Single-Gene Deletion Mutants of *Escherichia coli* with Altered Sensitivity to Bicyclomycin, an Inhibitor of Transcription Termination Factor Rho[▽]

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We have screened the entire KEIO collection of 3,985 single-gene knockouts in *Escherichia coli* for increased susceptibility or resistance to the antibiotic bicyclomycin (BCM), a potent inhibitor of the transcription termination factor Rho. We also compared the results to those of a recent study we conducted with a large set of antibiotics (A. Liu et al., *Antimicrob. Agents Chemother.* 54:1393-1403, 2010). We find that deletions of many different types of genes increase sensitivity to BCM. Some of these are involved in multidrug sensitivity/resistance, whereas others are specific for BCM. Mutations in a number of DNA recombination and repair genes increase BCM sensitivity, indicating that DNA damage leading to single- and double-strand breaks is a downstream effect of Rho inhibition. MDS42, which is deleted for all cryptic prophages and insertion elements (G. Posfai et al., *Science* 312:1044-1046, 2006), or W3102 deleted for the *rac* prophage-encoded *kil* gene, are partially resistant to BCM (C. J. Cardinale et al., *Science* 230:935-938, 2008). Deletion of cryptic prophages also overcomes the increased BCM sensitivity in some but not all mutants examined here. Deletion of the *hns* gene renders the cell more sensitive to BCM even in the Δ *kil* or MDS42 background. This suggests that BCM activates additional modes of cell death independent of Kil and that these could provide a target to potentiate BCM killing.

In *Escherichia coli*, the ATP-dependent RNA-DNA helicase Rho (2, 31) terminates transcription at numerous sites (5, 10, 34). A recent study (4) revealed that critical Rho-dependent *E. coli* terminators are found in the cryptic prophages. Thus, Rho, aided by its cofactors NusA and NusG, is required to terminate transcription upstream of toxic foreign genes. Rho is the target for the antibiotic bicyclomycin (BCM) that binds specifically to Rho and inhibits its action (21, 39). BCM enhances transcription of horizontally transferred genes, including the various cryptic prophage genes that are otherwise silenced (4). One of these, the *kil* gene of the cryptic *rac* prophage, is lethal to the cell when expressed (18) and represents a major cause of cell death upon Rho inactivation. Cells lacking the *kil* gene are still sensitive to BCM but only at higher concentrations than that for the wild type (4).

What other pathways render cells sensitive to Rho inactivation? Previous work identified a number of recombinational repair functions that sensitized cells to *rho* mutations (17). We recently showed that Rho prevents replication fork collapse caused by transcription overrunning replication, resulting in DNA double-strand breaks (36). We wished to determine if other functions were involved in protecting the cell from BCM (intrinsic resistance). We therefore screened the KEIO collec-

tion (1) of 3,985 individual *E. coli* gene deletion mutants to identify mutants hypersensitive to BCM. All KEIO deletions were deliberately constructed so that the *kan* promoter is directed downstream and the *kan* gene is followed by no known terminator. This eliminates effects from transcriptional polarity, although translational polarity may, in some cases, still be possible. These mutants were compared with those in a similar study we carried out with 22 other antibiotics (23). We show that many different types of functions protect against Rho inactivation and that mutants lacking some of these functions sensitize the cell to BCM even in the absence of Kil function and other cryptic prophage genes.

MATERIALS AND METHODS

Synergy experiments. BW25113 (11) was used in minimal medium A containing glucose (27). Approximately 10⁵ cells from a growing culture were inoculated into 5 ml of medium and grown overnight. The following antibiotics and the corresponding target concentrations were used: bicyclomycin (BCM), 9 μ g/ml; cephadrine (RAD), 11.5 μ g/ml; ciprofloxacin (CIP), 4 ng/ml; nitrofurantoin (NIT), 0.6 μ g/ml; chloramphenicol (CHL), 0.5 μ g/ml; tobramycin (TOB), 0.4 μ g/ml; triclosan (TRI), 25 ng/ml; fusidic acid (FUS), 110 μ g/ml; erythromycin (ERY), 5 μ g/ml; and neomycin (NEO), 1.5 μ g/ml. These target concentrations resulted in 60 to 80% growth compared with the growth in the absence of antibiotic in liquid culture. Four sets of triplicate cultures were prepared for each experiment: a minimal glucose medium control with no antibiotic, BCM alone, the antibiotic alone, and the combination of BCM with the antibiotic. The solvents for the stock solutions that were then diluted to prepare the final cultures were *N,N*-dimethylformamide for NIT and ethanol for CAM, ERY, and TRI. The control for these compounds also contained the same volumes of the respective solvent and the antibiotic used. Samples were analyzed using a Turner SP-830 spectrophotometer at a wavelength of 600 nm, using minimal medium A

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containing glucose as a blank. Samples with an optical density greater than 0.500 were diluted 1:4 in minimal glucose. Percent growth was determined by setting respective controls to 100% growth and determining the percent growth of the average of each set of triplicates. An interaction is defined as additive when the percent growth of the antibiotic pair is equal to the product of the two percent growth values for each antibiotic alone (37), here defined as the predicted additive effect (PAE). When the antibiotic pair has a percent growth significantly lower than the predictive additive effect, this is defined as a synergistic interaction.

High-throughput screening method. For further details, see the report by Liu et al. (23). Briefly, cultures from the KEIO collection, maintained on 45 96-well microtiter plates, were taken out of frozen glycerol stored at -80°C using the Deutz cryoreplicator (14), transferred to sterile microtiter wells containing 0.5 ml of Luria broth (LB), and incubated at 37°C overnight. Approximately 3 to 5 μl of the resulting saturated culture was transferred to microtiter wells with fresh LB medium containing 50 $\mu\text{g}/\text{ml}$ kanamycin to prevent the growth of contaminants (all strains taken from the KEIO collection are Kan^r). After 3 h of growth, the Deutz cryoreplicator was used to print microdrops of the subculture onto LB agar plates (with no kanamycin) containing various concentrations of BCM and incubated at 37°C overnight.

Construction of double mutants. The following strains (H. Mori and B. Warner, unpublished data) were used as P1 donors to transduce mutants (e.g., the *kil* mutant) from the KEIO collection (1) or strain MDS42 (30) chloramphenicol resistance: the *trxB::cat*, *fabF::cat*, *hns::cat*, *ydfA::cat*, *ydhT::cat*, *recA::cat*, *argO::cat*, and *dam::cat* strains. Each strain contains a chloramphenicol-resistant marker that replaces the deleted gene region. The methodology used is as described by Miller (27).

Chemicals. Chloramphenicol, nitrofurantoin, neomycin, tobramycin, erythromycin, fusidic acid, and triclosan were purchased from Sigma (St. Louis, MO). Ciprofloxacin was purchased from ICN Biomedicals, Inc. (Aurora, OH).

Screening for increased sensitivity to BCM. The entire KEIO collection of almost 4,000 single-gene knockout mutants was screened using the high-throughput method described above against three subinhibitory concentrations of BCM: 20, 23, and 25 $\mu\text{g}/\text{ml}$. The MIC of BCM for the starting strain (BW25113) (11) under these conditions is 30 $\mu\text{g}/\text{ml}$. Approximately 4×10^5 exponentially growing cells were spotted onto LB agar plates containing either 20, 23, or 25 $\mu\text{g}/\text{ml}$ BCM and allowed to grow overnight. Mutants displaying inhibited growth at any of the subinhibitory concentrations tested were determined to have increased susceptibility to BCM. All hypersensitive candidates were then repurified and retested using the same methodology.

Screening for increased resistance to BCM. The entire KEIO collection was also screened for mutants with increased resistance to BCM compared to that of the wild type. The KEIO collection was screened against three higher levels of BCM: 40, 45, and 50 $\mu\text{g}/\text{ml}$. All resistant candidates were then purified from single colonies and retested using the same methodology.

RESULTS

Screening for BCM hypersensitivity. The entire KEIO collection of 3,985 strains (1) was screened for mutants that are more sensitive than the wild type to 25 $\mu\text{g}/\text{ml}$ BCM, using a Deutz cryoreplicator (14) and 96-well plates (see Materials and Methods for details). Mutants showing increased susceptibility were then purified and retested against a series of BCM concentrations, yielding a set of 76 strains with significantly enhanced susceptibility to BCM. Figure 1 displays examples of the data. Figure 2 shows all of the mutants with increased BCM sensitivity. Colors and numbers indicate the different functions lacking in each mutant (see the legend to Fig. 2). The strongest sensitivities are indicated in darker colors. Quantitative values are provided in Table 1. Whereas Rho is the primary target of BCM (39), many different functions provide intrinsic resistance to BCM, including those concerned with DNA replication, recombination, and repair, as well as functions involved in cell wall and cell membrane synthesis, chaperoning, protein synthesis, and general metabolism. In addition, several genes encoding transcriptional regulators suppress BCM sensitivity. Figure 2 also summarizes the susceptibilities of the BCM-

FIG. 1. Sensitivity analysis of bicyclomycin (BCM)-treated *Escherichia coli* gene knockout mutants. (A) Effects of 25 $\mu\text{g}/\text{ml}$ BCM on *E. coli* growth. Ninety-six mutants from the KEIO collection were printed on LB agar plates with (right plate) and without (left plate) BCM. Two mutant strains on this plate, the *fabF* and *trxB* mutants, fail to grow on the plate containing 25 $\mu\text{g}/\text{ml}$ BCM. (B) Differences in susceptibility to BCM among several single-gene knockout mutant strains compared against wild-type BW25113 *E. coli* strain.

sensitive mutants to a set of other antibiotics (23) to identify those with reduced intrinsic resistance to multiple drugs (e.g., the *tolC*, *rimK*, *pgmB*, and *yciT* mutants, among others) or those with a more specific pattern of resistance, such as *trxA* and *trxB*, which are hypersensitive only to BCM and rifampin (RIF). Ten of the 83 mutants (18%), shown in bold type, display enhanced susceptibility only to BCM.

Effect of deleting cryptic prophage genes. BCM inhibition of Rho induces transcription read-through of many genes, including the *kil* gene of the cryptic *rac* prophage. *Kil* expression largely accounts for BCM sensitivity. Deletion of *kil* or of *kil* with all insertion elements and cryptic prophages (strain MDS42) yields significant resistance to BCM (4). We asked whether any of the BCM-sensitive mutants would be suppressed by deletions of cryptic prophages. Figure 3A confirms that deletion of *kil* increases resistance to BCM in otherwise wild-type *E. coli*. It also reveals that MDS42 is more BCM resistant than a strain deleted of *kil* alone. Figure 3B through D detail three mutations that increase wild-type *E. coli* susceptibility to BCM. With *argO* (Fig. 3B), both *kil* and the multiple deletions in MDS42 suppress the increased sensitivity and restore resistance to wild-type levels. On the other hand, *hns* strains remain sensitive to BCM in a *kil* or an MDS42 background (Fig. 3C). Interestingly, the BCM sensitivity of a *recA* strain is partially suppressed in an MDS42 background but not in a strain bearing only a *kil* deletion (Fig. 3D). We found that the *recA* mutant showed sensitivity in MG1655 (data not shown), which is isogenic with MDS42. Our interpretation is that the cryptic prophages contribute to the increased BCM sensitivity in *recA* strains.

	Gene	BCM	MDS	Categ	Gene Product Description
DNA replication, recombination, and repair	<i>dam</i>			1	DNA adenine methylase
	<i>recA</i>			1	DNA strand exchange and recombination protein
	<i>recB</i>			1	DNA helicase, dsDNA/ssDNA exonuclease
	<i>recC</i>			1	DNA helicase, dsDNA/ssDNA exonuclease
	<i>recF</i>			1	ssDNA and dsDNA binding, subunit of RecFOR complex
	<i>recO</i>			1	DNA repair protein; subunit of RecFOR complex
	<i>recR</i>			1	Recombination and repair protein, subunit of RecFOR complex
	<i>recG</i>			1	DNA helicase, resolution of Holliday junctions
	<i>recJ</i>			1	Single-stranded-DNA-specific exonuclease recJ
	<i>recN</i>			1	Recombination and repair protein
	<i>uvrC</i>			1	Excinuclease ABC, subunit C; UV damage repair
	<i>uvrD</i>			1	DNA-dependent ATPase I and helicase II
	<i>xseA</i>			1	Exodeoxyribonuclease VII large subunit
	yebG			1	conserved protein; DNA repair
	yjiY			1	CP4-57 prophage; predicted DNA repair protein
	rdgC			1	Replication fork repair protein
	<i>trxA</i>			1A	Thioredoxin I
	<i>trxB</i>			1A	Thioredoxin reductase
Transport, efflux, cell wall/membrane synthesis	<i>minC</i>			1A, 2	Septum site determining protein; cell division inhibitor
	<i>acrA</i>			2	AcrAB-TolC Multidrug Efflux transport, periplasmic lipoprotein subunit
	<i>tolC</i>			2	AcrAB-TolC Multidrug Efflux transport, outer membrane subunit
	<i>argO</i>			2	Arginine outward transport
	<i>envC</i>			2	Cytokinesis - murein hydrolase
	<i>csgG</i>			2	Putative curli production protein
	<i>ddlB</i>			2	D-alanine-D-alanine ligase, B subunit
	<i>dedD</i>			2	Putative lipoprotein - inner membrane
	nhaA			2	Sodium/proton transporter
	<i>nlpC</i>			2	NlpC-putative lipoprotein hydrolase
	<i>pal</i>			2	Peptidoglycan-associated lipoprotein precursor.
	pepN			2	Aminopeptidase N
	<i>pgpB</i>			2	Phosphatidylglycerophosphatase B
	<i>tatB</i>			2	Sec-independent protein translocase
	<i>tatC</i>			2	Sec-independent protein translocase
	<i>ybjO</i>			2	Predicted inner membrane protein.
	yciB			2	Probable intracellular septation protein.
	<i>ydcS</i>			2	Putative ABC transporter periplasmic binding protein
	<i>ydcX</i>			2	Predicted inner membrane protein.
Chaperones	<i>degP</i>			2A	Periplasmic serine protease and chaperone
	<i>dnaK</i>			2A	Chaperone protein Hsp70; DNA biosynthesis and heat shock
	<i>hscA</i>			2A	Chaperone, member of Hsp70 protein family
	<i>lon</i>			2A	DNA-binding, ATP-dependent protease
	<i>surA</i>			2A	Peptidyl-prolyl cis-trans isomerase and chaperone
	<i>hflK</i>			3	Regulator of FtsH protease
	rimI			3	Ribosomal-protein-alanine acetyltransferase
	<i>rimK</i>			3	Ribosomal protein S6 modification protein.
	<i>rplA</i>			3	50S ribosomal protein L1.
	<i>rpmG</i>			3	Subunit of 50S ribosomal subunit
Protein synthesis	rpmI			3	50S ribosomal subunit protein L35
	<i>rpmJ</i>			3	50S ribosomal protein L36
	<i>rpsF</i>			3	30S ribosomal protein S6.
	<i>penB</i>			3A	Poly(A) polymerase (Plasmid copy number protein).
	<i>rlmE</i>			3A	50S ribosomal protein L36
	<i>rppH</i>			3A	RNA pyrophosphohydrolase
	<i>cis</i>			4	Cardiolipin synthetase
	<i>dapF</i>			4	Diaminopimelate epimerase
	<i>fabF</i>			4	β -ketoacyl-ACP synthase
	<i>gntY</i>			4	Iron-sulfur cluster scaffold protein
Central Metabolic Reactions	<i>gpmM</i>			4	Phosphoglycerate mutase, cofactor independent
	<i>iscS</i>			4	Cysteine desulfurase monomer
	<i>ycjU</i>			4	β -phosphoglucosyltransferase
	<i>ygcO</i>			4	Predicted 4Fe-4S cluster-containing protein
	<i>zwf</i>			4	Glucose-6-phosphate 1-dehydrogenase.
	<i>cedA</i>			5	Cell division modulator
	<i>fur</i>			5	Ferric uptake regulation protein.
	<i>hns</i>			5	H-NS transcriptional dual regulator
	<i>phoP</i>			5	Transcriptional regulatory protein
	<i>yciT</i>			5	DNA-binding transcriptional regulator (deoT)
Prophage Regulation	<i>rzpQ</i>			6	Qin prophage; predicted protein
	<i>ydfA</i>			6	Qin prophage; predicted protein
	<i>ymlT</i>			6	e14 prophage; predicted DNA transcriptional regulator
	bdm			6	Biofilm-dependent modulation protein
	<i>ybaB</i>			7	Hypothetical protein ybaB
	<i>yciM</i>			7	Conserved protein yciM
	<i>ydhT</i>			7	Hypothetical protein ydhT
	ydjO			7	Predicted protein ydjO
	<i>yebV</i>			7	Predicted protein yebV

FIG. 2. Sensitivity profile for bicyclomycin (BCM). This sensitivity profile displays 76 single-gene knockout strains with increased sensitivity to BCM and their corresponding, collective sensitivity to 22 other antibiotics (MDS). These strains are organized by gene category, indicated by different colors: 1 (red), DNA replication, recombination, and repair; 1A (red), functions indirectly affecting category 1; 2 (green), transport, efflux, cell wall and cell membrane synthesis; 2A (teal), chaperones and functions related to category 2; 3 (orange), protein synthesis; 3A (orange), RNA processing; 4 (blue), central metabolic reactions; 5 (purple), regulation; 6 (yellow), prophage-encoded functions, cell adhesion; 7 (black), unassigned genes. Sensitivity to BCM is indicated with one of four levels of intensity: darker shades indicate stronger susceptibilities, lighter shades indicate medium susceptibilities, the lightest shades indicate medium-weak susceptibilities, and the lightest, halved shades indicate the weakest susceptibilities. The genes indicated in bold are uniquely sensitive to BCM. MDS indicates the degree of multidrug sensitivity of each BCM-sensitive strain. Darker brown shades indicate sensitivity to 8 or more other antibiotics, medium brown shades indicate sensitivity to 4 to 7 other antibiotics, light brown shades indicate sensitivity to 1 to 4 other antibiotics, and no shading indicates unique sensitivity to BCM. dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; ACP, acyl carrier protein.

TABLE 1. Bicyclomycin sensitivity of single mutants listed by level of susceptibility^a

Strain	MIC (μ g/ml)
BW25113.....	30
<i>fabF</i> mutant.....	≤ 20
<i>envC</i> mutant.....	≤ 20
<i>hns</i> mutant.....	≤ 20
<i>yciM</i> mutant.....	≤ 20
<i>yciB</i> mutant.....	20
<i>dnaK</i> mutant.....	20
<i>rimK</i> mutant.....	20
<i>ymfT</i> mutant.....	20
<i>rzpQ</i> mutant.....	20
<i>ydfA</i> mutant.....	≤ 23
<i>hscA</i> mutant.....	≤ 23
<i>rplA</i> mutant.....	≤ 23
<i>recO</i> mutant.....	≤ 23
<i>recA</i> mutant.....	≤ 23
<i>trxA</i> mutant.....	≤ 23
<i>trxB</i> mutant.....	≤ 23
<i>dapF</i> mutant.....	≤ 23
<i>yciT</i> mutant.....	≤ 23
<i>rlmE</i> mutant.....	≤ 23
<i>gpmM</i> mutant.....	≤ 23
<i>degP</i> mutant.....	≤ 23
<i>yebV</i> mutant.....	≤ 23
<i>minC</i> mutant.....	23
<i>surA</i> mutant.....	23
<i>lon</i> mutant.....	23
<i>rpsF</i> mutant.....	23
<i>pgpB</i> mutant.....	23
<i>rdgC</i> mutant.....	23
<i>ycjU</i> mutant.....	23
<i>ybaB</i> mutant.....	23
<i>csgG</i> mutant.....	23
<i>iscS</i> mutant.....	23
<i>ydhT</i> mutant.....	23
<i>tatC</i> mutant.....	≤ 25
<i>fur</i> mutant.....	≤ 25
<i>ydcX</i> mutant.....	≤ 25
<i>recJ</i> mutant.....	≤ 25
<i>uvrD</i> mutant.....	≤ 25
<i>pcnB</i> mutant.....	≤ 25
<i>tatB</i> mutant.....	≤ 25
<i>yffY</i> mutant.....	≤ 25
<i>ydcS</i> mutant.....	≤ 25
<i>phoP</i> mutant.....	≤ 25
<i>argO</i> mutant.....	25
<i>tolC</i> mutant.....	25
<i>pal</i> mutant.....	25
<i>cedA</i> mutant.....	25
<i>hflK</i> mutant.....	25
<i>ygcO</i> mutant.....	25
<i>yebG</i> mutant.....	25
<i>ybjO</i> mutant.....	25
<i>rpmI</i> mutant.....	25
<i>rimI</i> mutant.....	25
<i>dam</i> mutant.....	25
<i>nlpC</i> mutant.....	25
<i>xseA</i> mutant.....	> 25
<i>ddlB</i> mutant.....	> 25
<i>recN</i> mutant.....	> 25
<i>nhaA</i> mutant.....	> 25
<i>pepN</i> mutant.....	> 25
<i>gntY</i> mutant.....	> 25
<i>rpmJ</i> mutant.....	> 25
<i>rppH</i> mutant.....	> 25
<i>cis</i> mutant.....	> 25
<i>bdm</i> mutant.....	> 25
<i>ydjO</i> mutant.....	> 25
<i>rpmG</i> mutant.....	> 25
<i>zwf</i> mutant.....	> 25
<i>dedD</i> mutant.....	> 25
<i>recB</i> mutant.....	> 25
<i>recC</i> mutant.....	> 25
<i>recF</i> mutant.....	> 25
<i>recG</i> mutant.....	> 25
<i>recR</i> mutant.....	> 25
<i>uvrC</i> mutant.....	> 25
<i>acrA</i> mutant.....	> 25

^a Gene knockouts are from the KEIO collection, using BW25113 as the start-in strain. Strains listed in bold are uniquely sensitive to bicyclomycin.**Single-gene knockouts that increased resistance to BCM.**

We also screened the entire KEIO collection for mutants more resistant to BCM than the wild type. Table 2 lists mutants that are resistant to BCM concentrations of 40 μ g/ml or higher. Again, mutants defective in many types of functions are involved. Of particular note are mutants deleting the *e14* prophage and the *hfq* gene (see Discussion). The cutoff here of 40 μ g/ml is too high to detect the *kil* deletion in this collection, as this mutant does not grow above 32 μ g/ml BCM (see Fig. 3).

Pairwise interactions with other drugs. We also examined a number of pairwise interactions as described by Yeh et al. (37). Table 3 shows the results of testing subinhibitory concentrations of BCM in minimal medium with subinhibitory concentrations of nine different antibiotics. Only the two aminoglycosides tested (NEO, TOB) were synergistic with BCM.

DISCUSSION

We employed the antibiotic bicyclomycin (BCM), a potent, specific inhibitor of Rho (39), to identify *E. coli* mutants with changes in sensitivity to the drug. Cardinale et al. (4) used microarrays to show that BCM treatment preferentially increases the expression of genes derived from recent horizontal transfer, including cryptic prophage as well as noncoding intergenic regions. A major cause of death upon Rho inactivation is expression of the *kil* gene of the *rac* cryptic prophage, whose product, KilR, inhibits the essential cell division function FtsZ (7). Cells deleted of *kil* were relatively resistant to BCM, despite massive gene dysregulation due to read-through of terminators at the ends of operons. Our screen of a set of *E. coli* deletion mutants with altered BCM resistance (Fig. 1) defines the genes involved in intrinsic resistance to BCM and also provides information on the type of downstream events that occur after inactivation of Rho by BCM. Some of the gene deletions identified in Fig. 1 that increase sensitivity to BCM are involved in general intrinsic resistance to multiple antibiotics, as indicated by the intensity of color in the MDS (multidrug sensitivity) column. These include genes involved in recombination and recombinational repair of double-strand breaks (*recA*, *-B*, and *-C*), the main efflux pump in *E. coli* (*acrA*, *tolC*), genes involved in cell wall and cell membrane synthesis and integrity, and transporters and chaperones. This also includes several genes encoding ribosomal proteins (*rplA*, *rrmJ*) or their modification (*rimK*), a variety of transcriptional regulators (*fur*, *hns*, *yciT*), and genes with uncharacterized functions.

Recent work identified a number of recombinational repair functions that support resistance to BCM. It is proposed that Rho prevents replication fork collapse and double-strand breaks caused by replisome/RNA polymerase (RNAP) collisions (36). The numerous DNA recombination and repair functions in Fig. 1 support these findings. The *recA*, *-B*, and *-C* system repairs double-strand breaks, and the *recFORJ* system is involved in the repair of single-strand breaks (6, 24, 33). Mutants lacking the latter system increase sensitivity to only a select group of antibiotics, namely, those that damage DNA. Similarly, the recovery of *uvrC*, *uvrD*, and *xseA* in mutants (Fig. 1) further supports the idea that BCM treatment ultimately leads to DNA damage that is normally prevented by Rho. Interestingly, BCM does not induce the SOS system (36). We

FIG. 3. BCM sensitivity of mutant strains lacking *rac* and other cryptic prophages. (A) Multiply deleted MDS42 strain and Δkil strain tolerate higher concentrations of BCM than the wild type. (B) MDS42 and Δkil background counteract increased sensitivity caused by deletion in *argO* and restore wild-type resistance to BCM. (C) Neither MDS42 nor Δkil background can counteract increased sensitivity to BCM caused by deletion of *hns*. (D) MDS42 background counteracts increased sensitivity to BCM caused by deletion of *recA*, but Δkil background does not.

TABLE 2. Bicyclomycin-resistant single mutants^a

Strain	MIC ($\mu\text{g/ml}$)	Gene production description
BW25113	30	
<i>hfq</i> mutant	>50	RNA chaperone facilitating sRNA-mRNA pairing interactions
<i>acs</i> mutant	50	Acetyl-coenzyme A synthetase (AMP forming)
<i>proW</i> mutant	50	Proline ABC transporter
<i>rseB</i> mutant	50	Anti-sigma factor
<i>efeB</i> mutant	50	Peroxidase; cryptic ferrous iron transporter
<i>trmU</i> mutant	50	tRNA methyltransferase
<i>flhG</i> mutant	50	Flagellar motor switch protein
<i>flgI</i> mutant	50	Flagellar P-ring protein
<i>hinT</i> mutant	50	Purine nucleoside phosphoramidase
<i>ynfA</i> mutant	50	Inner membrane protein
<i>adhE</i> mutant	45	Alcohol/acetalddehyde dehydrogenase
<i>caiA</i> mutant	45	Crotonobetainyl-coenzyme A reductase
<i>ydjE</i> mutant	45	MFS transporter
<i>rhlE</i> mutant	45	ATP-dependent RNA helicase
<i>galE</i> mutant	45	UDP-glucose 4-epimerase
<i>yciU</i> mutant	45	Predicted protein
<i>yciS</i> mutant	45	Conserved inner membrane protein
<i>gloB</i> mutant	40	Glyoxalase II
<i>hybD</i> mutant	40	Maturation peptidase for hydrogenase 2
<i>yneJ</i> mutant	40	Predicted DNA-binding transcriptional regulator, LysR type
<i>greA</i> mutant	40	Transcription elongation factor
<i>lysS</i> mutant	40	Lysyl-tRNA synthetase
<i>ydgA</i> mutant	40	Conserved membrane protein
<i>ygdD</i> mutant	40	Conserved inner membrane protein
<i>ymfJ</i> mutant	40	e14 prophage
<i>seqA</i> mutant	40	Negative modulator of replication initiation
<i>poxA</i> mutant	40	Putative regulator of pyruvate oxidase

^a Gene knockouts are from the KEIO collection, using BW25113 as the start strain.

(36) have physically detected chromosomal breaks in cells treated with BCM. This underscores the complexities of antibiotic action (see, e.g., reference 19). Of particular interest are mutants that are specifically sensitive to BCM and not to any of the other antibiotics. Three of these appear to involve DNA repair proteins, the *rdgC*, *yebG*, and *yfjY* mutants. The ring-structured RdgC protein binds DNA and is associated with recombination and replication fork repair (3). Because it inhibits RecA-mediated DNA strand exchange reactions (15), it is considered to be a negative regulator of RecA action. Mahdi et al. (25) proposed that RecA drives the reversal of collapsed forks, rendering cells more dependent upon recombination to reestablish replication. In Fig. 1, we can see specific sensitivities within each category. For example, mutants lacking the thioredoxin/thioredoxin reductase system (the *trxA* and *trxB* mutants) (13) are specifically sensitive to BCM and RIF, and two mutants defective in septation, the *minC* and *yciB* mutants, are partially or completely specific for BCM. Mutants lacking the 50S ribosomal protein L36 (*rpmI* mutant) or the ribosomal protein alanine acetyltransferase (*rimI* mutant) are sensitive to only BCM among the antibiotics tested. Although many of the mutants defective in efflux pumps or membrane and cell wall synthesis and maintenance are multidrug sensitive, some members of this category are relatively specific, such as the *argO* mutant (strongly sensitive to BCM and CAM). Also, the pro-

TABLE 3. Pairwise interactions

Antibiotic	% BCM-only growth	% antibiotic-only growth	% predicted additive growth (PAE)	% BCM + antibiotic growth	Interaction
Cephadrine (RAD)	70.9	65.1	46.2	55.0	Additive
Ciprofloxacin (CIP)	74.8	82.6	61.8	48.7	Additive
Nitrofurantoin (NIT)	71.2	78.3	55.7	54.9	Additive
Chloramphenicol (CHL)	70.0	64.8	45.4	45.3	Additive
Triclosan (TRI)	68.9	84.2	58.0	56.4	Additive
Fusidic acid (FUS)	69.5	81.5	56.6	61.4	Additive
Erythromycin (ERY)	80.5	75.4	60.7	62.3	Additive
Neomycin (NEO)	78.6	68.5	53.8	5.2	Synergistic
Tobramycin (TOB)	77.9	72.7	56.6	10.0	Synergistic

tease-deficient *lon* mutant is hypersensitive only to BCM and metronidazole. This is consistent with the finding that Lon protease degrades Sula (28), a potent inhibitor of FtsZ (8, 20), reducing Sula's additive effect with BCM-stimulated expression of KilR. Highlighting the importance of FtsZ as a target of KilR is the finding that a deletion of *hfq* (22), a negative regulator of FtsZ (35, 38), yields cells that are more resistant to BCM (Table 2). For future applications, one might take advantage of these specific mutants in screening for antibiotics with properties similar to BCM.

Sozhamannan and Stitt (32) proposed a role for Rho in RNA degradation. Deletion of *pcnB*, encoding poly(A) polymerase I that polyadenylates RNAs, increased mRNA half-lives (29) and enhanced BCM sensitivity (Fig. 2). Moreover, deletion of *rppH*, which triggers mRNA degradation (12), also increased BCM sensitivity. Both of these results are consistent with the theory that Rho is involved in RNA turnover (32). It is possible that stabilization of the *kilR* transcript accounts for the hypersensitivity of these mutants, although an overall increase in RNA and RNA-DNA hybrids, toxic in Rho-deficient cells, is not ruled out.

We show that some of the increased susceptibilities to BCM could be overcome by deletion of *kil* alone or by deletion of all cryptic prophages and horizontally acquired genetic elements (strain MDS42). The deletions also increase BCM resistance in a wild-type background (4). Interestingly, in a BCM-sensitive mutant lacking the global regulator HNS, deletion of *kil* or placing the mutation in the MDS42 background did not enhance resistance. This raises the possibility of finding codrugs, or potentiators, of BCM by looking for inhibitors of, in this case, the HNS protein. Such an approach (9) might expand the use of BCM to other bacteria that lack the *rac* prophage. We (36) have studied a set of mutants, several of which are not in the KEIO collection, that also enhance the sensitivity of MDS42 to BCM. Figure 3 also shows that MDS42 is more resistant to BCM, in some circumstances, than strains lacking *kil* alone, suggesting that other *kil*-like-encoded functions missing in MDS42 contribute to BCM toxicity. A candidate for one of these functions might be the *ymfJ* gene of the *e14* prophage (26), since deletion of this gene increases resistance to BCM (Table 2). This prophage carries a *kil* analogue, which Cardinale et al. (4) found to be upregulated approximately 10-fold by BCM.

We tested pairwise combinations of BCM with 9 different antibiotics (Table 3). Yeh et al. (37) have classified antibiotics into groups based on the patterns of their pairwise interactions.

The data shown in Table 3, though not as extensive as that from Yeh et al., would at this point place BCM in the same grouping as CIP. This may not be surprising, given that BCM treatment results in expression of the Kil function that inhibits the cell division protein FtsZ and also inactivates Rho, leading to increased replisome/RNAP collisions and subsequent double-strand breaks (36), while CIP binds to DNA gyrase and blocks DNA synthesis, leading to double-strand breaks (16).

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REFERENCES

- Baba, T., et al. 2006. Construction of *Escherichia coli* K-12 in-frame single-gene knockout mutants: the Keio collection. *Mol. Syst. Biol.* **2**:2006.0008.
- Brennan, C. A., A. J. Dombroski, and T. Platt. 1987. Transcription termination factor Rho is an RNA-DNA helicase. *Cell* **48**:945–952.
- Briggs, G. S., et al. 2007. Ring structure of the *Escherichia coli* DNA-binding protein RdcC associated with recombination and replication fork repair. *J. Biol. Chem.* **282**:12353–12357.
- Cardinale, C. J., et al. 2008. Termination factor Rho and its cofactors NusA and NusG silence foreign DNA in *E. coli*. *Science* **320**:935–938.
- Ciampi, M. S. 2006. Rho-dependent terminators and transcription termination. *Microbiology* **152**:2515–2528.
- Clark, A. J., and K. B. Low. 1988. Pathways and systems of homologous recombination in *Escherichia coli*, p. 155–215. In K. B. Low (ed.), *The recombination of genetic material*. Academic Press, San Diego, CA.
- Conter, A., J.-P. Bouché, and M. Dassain. 1996. Identification of a new inhibitor of essential division gene *ftsZ* as the *kil* gene of defective prophage Rac. *J. Bacteriol.* **178**:5100–5104.
- Cordell, S. C., E. J. Robinson, and J. Lowe. 2005. Inhibition of the SOS cell division inhibitor SulaA and in complex with FtsZ. *Proc. Natl. Acad. Sci. U. S. A.* **100**:7889–7894.
- Cottarel, G., and J. Wierzbowski. 2007. Combination drugs, an emerging option for antibacterial therapy. *Trends Biotechnol.* **25**:547–555.
- Court, D., et al. 1980. Control of transcription termination: a Rho-dependent termination site in bacteriophage lambda. *J. Mol. Biol.* **138**:231–254.
- Datsenko, K. A., and B. L. Wanner. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U. S. A.* **97**:6640–6645.
- Deana, A., H. Celesnik, and J. G. Belasco. 2008. The bacterial enzyme RppH triggers messenger RNA degradation by 5' pyrophosphate removal. *Nature* **451**:355–358.
- Debarbieux, L., and J. Beckwith. 1998. The reductive enzyme thioredoxin 1 acts as an oxidant when it is exported to the *Escherichia coli* periplasm. *Proc. Natl. Acad. Sci. U. S. A.* **95**:10751–10756.
- Deutz, W. A., et al. 2000. Methods for intense aeration, growth, storage, and replication of bacterial strains in microtiter plates. *Appl. Environ. Microbiol.* **66**:2641–2646.
- Drees, J. C., S. Chittani-Pattu, D. R. McCaslin, R. B. Inman, and M. M. Cox. 2006. Inhibition of RecA protein function by the RdcG protein from *Escherichia coli*. *J. Biol. Chem.* **281**:4708–4717.
- Drlica, K., and X. Zhao. 1997. DNA gyrase, topoisomerase IV, and the 4-quinolones. *Microbiol. Mol. Biol. Rev.* **61**:377–392.
- Fassler, J. S., I. Tessman, and E. Tessman. 1985. Lethality of the double mutations *rho rep* and *rho ssb* in *Escherichia coli*. *J. Bacteriol.* **161**:609–614.

18. **Feinstein, S. I., and K. B. Low.** 1982. Zygotic induction of the *rac* locus can cause cell death in *E. coli*. *Mol. Gen. Genet.* **187**:231–235.
19. **Hancock, R. E. W.** 2007. The complexities of antibiotic action. *Mol. Syst. Biol.* **3**:142.
20. **Huisman, O., and R. D'Ari.** 1981. An inducible DNA replication-cell division coupling mechanism in *E. coli*. *Nature* **290**:797–799.
21. **Kohn, H., and W. Widger.** 2005. The molecular basis for the mode of action of bicyclomycin. *Curr. Drug Targets Infect. Disord.* **5**:273–295.
22. **Lee, T., and A. L. Feig.** 2008. The RNA binding protein Hfq interacts specifically with tRNAs. *RNA* **14**:514–523.
23. **Liu, A., et al.** 2010. Antibiotic sensitivity profiles determined with an *Escherichia coli* gene knockout collection: generating an antibiotic bar code. *Antimicrob. Agents Chemother.* **54**:1393–1403.
24. **Lloyd, R. G., and K. B. Low.** 1996. Homologous recombination, p. 2236–2255. *In* F. C. Neidhardt (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington, DC.
25. **Mahdi, A. A., C. Buckman, L. Harris, and R. G. Lloyd.** 2006. Rep and PriA helicase activities prevent RecA from provoking unnecessary recombination during replication fork repair. *Genes Dev.* **20**:2135–2147.
26. **Mehta, P., S. Casjens, and S. Krishnaswamy.** 2004. Analysis of the lambdoid prophage element ϕ 14 in the *E. coli* genome. *BMC Microbiol.* **4**:4.
27. **Miller, J. H.** 1992. A short course in bacterial genetics: a laboratory manual and handbook for *Escherichia coli* and related bacteria, p. 194–195. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
28. **Mizusawa, S., and S. Gottesman.** 1983. The *lon* gene controls the stability of *sulA* protein. *Proc. Natl. Acad. Sci. U. S. A.* **80**:358–362.
29. **Mohanty, B. K., and S. R. Kushner.** 1999. Analysis of the function of *Escherichia coli* poly(A) polymerase I in RNA metabolism. *Mol. Microbiol.* **34**:1094–1108.
30. **Posfai, G., et al.** 2006. Emergent properties of reduced genome *Escherichia coli*. *Science* **312**:1044–1046.
31. **Roberts, J. W.** 1969. Termination factor for RNA synthesis. *Nature* **224**:1168–1174.
32. **Sozhamannan, S., and B. L. Stitt.** 1997. Effects on mRNA degradation by *Escherichia coli* transcription termination factor Rho. *J. Mol. Biol.* **268**:689–703.
33. **Spies, M., and S. C. Kowalczykowski.** 2005. Homologous recombination by RecBCD and RecF pathways, p. 389–403. *In* N. P. Higgins (ed.), *The bacterial chromosome*. ASM Press, Washington, DC.
34. **Stewart, V., R. Landick, and C. Yanofsky.** 1986. Rho-dependent transcription termination in the tryptophanase operon leader region of *Escherichia coli* K-12. *J. Bacteriol.* **166**:217–223.
35. **Takada, A., M. Wachi, and K. Nagai.** 2009. Negative regulatory role of the *Escherichia coli* *hfq* gene in cell division. *Biochem. Biophys. Res. Commun.* **266**:579–583.
36. **Washburn, R. S., and M. E. Gottesman.** 2011. Transcription termination maintains chromosome integrity. *Proc. Natl. Acad. Sci. U. S. A.* **108**(2):792–797.
37. **Yeh, P., A. I. Tschumi, and R. Kishony.** 2006. Functional classification of drugs by properties of their pairwise interactions. *Nat. Genet.* **28**:489–494.
38. **Zambrano, N., et al.** 2009. Involvement of Hfq protein in the post-transcriptional regulation of *E. coli* bacterial cytoskeleton and cell division proteins. *Cell Cycle* **8**:2470–2472.
39. **Zwiefka, A., H. Kohn, and W. R. Widger.** 1993. Transcription termination factor Rho: the site of bicyclomycin inhibition in *Escherichia coli*. *Biochemistry* **32**:3564–3570.