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The Rcs Phosphorelay Is a Cell Envelope Stress Response Activated by Peptidoglycan Stress and Contributes to Intrinsic Antibiotic Resistance▽†

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Gram-negative bacteria possess stress responses to maintain the integrity of the cell envelope. Stress sensors monitor outer membrane permeability, envelope protein folding, and energization of the inner membrane. The systems used by gram-negative bacteria to sense and combat stress resulting from disruption of the peptidoglycan layer are not well characterized. The peptidoglycan layer is a single molecule that completely surrounds the cell and ensures its structural integrity. During cell growth, new peptidoglycan subunits are incorporated into the peptidoglycan layer by a series of enzymes called the penicillin-binding proteins (PBPs). To explore how gram-negative bacteria respond to peptidoglycan stress, global gene expression analysis was used to identify *Escherichia coli* stress responses activated following inhibition of specific PBPs by the β-lactam antibiotics amdinocillin (mecillinam) and cefsulodin. Inhibition of PBPs with different roles in peptidoglycan synthesis has different consequences for cell morphology and viability, suggesting that not all perturbations to the peptidoglycan layer generate equivalent stresses. We demonstrate that inhibition of different PBPs resulted in both shared and unique stress responses. The regulation of capsular synthesis (Rcs) phosphorelay was activated by inhibition of all PBPs tested. Furthermore, we show that activation of the Rcs phosphorelay increased survival in the presence of these antibiotics, independently of capsule synthesis. Both activation of the phosphorelay and survival required signal transduction via the outer membrane lipoprotein RcsF and the response regulator RcsB. We propose that the Rcs pathway responds to peptidoglycan damage and contributes to the intrinsic resistance of *E. coli* to β-lactam antibiotics.

The cell envelope is the hallmark of gram-negative bacteria. This complex and dynamic compartment performs critical functions for both environmental microbes and pathogens, protecting the organisms from hostile conditions in the environment and immune responses in a host. The outer membrane forms a barrier limiting the entry of foreign molecules, such as toxins and antimicrobial agents, while selectively allowing the entry of nutrients. Many processes central for the life of the organism occur in the inner membrane, including energy generation and transport of nutrients into and waste out of the cytoplasm. A thin layer of peptidoglycan lies in the periplasmic space between the inner and outer membranes and ensures the structural integrity of the cell by preventing osmolysis.

Gram-negative bacteria possess an array of stress responses that maintain the integrity of the cell envelope (11, 46, 48). In *Escherichia coli*, four transcriptional regulators mediate envelope stress responses: the CpxAR (Cpx) and BaeSR (Bae) two-component systems, the σ^E alternative sigma factor, and the phage shock protein (Psp) (11, 46, 48). Cpx and σ^E are activated in response to disruptions of the folding of envelope proteins and the integrity of the outer membrane, and Psp is activated by perturbations in the integrity and energization of the inner membrane (8, 11, 46). The role of the Bae response is not well understood, but it is activated by indole and some toxins that disrupt membrane integrity (45). Notably lacking from this array of stress responses is one that is activated by and combats stresses that affect the peptidoglycan layer.

Peptidoglycan (or murein) is a single mesh-like molecule formed by covalently cross-linked strands of peptidoglycan subunits that completely surrounds the cell (25). For the cell to grow and divide, murein hydrolases cut the peptidoglycan and penicillin-binding proteins (PBPs) ligate new strands into the existing peptidoglycan layer (Fig. 1) (25). These two processes must be carefully coordinated to prevent hydrolysis of the peptidoglycan (60). In *E. coli*, new peptidoglycan subunits are ligated into the existing peptidoglycan layer by the high-molecular-weight PBP enzymes, PBPs 1a, 1b, 1c, 2, and 3 (25). PBPs 2 and 3 are transpeptidases responsible for peptidoglycan growth during cell elongation and septation, respectively (Fig. 1), and are essential for viability (23, 25, 42). PBP 2 may also regulate some aspects of cell division, although this role is not fully understood (12). PBPs 1a and 1b are dual-function enzymes with both transglycosylase and transpeptidase activity and are required for insertion of new strands and cross-linking of the peptidoglycan layer during both elongation and septation (Fig. 1) (25). *E. coli* can survive in the absence of one of these enzymes but not both (56). The function of PBP 1c is not well characterized, and it is dispensable for viability (64). PBPs are thought to function in multiprotein complexes with murein hydrolases, and inhibition of one PBP often sensitizes the cells to inhibition of others (21, 25).

Given the dynamic nature of peptidoglycan synthesis and its importance for cell integrity, stress responses that sense the state of the peptidoglycan layer should be of critical importance. In addition to its intrinsic interest, identification of stress

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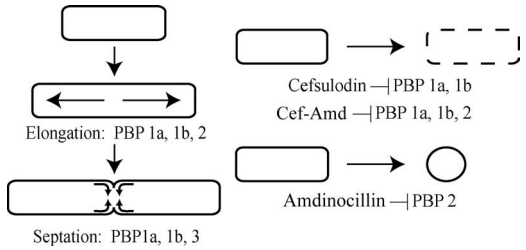

FIG. 1. General overview of the roles of essential PBPs in *E. coli* and the effects of inhibition of specific PBPs by antibiotics used in these studies. PBPs 1a and 1b are required for insertion of new strands and cross-linking of the peptidoglycan layer during elongation and septation, while PBP 2 is required for elongation and PBP 3 for septation. Cefsulodin inhibits PBPs 1a and 1b, causing cell lysis, while amdinocillin inhibits PBP 2, causing cells to become round and eventually stop dividing.

responses targeting this layer is of medical importance. Peptidoglycan is unique to prokaryotic cells, and several of the enzymes that construct it are essential for viability, making these enzymes important targets for antimicrobial chemotherapy (3). Therefore, peptidoglycan-sensing stress responses have the potential to protect bacteria against this important class of antibiotics.

In this work, we identified stress responses induced in *E. coli* as a result of damage to the peptidoglycan layer by examining the transcriptional response to a series of β-lactam antibiotics, each of which inhibits a different PBP and has different consequences for cell morphology and viability (Fig. 1). Because most stress responses are induced at the transcriptional level, comparison of the changes in gene expression following treatment of bacteria with different β-lactam antibiotics provides information about how the bacterium experiences peptidoglycan stress. We find that inhibition of peptidoglycan synthesis by the different antibiotic treatments elicited both shared and unique responses. Any perturbation to the peptidoglycan induced the Rcs phosphorelay, which is primarily known for its role in regulating synthesis of the colanic acid capsular exopolysaccharide (33). Moreover, the Rcs pathway enhanced bacterial survival in the presence of the antibiotics independently of capsule production.

MATERIALS AND METHODS

Strains, growth conditions, and antibiotics. The strains used in the study are listed in Table 1 and are all derivatives of *E. coli* strain MG1655. Mutant alleles *cpsE3*::Tn*10* (57), *rcsA*::Kan (35), and *rpoS*::Tn*10* (9) were transferred into recipient strains by using P1 transduction (38). Strains were grown in Luria Bertani (LB) broth at 37°C with agitation. Amdinocillin was provided by Leo Pharmaceutical Products, Ballerup, Denmark. Cefsulodin was purchased from Sigma Aldrich, St. Louis MO (catalog no. C8145).

Strain growth and sample preparation for microarray analysis. Cultures of SEA113 (MG1655) were grown in LB at 37°C overnight (14 to 18 h) with rotation. The overnight cultures were diluted in fresh media to an optical density at 600 nm (OD_{600}) of 0.02 and grown at 37°C with shaking in a gyratory water bath to an OD_{600} of 0.2, at which point antibiotics were added to the final concentrations indicated in the text. Samples were taken from both the treated and the untreated cultures at time points immediately before cell growth slowed due to antibiotic treatment (40 min after treatment with a combination of cefsulodin and amdinocillin [Cef-Amd], 10 min after cefsulodin treatment, and 60 min after amdinocillin treatment) and added to an ice-cold 5% phenol-ethanol solution to stop further transcription (47). Total RNA was isolated using the hot phenol method of RNA extraction (47). cDNA was prepared as described in the Affymetrix GeneChip manual (Affymetrix, Santa Clara, CA), with the exception that cDNA was labeled with biotin by using an Enzo BioArray terminal labeling kit (Enzo Life Sciences, Farmingdale, NY). RNA was prepared from four (Cef-Amd) or three (cefsulodin and amdinocillin) independent cultures, along with the corresponding untreated control cultures. Microarray experiments were also performed on cultures treated with amdinocillin and cefsulodin individually at the lower concentrations used in the combination treatment, but no changes in gene expression were detected.

Microarray procedures and data analysis. Biotinylated cDNA samples were hybridized to Affymetrix GeneChip microarrays, washed, stained, and scanned with the Affymetrix GeneChip instrument system according to the manufacturer's instructions. Samples from experiments with Cef-Amd were hybridized to Affymetrix *E. coli* Antisense arrays, while samples from experiments with amdinocillin and cefsulodin alone were hybridized to Affymetrix *E. coli* Genome 2.0 arrays. The Genome 2.0 arrays became available after the experiments with Cef-Amd samples were completed and replaced the older arrays, which are now available only by custom order. cDNA samples prepared from one Cef-Amd-treated culture and the corresponding control culture were hybridized to both types of arrays and produced comparable results.

The expression values for each gene were determined using robust multichip averaging and quantile normalization with the robust multichip averaging function of the Simpleaffy program for the *R* statistics environment (4, 18, 19, 26, 27, 62). The program Significance Analysis of Microarrays was used to identify genes with significant changes in gene expression (58). Significance Analysis of Microarrays was performed using two-way paired comparison, with false discovery rates of 0.958% for Cef-Amd, 0.828% for amdinocillin, and 1.67% for cefsulodin. The Genome 2.0 arrays have probes for genes not found on the older arrays, and probes were redesigned for many genes, so the number of genes affected by the combination of antibiotics could have been underestimated. To address this issue, genes with >2-fold alteration in expression, as determined from the Genome 2.0 array, were included in the list of genes affected by Cef-Amd if their expression was also significantly changed following treatment with the individual antibiotics. This analysis identified 12 additional genes with increased expression and 13 genes with decreased expression (see Table S1 in the supplemental material). These genes were members of regulons already shown to have altered expression with the Antisense arrays.

Bioinformatic identification of regulators. Genes were classified according to membership in known transcription factor regulons by using regulonDB (http://regulondb.ccg.unam.mx/), and information available in the literature (13, 14, 16, 22, 32, 40, 41, 44, 47, 61, 63).

Plating efficiency. Overnight cultures were serially diluted (1:10 dilutions) and 10-μl aliquots spotted onto LB agar containing 0.15 μg/ml amdinocillin, 45 μg/ml cefsulodin, 10 μg/ml cefsulodin, and 0.075 μg/ml amdinocillin or no antibiotics. These concentrations are slightly below the MIC for each treatment, and they were chosen because the wild-type (WT) strain was able to grow on the plates, although not as well as when plated on LB. This allowed us to see growth differences for mutants that decrease, increase, or do not change the suscepti-

TABLE 1. Strains used in this study

Strain	Relevant genotype	Source or reference
CAG45133	MG1655 Φλ(*rpoH*P3::*lacZ*) Δ*lacX74 rpoS*::Tn*10*	9
DH300	MG1655 Δ(*argF-lac*)*U169*; *rprA142-lacZ*	35
DH301	DH300 *rcsF*::Cm^r	36
DH310	DH300 *rcsA*::Kan^r	35
DH311	DH300 *rcsB*::Kan^r	35
DH366	DH300 *rcsC137 zei-10*::Cm^r	36
SEA001	MG1655 Φλ(*rpoH*P3::*lacZ*) Δ*lacX74*	1
SEA113	MG1655 (CAG45113)	Gross laboratory
SEA3141	DH366 *rcsA*::Kan^r	This study
SEA3143	DH366 *rpoS*::Tn*10*	This study
SEA3202	DH300 *cpsE3*::Tn*10*	This study
SG20043	MC4100 (*araD139*) Δ(*argF-lac*)*169* Δ*lon-100* λ$^-$ *flhD5301* *cpsE3*::Tn*10* Δ(*fruK-yeiR*)*725*(*fruA25*) *relA1* *rpsL150*(*strR*) *rbsR22 deoC1*	57

TABLE 2. Primers and probe used for TaqMan real-time PCR

Gene	Primer or probe	Sequence (5′ to 3′)
osmB	Forward primer	CCGACAGCTGCACCACCTA
	Reverse primer	TCTAAACGGGACCGCAACAC
	Fluorescent probe	TGTACCCAACGTACTGCCATCGGTCA
ymgG	Forward primer	ATGAACCGCACCACGAAAG
	Reverse primer	ACACCATTGCCAGTAAGTAAACCA
	Fluorescent probe	TGCATTGCTAGGCGCAGGCG
ydhA	Forward primer	GCGTTACACTGACGGAATCTATGT
	Reverse primer	TGCGGTCGCGTTTATAGACA
	Fluorescent probe	TCTGGTCGAAAGGCGATGAAGCG
entC	Forward primer	CGACAGTCCCTTCCAGCAA
	Reverse primer	GATGCCCTGCGCTTTGG
	Fluorescent probe	AACTCGCCGCGCTGTTTGCC
cpxP	Forward primer	CAACGCTGGCAGTCAGTTCA
	Reverse primer	ACCCGGATGCCAGTTATCG
	Fluorescent probe	AAGCCACGCTGCTGAAGTCGGTTC
icd	Forward primer	GCTGACCACTCCGGTTGGT
	Reverse primer	GGCAGATGTAGAGATCCAGTTCCT
	Fluorescent probe	TTCGCTCTCTGAACGTTGCCCTGC

bility of the strain to the antibiotics. After incubation at 37°C for 18 to 20 h, plating efficiencies were determined by comparing numbers of CFU/ml in the presence and absence of drugs.

β-Galactosidase assays. Cultures were grown and antibiotics added under the conditions used for the microarray experiments. β-Galactosidase assays were performed according to standard methods (38).

TaqMan quantitative real-time PCR. Gene expression was quantified by TaqMan quantitative real-time PCR using an Applied Biosystems 7300 real-time PCR system (Foster City, CA) at the Nucleic Acid Facility (Huck Institute, Pennsylvania State University). Primers and probes were designed for the genes of interest by using the real-time PCR probe/primer design software package Primer Express (version 2.0; Applied Biosystems). Prior to PCR, DNase-treated RNA was reverse transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Quantitative real-time PCR was performed using TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA). Primers and probes are listed in Table 2, and the probes were labeled with a 5′ 6-carboxyfluorescein reporter and a 3′ Black Hole quencher (Biosearch Tech, Novato, CA). Samples were amplified for 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Changes in expression were analyzed using ABI Prism SDS 1.2.2 software (Applied Biosystems). The *icd* (isocitrate dehydrogenase) gene was used for the reference sample, and its expression was not changed by antibiotic treatment. The changes shown are the averages for two biological replicates based on comparison with the *icd* reference gene. Similar results were obtained using *mdh* (malate dehydrogenase) as the reference gene.

Microarray data accession number. The microarray data were entered into the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE10160.

RESULTS

Identification of peptidoglycan-sensing stress responses. To identify peptidoglycan-sensing stress responses, we perturbed peptidoglycan synthesis by inhibiting specific PBPs with two β-lactam antibiotics, cefsulodin and amdinocillin (also known as mecillinam), used alone and in combination, and then monitored the resultant changes in gene expression. The antibiotic treatments had different consequences for cell morphology and viability. Individual antibiotics were added to growing cultures alone at their respective MICs (0.3 μg/ml for amdinocillin and 60 μg/ml for cefsulodin). Cefsulodin, which inhibits PBPs 1a and 1b, was bacteriolytic, causing rapid cell lysis without morphological changes (Fig. 1 and 2) (21, 51). Amdinocillin inhibits PBP 2, thereby blocking cell elongation (Fig. 1) (21, 51, 55). The amdinocillin-treated cells became round, and cell division stopped by 60 min after addition of the antibiotic, but the cells did not lyse (Fig. 2). Cells were also treated with both cefsulodin and amdinocillin simultaneously (10 μg/ml cefsulodin and 0.03 μg/ml amdinocillin). When used in combination (Cef-Amd), the antibiotics were effective at concentrations below their respective MICs and caused cell lysis after 40 min without a morphological change (Fig. 1 and 2). In contrast, similar effects on cell growth or viability were not observed when the antibiotics were added individually at these low concentrations (data not shown). This synergy has been noted previously, and the molecular basis for this effect is not fully understood (21, 51).

To focus on stress responses and minimize changes in gene expression due to cell death, samples from the treated cultures were collected immediately before the onset of lysis (10 min for cefsulodin and 40 min for Cef-Amd) or stasis (60 min for amdinocillin) and compared to samples taken from untreated cultures grown at the same time (Fig. 2). Since addition of cefsulodin and amdinocillin individually at the low concentrations did not cause lysis or stasis, samples were taken from these cultures at 40 min following antibiotic addition, the same time point used for the combination treatment. The expression levels of individual genes were determined using Affymetrix

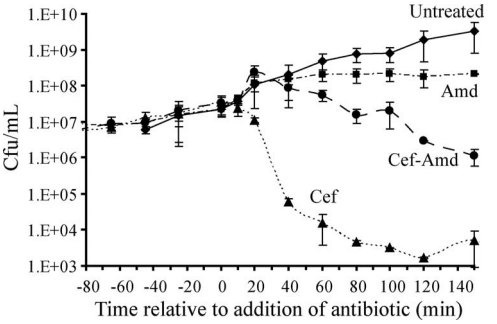

FIG. 2. Effect of antibiotics on growth of *E. coli*. Growth curves (CFU/ml as a function of time relative to antibiotic addition) are shown for Cef-Amd-treated (Cef-Amd; circles), amdinocillin-treated (Amd, squares), cefsulodin-treated (Cef, triangles), and untreated (diamonds) cultures. Overnight cultures of *E. coli* strain MG1655 (SEA113) were diluted to an OD_{600} of 0.02 in fresh LB and grown in a gyratory water bath at 37°C with shaking. When the cultures reached an OD_{600} of 0.2 (time zero), antibiotics were added to give the final concentrations indicated in the text. The numbers of CFU/ml are the averages for two replicate platings from each sample, and the error bars represent the standard deviations. Representative growth curves are shown. Samples were collected for microarray analysis immediately before growth stopped due to lysis or stasis. Cefsulodin-treated cells were collected at 10 min, Cef-Amd-treated cells were collected at 40 min, and amdinocillin-treated cells were collected at 60 min following addition of the respective antibiotics.

TABLE 3. Major stress regulons activated by antibiotic treatment

Regulator[a] and stress response type	Gene(s)
Cefsulodin	
Cell envelope	
Rcs	*osmB*, *rprA*, *ydhA*, *ymgG*
σ^E	*yiaL*
Other	
σ^S	*osmB*, *ydfO*, *ydiV*, *yfeT*, *ymgB*
CRP	*tnaC*, *yiaL*
Amdinocillin	
Cell envelope	
Rcs	*bdm*, *fcl*, *gmd*, *ivy*, *osmB*, *rprA*, *spy*, *wcaD*, *wza*, *ycfJ*, *ydhA*, *ygaC*, *yggG*, *ymgD*, *ymgG*, *ypeC*
CpxR	*cpxP*, *degP*, *spy*, *yebE*, *asnA**, *ivy**, *narG**, *osmB**, *rsxA**, *rsxB**, *ymgD**, *yncJ**
σ^E	*cca*, *degP*, *htrG*, *plsB*, *rpoE*, *rseA*, *rseB*, *rseC sbmA*, *yfeS*, *yhjJ*
BaeR	*spy*, *yiaD*, *malF*, *malG*
Other	
σ^{32}	*hslJ*
σ^S	*artJ*, *cpxP*, *ivy*, *osmB*
FNR	*fes*, *glpA*, *glpB*, *narG*, *tdcB*, *ybdZ*, *yecR*
Fur	*entA*, *entB*, *entC*, *entE*, *fepA*, *fes*, *fhuF*, *ybdB*, *ybdZ*, *ygaC*
CRP	*entA*, *entB*, *entC*, *entE*, *fepA*, *glpA*, *glpB*, *malF*, *malG*, *nanC*, *tdcB*, *uxuB*, *ybdB*
Cef-Amd	
Cell envelope	
Rcs	*bdm*, *fcl*, *galU*, *gmd*, *hha*, *ivy*, *osmB*, *osmC*, *rcsA*, *rcsB*, *rmf*, *rprA*, *spy*, *sra*, *ugd*, *wcaD*, *wcaE*, *wcaF*, *wza*, *wzc*, *yajI*, *ycfJ*, *ydhA*, *ygaC*, *ygdR*, *yggE*, *yggG*, *yjbJ*, *ymgD*, *ymgG*, *yohN*, *ypeC*, *ytfK*
CpxR	*cpxP*, *degP*, *htpX*, *spy*, *ybaJ*, *yccA*, *ycfS*, *ydeH*, *yebE*, *ivy**, *ompX**, *osmB**, *rcsA**, *rmf**, *ygaM**, *ymgD**, *yncJ**, *yqaE**
σ^E	*degP*, *fkpA*, *htrG*, *lon*
BaeR	*spy*, *yiaD*
Other	
σ^{32}	*bssS*, *clpB*, *hslJ*, *hslU*, *htpG*, *htpX*, *ibpA*, *ibpB*, *lon*
σ^S	*cpxP*, *dacC*, *dps*, *ivy*, *ompX*, *osmB*, *osmC*, *osmE*, *sra*, *sufB*, *yaeR*, *ybjP*, *yegS*, *ygaM*, *ygaU*, *ygdI*, *yggE*, *yjbJ*, *ymgB*, *yqaE*, *yftK*
FNR	*fes*, *ybdZ*
Fur	*entA*, *entB*, *entC*, *entE*, *fepA*, *fes*, *nrdH*, *nrdI*, *sufB*, *ybdB*, *ybdZ*, *ygaC*
CRP	*entA*, *entB*, *entC*, *entE*, *fepA*, *ybdB*

[a] Regulon members were derived from information in RegulonDB (http://regulondb.ccg.unam.mx/) and the literature (13, 14, 16, 22, 32, 40, 41, 44, 47, 61, 63). Many of these studies are microarray studies and do not distinguish between direct and indirect effects on regulation. Major stress pathways are shown. For a complete list of regulators, see Table S1 in the supplemental material. *, genes predicted to have a CpxR binding site but for which CpxR regulation has not been experimentally verified.

GeneChip microarrays; genes with significantly altered expression (identified using the program Significance Analysis of Microarrays [58]) are listed in Table S1 in the supplemental material. Statistically significant changes in gene expression were found for treatment with MIC levels of the individual antibiotics and the Cef-Amd combination. However, none were detected for cultures treated with cefsulodin or amdinocillin individually at the low concentrations (data not shown). To identify stress responses activated by each antibiotic treatment, significantly responding genes were classified according to membership in known stress regulons (Table 3; also see Table S1 and Fig. S1 in the supplemental material). Most responding genes belong to identified regulons. Interestingly, members of the Rcs regulon were induced by all three treatments. Only a minority of genes could not be assigned to a known regulator (see Table S1 in the supplemental material).

Cefsulodin-induced changes in gene expression. Inhibition of PBPs 1a and 1b by cefsulodin had fewer effects on gene expression and induced smaller changes than those observed for the other treatments (see Table S1 in the supplemental material). Treatment with cefsulodin increased the expression levels of only 20 genes and did not result in significantly decreased expression of any gene (Fig. 3; also see Table S1 in the supplemental material). Only the Rcs and σ^S stress responses, which regulate exopolysaccharide synthesis and the general stress response, respectively, had more than two regulon members with increased expression (Table 3; also see Fig. S1 in the supplemental material).

Amdinocillin-induced changes in gene expression. Inhibition of PBP 2 by amdinocillin activated more genes and stress responses, including members of the Cpx, σ^E, and Rcs regulons (Fig. 3 and Table 3; also see Fig. S1 and Table S1 in the supplemental material). Several genes in the FNR, Fur, and Crp regulons, stress responses not directly targeted to the cell envelope, were also activated, suggesting that the cells experienced iron limitation and oxidative stress (Table 3; also see

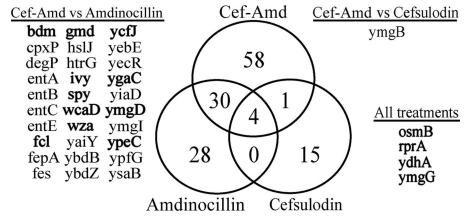

FIG. 3. Venn diagram of genes with increased expression due to inhibition of PBPs 1a and 1b by cefsulodin, PBP 2 by amdinocillin, and all three PBPs by the Cef-Amd combination. The numbers in the intersecting sections refer to the number of genes induced in common, while the numbers outside of these sections refer to the number of genes induced by that treatment alone. Genes whose expression levels were increased by two or more treatments are listed under the indicated headings (all treatments, Cef-Amd versus amdinocillin, and Cef-Amd versus cefsulodin). Genes in bold are members of the Rcs regulon.

TABLE 4. Changes in gene expression induced by antibiotic treatments

Gene	Fold change for indicated assay and treatment					
	Cef-Amd		Amdinocillin		Cefsulodin	
	qPCR	Microarray[a]	qPCR	Microarray	qPCR	Microarray
osmB	118.26	18.97	11.96	5.03	2.18	2.46
ydhA	12.87	4.08	3.46	2.50	1.41	2.04
ymgG	52.87	6.70	26.94	11.55	1.58	1.78
entC	7.96	4.10	2.66	1.75	1.02	1.03
cpxP	2.99	3.02	2.94	2.16	1.06	1.24

[a] *osmB*, *ydhA*, and *ymgG* had greater changes in expression on the Genome 2.0 arrays, comparable to those shown by TaqMan quantitative real-time PCR (qPCR), due to redesigned probes on the new arrays. Data from the antisense arrays for these genes are reported; see Materials and Methods for more information.

Fig. S1 in the supplemental material). Amdinocillin treatment decreased the expression levels of genes required for flagellar synthesis, including the "master regulators" of flagellum gene expression, *flhDC* (see Table S1 in the supplemental material). The Rcs pathway is known to inhibit transcription of the *flhDC* operon; thus, it is likely that the flagellar genes were downregulated as a result of activation of the Rcs phosphorelay and inhibition of *flhDC* expression (17).

Cef-Amd-induced changes in gene expression. Simultaneous inhibition of PBPs 1a, 1b, and 2 by Cef-Amd caused the most extensive alterations in gene expression. Many of the same stress responses were activated, including Rcs, Cpx, and Fur, but more genes in the respective regulons had increased expression upon Cef-Amd treatment than upon treatment with the individual antibiotics (Fig. 3 and Table 3; also see Fig. S1 and Table S1 in the supplemental material). In addition to the shared responses, the σ^{32} cytoplasmic stress response was specifically activated by Cef-Amd treatment (Table 3; also see Fig. S1 in the supplemental material). Recent analysis of the σ^{32} regulon suggests that it combats inner membrane stress as well as cytoplasmic stress (41). In contrast to what was found for treatment with amdinocillin alone, the σ^{E} and FNR responses were not significantly activated. Both activation of the σ^{32} response by Cef-Amd and activation of the σ^{E} response by amdinocillin but not the other treatments were verified using *lacZ* reporter fusions to promoters dependent on each sigma factor (data not shown). Cef-Amd treatment also decreased the expression levels of the genes encoding flagellar proteins, including *flhDC* (see Table S1 in the supplemental material).

Shared responses. There was significantly more overlap between the amdinocillin and Cef-Amd gene expression profiles than between the cefsulodin and Cef-Amd profiles. Only one gene, *ymgB*, was induced exclusively by cefsulodin and Cef-Amd, while 34 genes were induced in common by amdinocillin and Cef-Amd (Fig. 3). Over half of these genes were in the Rcs regulon, and many genes were among the most highly induced by either treatment (Fig. 3 and Table 3; also see Fig. S1 and Table S1 in the supplemental material). The majority of the remaining genes induced in common were in the Cpx and Fur regulons (Fig. 3 and Table 3; also see Fig. S1 in the supplemental material). The RNA levels of four genes, *rprA*, *ydhA*, *ymgG*, and *osmB*, were increased in all three experiments (Fig. 3). All four of these genes are regulated by the Rcs phosphorelay, suggesting that the Rcs system is a global response to peptidoglycan stress (5, 16, 22, 35). *osmB* is also regulated by the general stress factor σ^{S}, suggesting that σ^{S} may also have a role in a shared response to peptidoglycan stress (5, 24).

The changes in expression for the four genes whose RNA levels increased in all three experiments were confirmed using TaqMan quantitative real-time PCR for *ydhA*, *ymgG*, and *osmB* (Table 4) and using a *lacZ* reporter fusion for *rprA* (Fig. 4). The *rprA* gene is short, and suitable TaqMan probes could not be identified. Changes in gene expression for a representative gene in the Cpx regulon, *cpxP*, and one in the Fur regulon, *entC*, were also examined by real-time PCR (Table 4). In each instance, the real-time PCR results confirmed the microarray results. For the cefsulodin-treated cells, the changes in expression were consistently small, but the values were reproducible. The microarray results for *rprA* were supported as well by results from experiments with the *rprA-lacZ* fusion. Amdinocillin and Cef-Amd treatment increased *rprA-lacZ* reporter activity six- to sevenfold (Fig. 4). Cefsulodin treatment increased reporter gene expression only 1.5-fold, compared to 2.7-fold measured in the array experiments (Fig. 4). Measurements of β-galactosidase activity could underestimate the extent of activation by cefsulodin, because

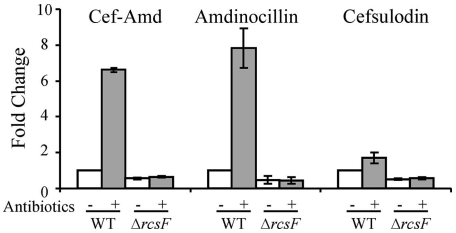

FIG. 4. Inhibition of peptidoglycan synthesis by Cef-Amd, amdinocillin, and cefsulodin activates the Rcs pathway via RcsF. WT and Δ*rcsF* strains with the $RcsB_2$-dependent *rprA-lacZ* reporter were treated with antibiotics at the concentrations used for the microarray experiments. Samples were collected at 40, 60, and 20 min following Cef-Amd, amdinocillin, and cefsulodin treatment, respectively. The change in β-galactosidase activity was determined with respect to the untreated WT culture, and average values with standard deviations for a minimum of five experiments are shown. The basal level of reporter gene activity is lower in the Δ*rcsF* strain than in the WT strain and is no longer inducible.

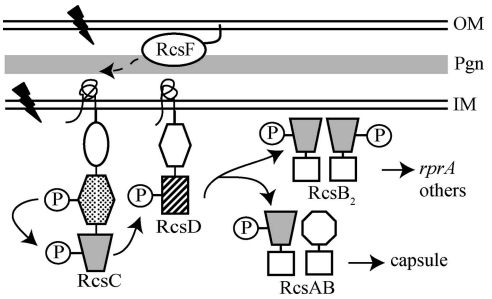

FIG. 5. The Rcs phosphorelay. Signals, indicated by lightening bolts, activate the pathway at RcsF or RcsC and initiate the phosphorelay. The direction of phosphate transfer from RcsC to RcsD to RcsB is shown. OM, outer membrane; IM inner membrane; Pgn, peptidoglycan.

rapid lysis may affect protein production. However, as noted above, induction of other Rcs regulon members (*osmB*, *ydhA*, and *ymgG*) by cefsulodin was confirmed by real-time PCR (Table 4).

The Rcs phosphorelay enhances survival in the presence of antibiotics that inhibit peptidoglycan synthesis. The results from the microarray experiments clearly point to the Rcs phosphorelay as a key sensor of the state of the peptidoglycan layer. The Rcs pathway is activated by autophosphorylation of the hybrid sensor kinase RcsC, which then phosphorylates the histidine phosphotransferase RcsD (Fig. 5). RcsD passes the phosphate moiety to the response regulator RcsB, which binds to DNA as a homodimer ($RcsB_2$) or a heterodimer with the protein RcsA (RcsAB) (Fig. 5) (33). RcsAB activates the transcription of genes required for synthesis of the colanic acid capsular exopolysaccharide (33). The $RcsB_2$ regulon is not yet fully elucidated and consists of genes unrelated to the capsule, including *rprA*, the small RNA regulator of *rpoS* (the gene encoding σ^S) translation (34, 35). Signals that activate the Rcs phosphorelay pass through either RcsC in the inner membrane or the outer membrane lipoprotein RcsF (Fig. 5) (7, 36).

If the Rcs response protects the cell from deleterious effects resulting from inhibition of peptidoglycan synthesis, then inactivation of the pathway should increase the sensitivity of the bacteria to the antibiotics. To test this hypothesis, we measured the plating efficiency of a Δ*rcsB* strain on plates containing cefsulodin (45 μg/ml), amdinocillin (0.15 μg/ml), or Cef-Amd (10 and 0.075 μg/ml, respectively). The WT strain was able to grow somewhat in the presence of these concentrations of antibiotics, although growth was reduced compared to that on LB alone. By using these concentrations of antibiotics, we were able to evaluate mutants that increased, decreased, or did not change the susceptibilities to the antibiotics. The Δ*rcsB* strain grew as well as the WT strain on LB alone but was not able to grow on medium containing any of the antibiotics, indicating that RcsB activity was indeed required for the bacteria to survive in the presence of the antibiotics (Fig. 6).

The observation that deletion of *rcsB* reduced the ability of the bacteria to survive in the presence of these antibiotics suggested that activation of the pathway might increase survival. To test this idea, we used a strain containing a constitutively active allele of *rcsC*, *rcsC137* (20, 36). Colonies formed by the *rcsC137* strain were extremely mucoid due to constitutive production of the capsule and grew better than the WT strain on amdinocillin and Cef-Amd, with plating efficiencies close to 1 (Fig. 6). Although activation of Rcs significantly increased survival on amdinocillin and Cef-Amd, it did not enhance survival on cefsulodin (Fig. 6).

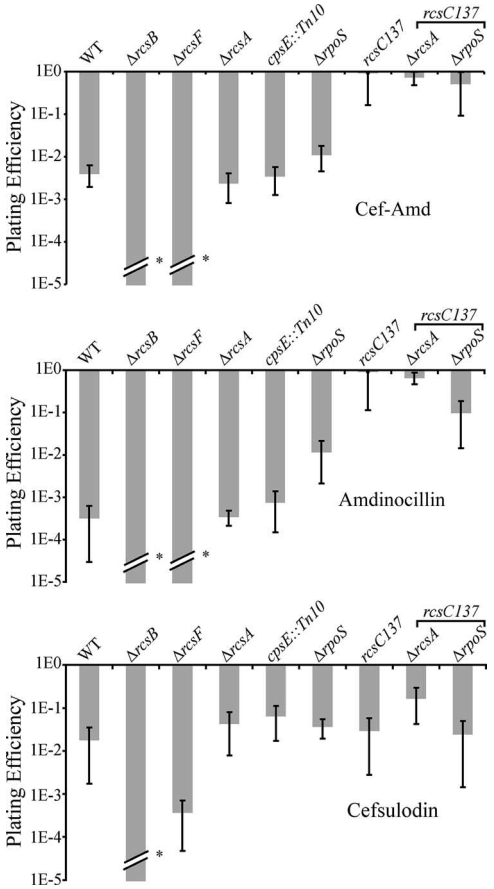

FIG. 6. The Rcs phosphorelay increases survival in the presence of antibiotics, independently of capsule synthesis and σ^S. Plating efficiencies comparing numbers of CFU/ml in the presence and absence of antibiotic are displayed. Average values and standard deviations for at least three experiments are presented. Antibiotics were used in LB agar plates at the following concentrations: 0.075 μg/ml amdinocillin and 10 μg/ml cefsulodin (top), 0.15 μg/ml amdinocillin (middle), and 45 μg/ml cefsulodin (bottom). Strains marked with an asterisk did not form colonies on plates containing antibiotics (plating efficiency = 0).

RcsA and the capsule are not required for enhanced survival in the presence of antibiotics. Increased production of the exopolysaccharide capsule due to transcriptional activation of the colanic acid biosynthetic genes by RcsAB could be responsible for survival by osmotically stabilizing the cells or reducing entry of antibiotics. Therefore, we tested the growth of Δ*rcsA* and Δ*rcsA rcsC137* strains in the presence of antibiotics. Deletion of the *rcsA* gene in either background had no effect on the plating efficiencies with any of the antibiotics (Fig. 6), even though the Δ*rcsA rcsC137* strain was no longer mucoid. In strains lacking *rcsA*, some residual capsule synthesis remains, due to activation by $RcsB_2$ (6). To determine whether the residual capsule synthesis was responsible for survival, we examined the plating efficiency of a strain with the *cpsE3*::Tn*10* allele, which disrupts the expression of several enzymes re-

TABLE 5. Changes in expression[a] induced by Cef-Amd in WT and Δ*rcsF* strains

Strain	Fold change for indicated gene			
	osmB	*ymgG*	*ydhA*	*cpxP*
WT	118.26	52.87	12.87	2.99
Δ*rcsF* strain	0.99	1.08	0.79	2.27

[a] Measurements were made using TaqMan quantitative real-time PCR.

quired for capsule synthesis (57). Even though these strains cannot produce the capsule, their plating efficiency in the presence of antibiotics was the same as that for the WT strain (Fig. 6). These results indicate that genes regulated by the $RcsB_2$ homodimer are important for resistance and eliminate RcsAB and increased colanic acid synthesis as mediators of resistance.

RcsF is required for signal transduction following inhibition of peptidoglycan synthesis. Inducers of the Rcs pathway signal through the RcsF lipoprotein in the outer membrane or RcsC in the inner membrane (Fig. 5) (7, 36). To determine which pathway transduces information about the state of the peptidoglycan, we monitored the activation of the phosphorelay by using the $RcsB_2$-regulated *rprA-lacZ* reporter fusion in a Δ*rcsF* genetic background following antibiotic treatment (Fig. 4). Reporter gene activity was not induced in the Δ*rcsF* strain by any of the antibiotics. In addition, we monitored the activation of the Rcs regulon members *osmB*, *ymgG*, and *ydhA* and the Rcs-independent gene *cpxP* in the Δ*rcsF* genetic background following Cef-Amd treatment using TaqMan quantitative real-time PCR. The *osmB*, *ymgG*, and *ydhA* genes were not induced by Cef-Amd treatment in the Δ*rcsF* strain, while the *cpxP* gene was still induced (Table 5). These results indicate that signal transduction through RcsF is required for induction of Rcs regulon members following inhibition of peptidoglycan synthesis and confirm that induction of *osmB*, *ydhA*, *ymgG*, and *rprA* is dependent on the the Rcs pathway.

If RcsF is required to transduce the signal generated by inhibition of peptidoglycan synthesis, then it should be required for survival in the presence of these antibiotics. Consistent with this hypothesis, the Δ*rcsF* strain had a phenotype similar to that of the Δ*rcsB* strain; it was not able to form colonies in the presence of amdinocillin or Cef-Amd (Fig. 6). The Δ*rcsF* mutant could grow on cefsulodin, but the plating efficiency was reduced ~30-fold compared to that of the WT strain (Fig. 6). These results indicate that signal transduction through RcsF is required to activate the phosphorelay.

σ^S is not required for survival in the presence of antibiotics. Because RprA positively regulates σ^S production, activation of the Rcs pathway could increase survival in the presence of the antibiotics via σ^S. Indeed, members of the σ^S regulon were induced by all three treatments. To test this hypothesis, we examined the plating efficiencies of both Δ*rpoS* and Δ*rpoS rcsC137* strains. If σ^S were required for resistance, then deleting the gene encoding σ^S, *rpoS*, should reduce the plating efficiencies in the presence of the antibiotics. However, the plating efficiencies of the Δ*rpoS* strains were not affected on any of the antibiotics, indicating that the Rcs pathway does not act through σ^S (Fig. 6).

DISCUSSION

E. coli possess extracytoplasmic stress responses that are activated in response to disruptions in the integrity of the inner and outer membranes and cell envelope proteins (46, 48). In this work, we demonstrate that *E. coli* also responds to disruptions in the integrity of the peptidoglycan layer resulting from inhibition of the major high-molecular-weight PBPs 1a, 1b, and 2, which catalyze the assembly of the peptidoglycan layer, and that the Rcs phosphorelay plays a major role in this response. Changes in *E. coli* gene expression have also been measured following inhibition of the essential PBP, PBP 3, by aztreonam (2) and inhibition of seven PBPs, including PBPs 1a, 1b, 2, and 3, by ampicillin (31). The expression levels of genes in the Rcs regulon also increased following aztreonam and ampicillin treatment, supporting our conclusion that the Rcs response senses the effects of peptidoglycan stress. As observed with amdinocillin, cefsulodin, and Cef-Amd, genes in the Rcs regulon were among the most highly induced and constituted a significant fraction of the induced genes. No other stress responses were activated by all five antibiotic treatments (cefsulodin, amdinocillin, Cef-Amd, aztreonam, and ampicillin).

Activation of the Rcs phosphorelay does not appear to be a nonspecific consequence of antibiotic-induced cell death or inhibition of growth. In microarray experiments examining changes in gene expression following treatment of *E. coli* with antibiotics that do not target the peptidoglycan (the protein synthesis inhibitors 4-azaleucine, mupirocin, kasugamycin, and puromycin [49] and the DNA gyrase inhibitors norfloxacin and ofloxacin [29, 31]), very few members of the Rcs regulon were induced, and those that were constituted only a small percentage of the total number of genes with increased expression. In addition, these genes were not among the most highly induced, and several are also regulated by σ^S (29, 31, 49, 61).

In addition to treatment with these β-lactam antibiotics, several other conditions that directly perturb the peptidoglycan have been noted to activate the Rcs pathway. These conditions include deletion of PBP 1b (50) and mislocalization of the AmiA and AmiC murein hydrolases to the cytoplasm due to disruption of the twin arginine transport system (28). These observations indicate that the Rcs phosphorelay likely senses stress resulting from perturbations of the peptidoglycan layer rather than sensing the antibiotics themselves. Deletion of PBP1b and the mislocalization of AmiA and AmiC are not lethal (28, 50), indicating that the Rcs pathway is not simply activated by impending cell lysis or inhibition of growth caused by disruption of the peptidoglycan.

The outer membrane lipoprotein RcsF is required to activate the Rcs pathway following inhibition of peptidoglycan synthesis. However, the nature of the inducing signal(s) and the way that RcsF in the outer membrane transmits information to RcsC in the inner membrane are not well understood for any known activator of the pathway. In general, inducers of the Rcs phosphorelay that act through RcsF perturb the outer membrane, where RcsF is localized (7, 36, 52). In addition, the Rcs pathway is activated by osmotic shock and the antimicrobial peptide polymyxin B, which disorganizes bacterial membranes, although RcsF dependence was not investigated for these inducers (15, 53). Taken together, these results suggest that the Rcs pathway is activated by downstream effects result-

ing from inhibition of peptidoglycan synthesis that perturb the envelope membranes, possibly due to alterations in the osmotic or turgor pressure. Alternatively, we cannot eliminate the possibility that the Rcs phosphorelay senses alterations in the integrity of the peptidoglycan layer directly, perhaps through interactions with peptidoglycan itself or with other outer membrane lipoproteins that bind to the peptidoglycan layer (39, 60). Regardless of the nature of the inducing signal, activation of the Rcs pathway enhances survival in the presence of the antibiotics and mutants unable to activate the pathway are more sensitive to the antibiotics.

How might the Rcs regulon enhance the ability of *E. coli* to grow in the presence of these β-lactam antibiotics? Recent observations with L-form bacteria generated by treatment of cells with high levels of cefsulodin show that cells lacking over 90% of their peptidoglycan required capsule production mediated by *rcsA* for survival (30). In contrast, enhanced survival in the presence of lower levels of antibiotics used here does not depend on *rcsA* or capsule synthesis, indicating that genes transcribed by $RcsB_2$ are responsible for the phenotype. Most of the genes in the Rcs regulon not involved in capsular synthesis are of unknown functions, although many are predicted to be associated with the cell envelope (16, 22, 28). Key candidates for genes that are important for resisting the effects of antibiotics are the genes induced by all treatments, *rprA*, *ydhA*, *osmB*, and *ymgG*. Although we demonstrated that the one known target of RprA, σ^S, was not required, RprA may have additional targets important for survival. The proteins encoded by the three other genes are each predicted to be localized to the cell envelope, but little is known about their functions. Interestingly, recent studies with *Salmonella enterica* showed that RcsB but not RcsA was required for survival following polymyxin B treatment, similar to the results that we obtained with β-lactam antibiotics (15).

Another hypothesis for how the Rcs pathway protects the cell from killing by the antibiotics is that the Rcs pathway strengthens the outer membrane permeability barrier, preventing entry of the antibiotics into the cell. However, the Δ*rcsB* and Δ*rcsF* strains, which were more sensitive to the β-lactam antibiotics used here, do not have permeability defects. These strains did not show increased sensitivity to small molecules, such as rifampin, detergents, and bile salts, which are more toxic for cells with increased permeability (data not shown).

The experiments described in this paper were designed to identify stress responses that sense and respond to the effects of stress in the peptidoglycan layer. The antibiotics were used at MIC concentrations (or near for the combination) and resulted in significantly different outcomes for the cells, providing us with the opportunity to detect a potentially diverse array of changes in gene expression. Since stress responses have particular inducing signals (48), many of which are known, activation of specific stress responses provides information about the types of stress caused by inhibition of the PBPs in question. Comparison of the changes in gene expression elicited by the different treatments demonstrates that inhibiting peptidoglycan synthesis results in both shared and distinct effects on cellular physiology, depending on which enzymes are inhibited and/or what the time course of inhibition is.

Interestingly, the responses were not correlated with the ultimate outcome for the cell, lysis versus stasis. Amdinocillin is bactericidal at high concentrations (59), but in our studies, amdinocillin was used at a concentration that was bacteriostatic, while cefsulodin and Cef-Amd were bacteriolytic. Amdinocillin, rather than cefsulodin, altered more genes in common with Cef-Amd. This result suggests that both treatments cause similar alterations in physiology, even though Cef-Amd-treated cells ultimately lyse while amdinocillin-treated cells do not. The primary overlapping sets of genes for the amdinocillin and Cef-Amd treatments were members of the Rcs, Cpx, and Fur regulons. We noticed that Cef-Amd consistently induced more members of these regulons than amdinocillin. The basis for this difference is not known. Genes can be activated to different extents due to variations in the activator binding sites upstream of individual genes and/or to interactions with other regulators. For example, a number of the Rcs regulon members activated by Cef-Amd but not amdinocillin are also in the σ^S regulon (61) and could be induced by contributions from σ^S (Table 3; also see Fig. S1 in the supplemental material).

The observation that cefsulodin did not activate stress responses to the same extent as the other treatments indicates that inhibiting PBPs 1a and 1b, such that rapid lysis ensues, has markedly different effects on physiology. We cannot distinguish whether the differences in gene expression between cefsulodin and the other treatments are due to inhibition of PBPs 1a and 1b specifically or would be observed if other PBPs were inhibited such that rapid lysis ensued. Nevertheless, it is clear that rapid lysis results in different changes in gene expression compared with lysis that occurs after a more extended period of growth in the presence of antibiotics. Additional experiments examining the changes in gene expression using a lower concentration of cefsulodin, which results in lysis after two or three generations of growth, might help to address this issue.

Inhibition of PBP 3 has been shown to activate the DpiAB two-component system, which in turn activates the SOS response and *sulA*, an inhibitor of cell division that blocks septal ring formation by FtsZ (37). This mechanism has been hypothesized to contribute to persistence in the presence of antibiotics by halting cell growth (37). The *dpiAB* genes and SOS regulon members did not have increased expression in our experiments, consistent with earlier results indicating that this response is specific for inhibition of PBP 3 (37). These results indicate that inhibition of PBP 3 generates a unique signal not produced by inhibition of PBP 1a, 1b, or 2. The DpiAB response provides a mechanism for halting septal ring formation when PBP 3 is inactivated and cannot direct septal peptidoglycan synthesis. Similarly, inhibition of PBP 2 specifically induced the σ^E envelope stress response, suggesting that σ^E might also mediate a protective response.

Another notable finding is that *E. coli* does not appear to possess a feedback response for altering the expression levels of genes involved in cell growth and division to compensate for or adapt to inhibition of PBPs 1a, 1b, and 2. In fact, the only PBP observed in the array data was *dacC*, encoding the nonessential protein PBP 6, which had a slight increase in gene expression due to treatment with Cef-Amd (see Table S1 in the supplemental material). Instead, the transcriptional responses are directed at coping with the downstream effects caused by stresses resulting from inhibition of peptidoglycan biosynthesis. If such feedback mechanisms do exist, they act at posttran-

scriptional levels that cannot be detected by microarray analyses or are mediated by genes of unknown functions.

In addition to the Rcs pathway, several other stress responses were activated by multiple treatments. One of these is the Cpx cell envelope stress response pathway. Members of the Cpx regulon had increased expression following addition of amdinocillin, Cef-Amd, and ampicillin but not aztreonam or cefsulodin. Interestingly, this pathway can also be activated by an outer membrane lipoprotein, NlpE (10, 43, 54). We do not yet know whether NlpE is required for sensing the effects of antibiotics. Preliminary results show that Δ*cpxAR* strains, lacking this sensory module, are more sensitive to amdinocillin and Cef-Amd.

Fundamental cell envelope stress responses, such as the Rcs pathway, are unlikely to have evolved specifically to combat damage to the peptidoglycan layer caused by β-lactam antibiotics and do not confer dramatically increased resistance like inducible β-lactamase systems, which produce an enzyme that hydrolyzes β-lactam antibiotics. The natural role of the Rcs response and those like it in combating peptidoglycan stress is likely to be in response to stresses that occur stochastically as a function of normal growth during construction of the peptidoglycan layer. The Rcs pathway, which is conserved in a variety of enteric pathogens, can protect the bacterium from damage to other components of the cell envelope as well, and we do not yet know if these functions are distinct or if similar signals are sensed (15, 33). While the Rcs response does not confer dramatically increased drug resistance, it does enhance growth in the presence of concentrations of the drug near or below the MIC, increasing the amount of drug required to kill the bacteria. During antibiotic treatment of bacterial infections, intrinsic peptidoglycan stress-sensing responses like the Rcs pathway may allow the bacteria to survive longer in the presence of the antibiotic or in a niche in the body where the antibiotic concentrations do not reach maximal levels. Therefore, these responses serve important functions that have the potential to significantly impact antibiotic chemotherapy and bacterial drug resistance.

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