymatic activities. Here, we show that the loss of one specific combination of LMW PBPs activates two stress responses, which suggests that these and other ostensibly redundant PBPs may have somewhat more individual physiological roles than is now appreciated. Specifically, the Rcs and Cpx responses are triggered by removing one D,D-carboxypeptidase (PBP5) and three of the four *E. coli* endopeptidases. No other combination of PBP deletions reproduced this phenotype, indicating that these enzymes exhibit a great deal of selectivity as they interact with their substrates. For example, we conclude that *in vivo*, PBP4, PBP7, AmpH, and MepA must have small but physiologically relevant differences in endopeptidase activity or substrate specificity, so that these enzymes may not overlap in function or be as superfluous as has been assumed.

Another question is why multiple LMW PBPs should have to be inactivated before the Rcs and Cpx responses are highly induced. Because many β-lactam antibiotics inhibit multiple PBPs, deleting four PBPs may reproduce the cell wall modifications that accompany exposure to β-lactams. We speculate that this requirement may allow *E. coli* to monitor cell wall damage in order to activate stress responses only when the damage becomes sufficiently severe, as reflected by the simultaneous inactivation of multiple PBPs.

Finally, why might the Rcs and Cpx responses be activated by PBP inactivation? Other than the antilysozyme proteins Ivy and MliC (35), no known Rcs-induced proteins are related to peptidoglycan synthesis or modification (10). Thus, it is not clear that the Rcs system is set up to repair damage to the peptidoglycan or whether this kind of damage serves only as a convenient signal. On the other hand, the Cpx response stimulates the expression of two peptidoglycan amidase genes (*amiA* and *amiC*) (42), as well as that of at least four other cell wall-associated genes (*dacC*, *slt*, *ycbB*, and *ygaU*) (43), so this response may actively repair cell wall damage.

The Rcs and Cpx stress responses synergistically inhibit motility. Removing the Rcs response did not appreciably affect activation of the Cpx pathway in *E. coli* CS448-3, but removing CpxR returned Rcs reporter expression to its basal level, indicating that Rcs activity depends on CpxR in this mutant. This phenotype has not been reported previously, and we are characterizing it further. One possibility is that expression of a Cpx-regulated gene is required to activate Rcs; alternately, although unlikely, in the absence of CpxR, CpxA may dephosphorylate RcsB and thus inhibit the Rcs response. The latter scenario might reflect a natural mechanism that keeps the level of phosphorylated RcsB in check, or it may simply be an artifact caused by removing CpxR. In any case, both the Rcs and Cpx responses must be activated before migration is inhibited completely; that is, the CS448-3 mutant can migrate if only one or the other response is disabled. The simplest explanation is that neither of these inhibitory events is fully effective on its own. Such regulation is not unprecedented. For example, a high level of *spy* gene expression requires that both the Cpx and the Bae stress responses be activated (44). Thus, *E. coli* is able to fine-tune transcription levels and cellular physiology based on environmental stimuli, the degree of stress-induced damage, and the specific stress responses that become activated.

Conclusion. Peptidoglycan or its modified components can serve as biological signals in several different species and systems. For example, peptidoglycan fragments induce sporulation in *Bacillus subtilis* (45, 46), alert the innate immune system (47, 48), act as virulence factors in *Bordetella pertussis* (49), and induce the production of β-lactamase (50, 51). We now report that peptidoglycan modifications activate the Rcs and Cpx stress responses in a system that may help integrate and improve our understanding of how cell envelope stress is sensed and conveyed to the requisite genetic regulators.

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