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Determination of Antibiotic Hypersensitivity among 4,000 Single-Gene-Knockout Mutants of *Escherichia coli*▽†

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We have tested the entire Keio collection of close to 4,000 single-gene knockouts in *Escherichia coli* for increased susceptibility to one of seven different antibiotics (ciprofloxacin, rifampin, vancomycin, ampicillin, sulfamethoxazole, gentamicin, or metronidazole). We used high-throughput screening of several subinhibitory concentrations of each antibiotic and reduced more than 65,000 data points to a set of 140 strains that display significantly increased sensitivities to at least one of the antibiotics, determining the MIC in each case. These data provide targets for the design of "codrugs" that can potentiate existing antibiotics. We have made a number of double mutants with greatly increased sensitivity to ciprofloxacin, and these overcome the resistance generated by certain *gyrA* mutations. Many of the gene knockouts in *E. coli* are hypersensitive to more than one antibiotic. Together, all of these data allow us to outline the cell's "intrinsic resistome," which provides innate resistance to antibiotics.

Antibiotics have had a major impact over the past 6 decades in the fight against infectious diseases (for a review by Davies, see reference 11). However, the spread of antibiotic-resistant microorganisms has reached an alarming point (1, 11, 35), prompting renewed efforts to find new antibiotics by detecting new targets through genomics, altering existing antibiotics, screening chemical (e.g., see reference 9) or peptide (21, 31) libraries for specific inhibitors (e.g., see reference 9), or finding new sources of antibiotics via metagenomics (e.g., see reference 53). While these lines of investigation show great promise, additional approaches are constantly being sought to yield a new generation of useful antimicrobial compounds. For instance, focusing on species-specific antibiotics rather than broad-spectrum antibiotics can result in important new agents (38), as could targeting bacterial transcription factors (5) or different processes, such as Holliday junction processing (21, 31) and quorum sensing (24), or even targeting host factors that support pathogen growth (33). Another approach, examined here, involves potentiating existing antibiotics by identifying targets for increasing susceptibility to specific antimicrobials. There are precedents for using such combinational therapy. For example, inhibitors of β-lactamase have been used together with β-lactam antibiotics (for a review by Buynak, see reference 7) and inhibitors of efflux pumps together with tetracycline in *Escherichia coli* (46) and with levofloxacin in *Pseudomonas aeruginosa* (37). In the case of chemotherapeutics, zebularine, a cytosine analog (41) and mutagen (34) that is converted in vivo to an inhibitor of cytosine deaminase (41), is used in combination with certain cytosine deaminase-susceptible cytosine-based drugs (14, 40). With regard to finding new targets for this type of approach, a number of genes that increase the sensitivities of microorganisms to different antibiotics have been identified (e.g., for a review by Drlica and Zhao, see reference 15; see also reference 42), but only recently have tools been available for systematic searches for these potential targets. A transposon library has been used to detect genes responsible for increased sensitivity to one of the antibiotics in a set of antibiotics in *Acinetobacter baylyi* (20), and a yeast deletion library has been screened against a set of DNA-damaging agents (58) and also a set of over 400 small molecules (25). In the work reported here, we used high-throughput screening of an *E. coli* knockout collection of close to 4,000 strains, each with a different gene inactivated (3), to look for mutants that are more susceptible to one of seven different antibiotics (see Table 1). We identify mutants with increased and decreased susceptibilities to ciprofloxacin (CPR) and those with increased susceptibilities to rifampin (RIF), vancomycin (VAN), ampicillin (AMP), sulfamethoxazole (SFX), gentamicin (GEN), and metronidazole (MET). In the case of CPR, VAN, and MET, we show that certain combinations of two mutations result in mutants with even greater susceptibilities. Moreover, for CPR, some of these mutations can counteract the resistance conferred by certain *gyrA* alleles. These results help to define new combinational drug targets and lay the groundwork for typing antibiotics by their resulting "sensitivity profile."

MATERIALS AND METHODS

***E. coli* strains.** The Keio collection is as described by Baba et al. (3), made from the starting strain BW25113 (10). This strain (*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH533}$ Δ*rhaBAD*$_{LD78}$) is the starting strain used in the experiments reported here, unless otherwise stated. (Strains in the Keio collection with a JW designation, e.g., JW5115, JW5360, JW5474, carry deletions of sequences that were originally designated as open reading frames but were then demoted to an uncertain status.) We used the following strains for donors of the indicated markers for P1 transduction: CSH126 (Δ*recA-srl*)*306 srl*::Tn*10-84* (44); P90C *uvrD*::mini-Tn*10* (44; J. H. Miller, K. Kim, A. Liu, and C. Tamae, unpublished data); RJ3460 *fis*::*cat* (gift from Reid Johnson); CGSC6911 *tolC6*::mini-Tn*10* (*E. coli* Genetic Stock Center); CGSC7550 *ruvC67*::*cat* (*E. coli* Genetic Stock Center); and CGSC7553 *recG265*::*cat* (*E. coli* Genetic Stock Center). DY330 (64) was converted to *recC*::*cat* by the procedure described below and was used as a donor for P1 transduction.

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TABLE 1. List of antibiotics

Category	Example used	Primary target[a]	Process affected
Fluoroquinolone	CPR	DNA gyrase	DNA replication
Glycopeptide	VAN	NAM/NAG peptides	Cell wall synthesis
Rifamycin	RIF	RNA polymerase	Transcription
Penicillin	AMP	Transpeptidase	Cell wall synthesis
Sulfonamide	SFX	Dihydropteroate synthetase	Folate synthesis
Aminoglycoside	GEN	30S subunit	Translation
Nitroimidazole	MET	DNA	DNA structure

[a] NAM, *N*-acetylmuramic acid; NAG, *N*-acetylglucosamine.

***E. coli* genetic methods.** Unless otherwise stated, all genetic methods are as described by Miller (44), including P1 transduction and mutagenesis with 2-aminopurine (700 μg/ml) (see also reference 39 for exact details).

Use of the Deutz cryoreplicator. The Deutz cryoreplicator (12) contains 96 prongs on individual springs, allowing its frequent application to frozen glycerol cultures. The Keio collection (3) is maintained on 45 96-well microtiter plates and stored at −80°C in glycerol. Material from frozen microtiter plates was transferred to microtiter wells with 0.5 ml of LB medium, which were incubated overnight, and then the replicator was used to transfer a microdrop to microtiter wells with fresh LB medium containing 50 μg/ml kanamycin to prevent the growth of contaminants. (All of the strains in the Keio collection are Kan^r.) After 3 to 4 h of growth, these plates were printed onto LB plates with different concentrations of different antibiotics. For the initial screening, kanamycin was present in the plates, but for all retests, Etests, and MIC determinations, kanamycin was not present. We screened four concentrations of CPR (5, 10, 15, and 25 ng/ml), two each of VAN (100 μg/ml and 150 μg/ml), RIF (7.5 μg/ml and 10 μg/ml), and GEN (0.3 μg/ml and 0.5 μg/ml), and one each of AMP (2 μg/ml) and SFX (400 μg/ml) as well as a control medium with no antibiotic. We initially screened 27 of the 45 microtiter plates in the collection with four different concentrations of MET (500, 600, 700, and 800 μg/ml) and the remaining 18 plates with 600 μg/ml.

Determination of MIC. MICs (2) were determined either by applying 10^4 to 10^5 CFU onto an LB plate with the appropriate concentration of the desired antibiotic and examining the plate after overnight incubation at 37°C or by Etests. In all cases, tests were performed on repurified strains. (In a few cases, MICs were confirmed by liquid culture tests, starting from an initial inoculum of 10^5 cells [2].) Etests were obtained from AB Biodisk North America Inc. (Piscataway, NJ). The appropriate test material was applied to a freshly plated lawn of an exponential culture of the desired strain on LB plates and incubated overnight at 37°C. Most of the mutants in the Keio collection grow indistinguishably from the starting strain on LB plates under the conditions tested. However, a number of mutants grow significantly more slowly (e.g., *rnt*, *lptB*, *tonB*, *ubiG*, and *lpdA* mutants), leading to potential artifacts in determining inhibition by antibiotics. In principle, all true inhibition by an antibiotic represents an inhibition of growth of a mutant to a greater degree than the inhibition of growth of the starting strain. MICs were therefore confirmed by optical density measurements of growth in liquid culture for all mutants that grow significantly more slowly than the starting strain in LB medium (without antibiotic).

Construction of *E. coli* knockout mutants. The procedure for constructing *E. coli* single-knockout mutants is essentially the same as described by Yu et al. (64). Briefly, a PCR product was prepared, one which contained an antibiotic resistance gene cassette, in this case the *cat* gene, flanked at both ends by 50-bp DNA sequences that are identical to sequences flanking the open reading frame of the target gene, in this case *recC*. The purified PCR product was used to transform the recombination-proficient *E. coli* strain DY330 (64). The transformants that were resistant to the antibiotic selection were further purified on the LB plates and were verified for the loss of the target gene by PCR. Standard P1 transduction was used to transfer the knockout allele from DY330 to a desired genetic background for analysis of its phenotype.

DNA sequencing of *gyrA*. We isolated a series of mutants resistant to high (100 and 500 ng/ml) concentrations of CPR, using BW25113 as the starting strain. We sequenced the relevant part of the *gyrA* gene (see Materials and Methods for details), and found four different mutations (generating S83L, D87G, G81D, and D87N), two of which have been described previously (4). One of these, resulting in a serine-to-leucine change at position 83 in the GyrA protein, confers resistance to CPR up to a concentration of 750 ng/ml. A second mutation, resulting in D87G, has also been detected in *E. coli*, although the two other mutations (G81D and D87N), have not been described previously. The protocol used to PCR amplify directly from a single colony was adapted from Haldimann and Wanner (22). Isolated colonies were resuspended in 50 μl of water. One microliter of the suspension was used to PCR amplify the *gyrA* gene by using the GyrA1 primer, 5′-TTATGGTTTACCGGCGAT-3′, and the GyrA2 primer, 5′-ACGACCGTTAATGATTGCC-3′.

The PCR product was purified using the MinElut PCR purification kit (Qiagen, Valencia, CA). The purified PCR products were then sequenced using the GyrA1 primer. The DNA sequencing reactions were carried out using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) on iCycler thermal cyclers (Bio-Rad), and the sequencing was performed on Applied Biosystems 3730 DNA analyzers at the UCLA Genotyping and Sequencing Core.

Chemicals. Kanamycin, tetracycline, chloramphenicol, RIF, VAN, SFX, GEN, AMP, and MET were purchased from Sigma (St. Louis, MO). CPR was purchased from ICN Biomedicals, Inc., Aurora, OH.

RESULTS AND DISCUSSION

Screening. We screened the entire Keio collection of close to 4,000 strains (3) for mutants that are more sensitive than the wild type to one or more antibiotics of a set of seven different antibiotics (Table 1), CPR, VAN, RIF, AMP, SFX, GEN, and MET, at several different concentrations (see Materials and Methods for details). Figure 1 shows an example. We also

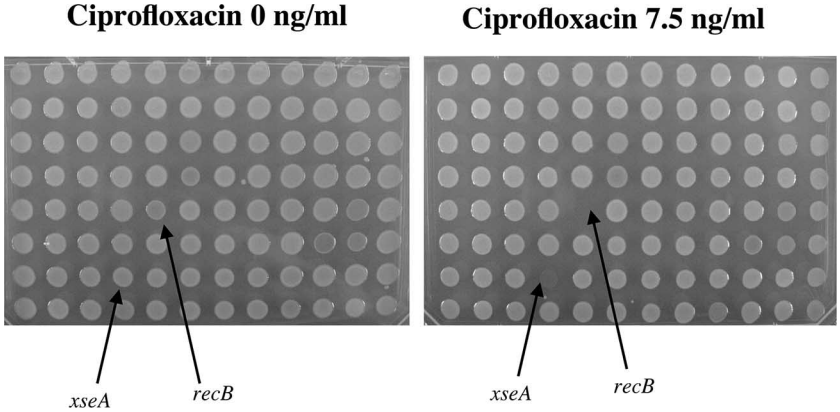
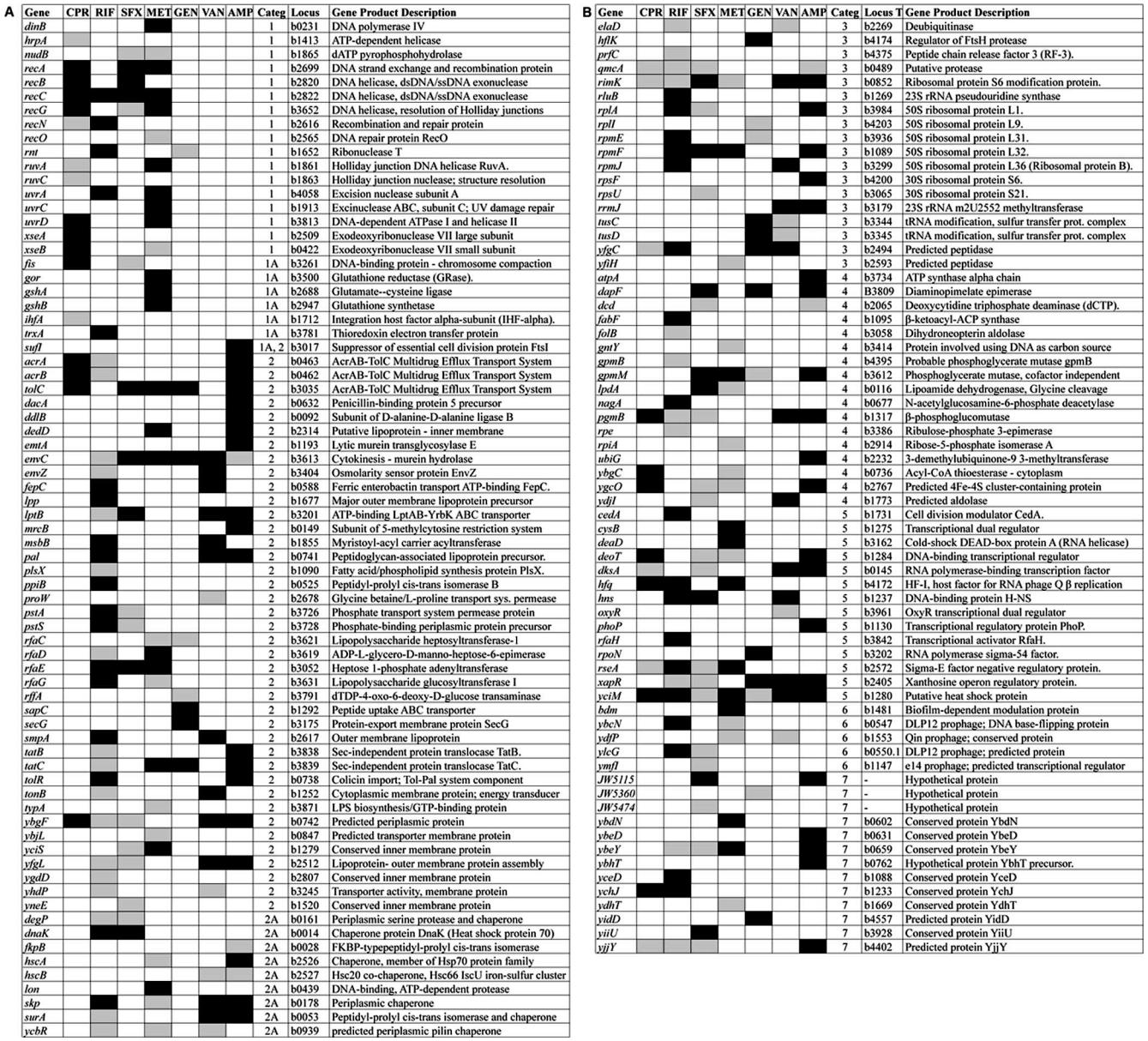


FIG. 1. Effect of 7.5 ng/ml CPR on *Escherichia coli*. Ninety-six mutants from the Keio collection were printed onto LB plates with no CPR (left panel) and LB plates with 7.5 ng/ml CPR (right panel). Here, only two mutants, with knockouts of the *xseA* and *recB* genes, fail to grow on the plate with 7.5 ng/ml CPR. Further tests on purified colonies confirm their hypersensitivities.



A	Gene	CPR	RIF	SFX	MET	GEN	VAN	AMP	Categ	Locus	Gene Product Description
	dinB				■				1	b0231	DNA polymerase IV
	hrpA	▒							1	b1413	ATP-dependent helicase
	nudB			▒	▒				1	b1865	dATP pyrophosphohydrolase
	recA	■		■	■				1	b2699	DNA strand exchange and recombination protein
	recB	■		■	■				1	b2820	DNA helicase, dsDNA/ssDNA exonuclease
	recC	■	■	▒	■				1	b2822	DNA helicase, dsDNA/ssDNA exonuclease
	recG	■		▒					1	b3652	DNA helicase, resolution of Holliday junctions
	recN	▒	■						1	b2616	Recombination and repair protein
	recO				▒				1	b2565	DNA repair protein RecO
	rnt		■			▒			1	b1652	Ribonuclease T
	ruvA	▒			■				1	b1861	Holliday junction DNA helicase RuvA.
	ruvC	▒							1	b1863	Holliday junction nuclease; structure resolution
	uvrA		■		■				1	b4058	Excision nuclease subunit A
	uvrC				■				1	b1913	Excinuclease ABC, subunit C; UV damage repair
	uvrD	■			■				1	b3813	DNA-dependent ATPase I and helicase II
	xseA	■							1	b2509	Exodeoxyribonuclease VII large subunit
	xseB	■			▒				1	b0422	Exodeoxyribonuclease VII small subunit
	fis	■		▒					1A	b3261	DNA-binding protein - chromosome compaction
	gor				■				1A	b3500	Glutathione reductase (GRase).
	gshA				■				1A	b2688	Glutamate--cysteine ligase
	gshB				■				1A	b2947	Glutathione synthetase
	ihfA	▒							1A	b1712	Integration host factor alpha-subunit (IHF-alpha).
	trxA		■						1A	b3781	Thioredoxin electron transfer protein
	sulI							■	1A, 2	b3017	Suppressor of essential cell division protein FtsI
	acrA	■	▒					■	2	b0463	AcrAB-TolC Multidrug Efflux Transport System
	acrB	■	▒					■	2	b0462	AcrAB-TolC Multidrug Efflux Transport System
	tolC	■		■	■	■		■	2	b3035	AcrAB-TolC Multidrug Efflux Transport System
	dacA							■	2	b0632	Penicillin-binding protein 5 precursor
	ddlB							■	2	b0092	Subunit of D-alanine-D-alanine ligase B
	dedD				■			■	2	b2314	Putative lipoprotein - inner membrane
	emtA							■	2	b1193	Lytic murein transglycosylase E
	envC		▒	■	■	■	■	▒	2	b3613	Cytokinesis - murein hydrolase
	envZ		▒				■	■	2	b3404	Osmolarity sensor protein EnvZ
	fepC		■				■	■	2	b0588	Ferric enterobactin transport ATP-binding FepC.
	lpp		■					■	2	b1677	Major outer membrane lipoprotein precursor
	lptB		▒	■			■	■	2	b3201	ATP-binding LptAB-YrbK ABC transporter
	mrcB							■	2	b0149	Subunit of 5-methylcytosine restriction system
	msbB		■				■	■	2	b1855	Myristoyl-acyl carrier acyltransferase
	pal		■				■	■	2	b0741	Peptidoglycan-associated lipoprotein precursor.
	plsX		▒						2	b1090	Fatty acid/phospholipid synthesis protein PlsX.
	ppiB		■						2	b0525	Peptidyl-prolyl cis-trans isomerase B
	proW						▒		2	b2678	Glycine betaine/L-proline transport sys. permease
	pstA		■	▒					2	b3726	Phosphate transport system permease protein
	pstS		■	▒					2	b3728	Phosphate-binding periplasmic protein precursor
	rfaC				▒	▒			2	b3621	Lipopolysaccharide heptosyltransferase-1
	rfaD		▒		■				2	b3619	ADP-L-glycero-D-manno-heptose-6-epimerase
	rfaE		■	■					2	b3052	Heptose 1-phosphate adenyltransferase
	rfaG		■		▒				2	b3631	Lipopolysaccharide glucosyltransferase I
	rffA					▒			2	b3791	dTDP-4-oxo-6-deoxy-D-glucose transaminase
	sapC					■			2	b1292	Peptide uptake ABC transporter
	secG						■		2	b3175	Protein-export membrane protein SecG
	smpA		■					■	2	b2617	Outer membrane lipoprotein
	tatB		▒					■	2	b3838	Sec-independent protein translocase TatB.
	tatC		▒		■	■		■	2	b3839	Sec-independent protein translocase TatC.
	tolR		■					■	2	b0738	Colicin import; Tol-Pal system component
	tonB		▒				■		2	b1252	Cytoplasmic membrane protein; energy transducer
	typA							■	2	b3871	LPS biosynthesis/GTP-binding protein
	ybgF	■	▒	▒			■	■	2	b0742	Predicted periplasmic protein
	ybjL								2	b0847	Predicted transporter membrane protein
	yciS			▒	■				2	b1279	Conserved inner membrane protein
	yfgL		▒	▒			■	■	2	b2512	Lipoprotein- outer membrane protein assembly
	ygdD		▒						2	b2807	Conserved inner membrane protein
	yhdP		▒				▒		2	b3245	Transporter activity, membrane protein
	yneE		▒	▒					2	b1520	Conserved inner membrane protein
	degP		▒	▒					2A	b0161	Periplasmic serine protease and chaperone
	dnaK		■	■					2A	b0014	Chaperone protein DnaK (Heat shock protein 70)
	fkpB							▒	2A	b0028	FKBP-typepeptidyl-prolyl cis-trans isomerase
	hscA				▒			■	2A	b2526	Chaperone, member of Hsp70 protein family
	hscB						▒	▒	2A	b2527	Hsc20 co-chaperone, Hsc66 IscU iron-sulfur cluster
	lon				■				2A	b0439	DNA-binding, ATP-dependent protease
	skp		■		▒		■	■	2A	b0178	Periplasmic chaperone
	surA		▒				■	■	2A	b0053	Peptidyl-prolyl cis-trans isomerase and chaperone
	ycbR		▒		▒		▒		2A	b0939	predicted periplasmic pilin chaperone

B	Gene	CPR	RIF	SFX	MET	GEN	VAN	AMP	Categ	Locus T	Gene Product Description
	elaD		▒						3	b2269	Deubiquitinase
	hflK					■			3	b4174	Regulator of FtsH protease
	prfC		▒						3	b4375	Peptide chain release factor 3 (RF-3).
	qmcA	▒	▒	▒				▒	3	b0489	Putative protease
	rimK	▒	▒	■	▒		■	■	3	b0852	Ribosomal protein S6 modification protein.
	rluB		■						3	b1269	23S rRNA pseudouridine synthase
	rplA		■	▒				■	3	b3984	50S ribosomal protein L1.
	rplI					▒			3	b4203	50S ribosomal protein L9.
	rpmE		■			▒			3	b3936	50S ribosomal protein L31.
	rpmF		■	■	■			■	3	b1089	50S ribosomal protein L32.
	rpmJ		■				■		3	b3299	50S ribosomal protein L36 (Ribosomal protein B).
	rpsF							■	3	b4200	30S ribosomal protein S6.
	rpsU			▒					3	b3065	30S ribosomal protein S21.
	rrmJ						■	■	3	b3179	23S rRNA m2U2552 methyltransferase
	tusC					■	▒		3	b3344	tRNA modification, sulfur transfer prot. complex
	tusD					■	▒		3	b3345	tRNA modification, sulfur transfer prot. complex
	yfgC	▒	■			■	■		3	b2494	Predicted peptidase
	yfiH				▒				3	b2593	Predicted peptidase
	atpA							■	4	b3734	ATP synthase alpha chain
	dapF			■		■		■	4	B3809	Diaminopimelate epimerase
	dcd			▒				▒	4	b2065	Deoxycytidine triphosphate deaminase (dCTP).
	fabF		■						4	b1095	β-ketoacyl-ACP synthase
	folB		▒						4	b3058	Dihydroneopterin aldolase
	gntY				▒				4	b3414	Protein involved using DNA as carbon source
	gpmB		▒						4	b4395	Probable phosphoglycerate mutase gpmB
	gpmM			■	■	▒		■	4	b3612	Phosphoglycerate mutase, cofactor independent
	lpdA			■	▒				4	b0116	Lipoamide dehydrogenase, Glycine cleavage
	nagA		■						4	b0677	N-acetylglucosamine-6-phosphate deacetylase
	pgmB	■		▒			■	■	4	b1317	β-phosphoglucomutase
	rpe		▒						4	b3386	Ribulose-phosphate 3-epimerase
	rpiA				▒				4	b2914	Ribose-5-phosphate isomerase A
	ubiG							■	4	b2232	3-demethylubiquinone-9 3-methyltransferase
	ybgC	■				▒			4	b0736	Acyl-CoA thioesterase - cytoplasm
	ygcO			▒		▒		■	4	b2767	Predicted 4Fe-4S cluster-containing protein
	ydjI						■		4	b1773	Predicted aldolase
	cedA		■						5	b1731	Cell division modulator CedA.
	cysB				■				5	b1275	Transcriptional dual regulator
	deaD								5	b3162	Cold-shock DEAD-box protein A (RNA helicase)
	deoT	■		▒	▒			■	5	b1284	DNA-binding transcriptional regulator
	dksA			▒			■	■	5	b0145	RNA polymerase-binding transcription factor
	hfq	■	■		■				5	b4172	HF-I, host factor for RNA phage Q β replication
	hns		■	■			■		5	b1237	DNA-binding protein H-NS
	oxyR						▒		5	b3961	OxyR transcriptional dual regulator
	phoP							■	5	b1130	Transcriptional regulatory protein PhoP.
	rfaH		■						5	b3842	Transcriptional activator RfaH.
	rpoN					■			5	b3202	RNA polymerase sigma-54 factor.
	rseA	▒	▒	▒	■				5	b2572	Sigma-E factor negative regulatory protein.
	xapR		▒	▒		■	■	■	5	b2405	Xanthosine operon regulatory protein.
	yciM	■	■	▒	■	▒	■	■	5	b1280	Putative heat shock protein
	bdm				■				6	b1481	Biofilm-dependent modulation protein
	ybcN		■		▒				6	b0547	DLP12 prophage; DNA base-flipping protein
	ydfP				▒		▒		6	b1553	Qin prophage; conserved protein
	ylcG		■		▒				6	b0550.1	DLP12 prophage; predicted protein
	ymfI				▒				6	b1147	e14 prophage; predicted transcriptional regulator
	JW5115			■				■	7	-	Hypothetical protein
	JW5360					▒			7	-	Hypothetical protein
	JW5474				▒				7	-	Hypothetical protein
	ybdN				■				7	b0602	Conserved protein YbdN
	ybeD							■	7	b0631	Conserved protein YbeD
	ybeY		▒	▒	■			■	7	b0659	Conserved protein YbeY
	ybhT							■	7	b0762	Hypothetical protein YbhT precursor.
	yceD		■						7	b1088	Conserved protein YceD
	ychJ	■	■						7	b1233	Conserved protein YchJ
	ydhT				▒				7	b1669	Conserved protein YdhT
	yidD					■			7	b4557	Predicted protein YidD
	yiiU			■					7	b3928	Conserved protein YiiU
	yjjY	▒	▒	▒				■	7	b4402	Predicted protein YjjY

FIG. 2. Gene knockouts affecting increased sensitivity to one or more of the seven antibiotics tested. Strong sensitivity to a particular antibiotic is indicated by a black panel, while weaker sensitivities are indicated with a gray panel. See tables in the supplemental material for numerical values of MICs. The locus tags refer to the accession number (http://www.ecocyc.org). Categories are as follows: category 1, DNA replication, recombination, and repair; category 1A, functions indirectly affecting category 1; category 2, transport, efflux, cell wall, and cell membrane synthesis; category 2A, chaperones and functions related to category 2; category 3, protein synthesis; category 4, general metabolic reactions; category 5, regulation; category 6, prophage-encoded genes and cell adhesion; and category 7, unassigned genes.

tested for mutants resistant to CPR. This generated an initial set of close to 65,000 data points. Mutants showing increased susceptibility (or in the case of CPR, resistance or susceptibility) were then purified and retested from single colonies by several methods to determine the MIC. Figure 2 incorporates all of the data (see the supplemental material for detailed charts), and selected examples are given in subsequent tables. The mutants in Fig. 2 have been arranged according to the type of function affected, with black bars indicating the strongest effects. We see several types of mutants in most cases. Namely, some hypersensitive mutants are specific for each class of antibiotic and reflect the initial target and process affected. Other sensitivities probably arise from secondary targets and indirect effects and reflect the complexities of antibiotic action (see below; also see references 6 and 23). Also, some mutants show up on the sensitivity profiles of several different antibiotics and represent functions involved in general intrinsic resistance.

CPR. CPR is a fluoroquinolone that binds to DNA gyrase and topoisomerase IV (for a review by Drlica and Zhao, see reference 15). DNA gyrase, which controls DNA supercoiling, is encoded by the *gyrA* and *gyrB* genes in *E. coli*. CPR-bound gyrase forms a complex with DNA that blocks replication and

TABLE 2. CPR sensitivities of single and double mutants[a]

Strain	MIC (ng/ml)
BW25113	16–20
fis mutant	6
ruvC mutant	8
fis ruvC mutant	2
tolC mutant	5
fis tolC mutant	0.2
recA mutant	2
fis recA mutant	1
xseA mutant	6
recC mutant	8
xseA recC mutant	1.3
recC tolC mutant	0.3

[a] Gene knockout mutants from Keio collection (3) using BW25113 (10) as the starting strain.

TABLE 3. Effect of gene knockouts in CPR-resistant mutants

Strain[a]	MIC (ng/ml)
BW25113 (S83L)	750
recC mutant	500
fis mutant	300
xseA mutant	250
recA mutant	125
tolC mutant	64
tolC recC mutant	20

[a] Gene knockout mutants from Keio collection transduced into strain BW25113 carrying a *gyrA* mutation [BW25113 (S83L)] that results in a S→L change at residue 83 of DNA gyrase A.

also results in the exposure of double-stranded DNA breaks. It is not surprising, therefore, that some of the strongest-susceptibility mutants are defective in genes involved in DNA binding, replication, and repair functions and in the repair of double-stranded breaks. New findings include the *fis*, *xseA*, and *xseB* genes leading to hypersusceptible phenotypes when inactivated. FIS is the most abundant of the DNA binding proteins (HU, IHF, HNS, FIS) that are associated with the bacterial nucleoid and is believed to play a role in compaction by bending the DNA (56). The *xseAB* genes encode ExoVII (8). It has been reported that *recA* and *recB* mutants are more susceptible to nalidixic acid, a related quinoline antibiotic (42). We find that *recA* and *recC* mutants are particularly sensitive to CPR (Fig. 2 and Table 2), with *recB* mutants also being sensitive (Fig. 2). This may be related to the repair of double-stranded breaks or, in the case of *recA* mutants, be a response to the generation of reactive oxygen species that have been postulated to be involved in cell death by bactericidal antibiotics (32). Mutants that are more sensitive to CPR as well as a set of other antibiotics (Fig. 2) are defective in cell wall and membrane synthesis and in efflux pumps (*tolC*). Previous work with *E. coli* had shown that *polA* and *uvrD* mutants have increased sensitivity to nalidixic acid (for a review by Drlica and Zhao, see reference 15), and recent studies revealed several genes in *Acinetobacter baylyi* that result in increased sensitivity to CPR, although only the *acrB* gene gave similar results in *E. coli* when the homologous gene knockouts were tested (20).

In the work reported here, the mutants with the strongest effects are close to 1 order of magnitude more sensitive than the starting strain to CPR. Double mutants with certain combinations of mutations, such as *fis tolC* and *recC tolC* (Table 2), approach sensitivities that are 2 orders of magnitude greater. The increased sensitivity can also counteract the effect of mutations that increase resistance to CPR, such as *gyrA* mutations. The change S83L in the gyrase A subunit is a single-step mutation that confers a high level of CPR resistance in *E. coli* (4). We rederived this mutation in strain BW25113 (see Materials and Methods). We used P1 transduction to transfer several of the gene knockouts conferring increased susceptibility (*recC*, *fis*, *xseA*, *recA*, and *tolC*) into BW25113 containing the S83L CPR-resistance-generating *gyrA* mutation. Table 3 shows the results. Clearly, the increased resistance to CPR can be partially reversed by sensitive single-step mutants and totally reversed by certain double-mutation combinations, for instance, *tolC recC*.

Table 4 shows the single-gene-knockout mutants that have measurably higher levels of CPR resistance than the wild type. Although close to 200 mutants were detected in the initial screen, a much smaller number of mutants show reproducible effects when examined more closely. None of the single-gene knockouts confer resistance to CPR at the levels seen for strains with certain point mutations in the *gyrA* gene (4; see below).

Sensitivity to VAN. VAN, a glycopeptide antibiotic that is effective on many gram-positive bacteria, does not normally affect gram-negative bacteria because it cannot sufficiently penetrate the outer cell membrane (47), which excludes many small molecules (e.g., see references 28, 50, and 51). Once in the cell, VAN interferes with the synthesis of peptidoglycan, the major structural component of the cell wall, specifically inhibiting peptidoglycan polymerase and transpeptidation reactions. It operates at an earlier stage in peptidoglycan biosynthesis than AMP. Although these latter enzymes are the primary targets once inside the cell, for a gram-negative

TABLE 4. CPR-resistant strains[a]

Locus tag[b]	Gene	Gene product	MIC (ng/ml) determined by indicated method	
			Etest	Plating[c]
b2496	*hda*	Regulator of DnaA that prevents premature reinitiation of DNA replication	32	—
b2688	*gshA*	Glutamate-cysteine ligase	32	—
b1133	*mnmA*	tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase	23	25
b1385	*feaB*	Phenylacetaldehyde dehydrogenase	16	25
b1072	*flgA*	Flagella basal body P-ring formation protein	23	—
b1261	*trpB*	Tryptophan synthase beta chain	16	25
b1449	*yncB*	Predicted oxidoreductase, Zn-dependent and NADP-binding	16	25
b0683	*fur*	Ferric uptake regulation, transcriptional dual regulator	23	25
b2530	*iscS*	Cysteine desulfurase monomer	32	—
b1506	JW5244	Hypothetical protein	25	—

[a] MICs for BW25113 by Etest and plating are 16 and 20 ng/ml, respectively. Also, see footnote for Table 2.
[b] Locus tags refer to the accession numbers (http://www.ecocyc.org).
[c] —, not tested with this method.

bacterium such as *E. coli*, we would expect to see mutants that facilitate permeation of VAN well represented among the strains with increased sensitivity. We did detect numerous mutants that render *E. coli* susceptible to VAN at concentrations (100 μg/ml to 150 μg/ml) that do not affect the starting strain BW25113, which has a MIC of 500 μg/ml. The *smpA* mutant displays a MIC of 70 μg/ml. In one exceptional case, in a mutant with a *surA* defect, the MIC is lowered to 4 μg/ml, and the *surA smpA* double mutant displays a MIC of 1.5 μg/ml. (The increased sensitivities to VAN and certain other agents, resulting from *surA* defects, have been noted previously, although not quantified in this manner, as has an enhanced sensitivity in the *surA fkpA ppiA ppiD* quadruple mutant [28].) The SurA protein is one of four *cis-trans* proyl isomerases that aid protein folding in the periplasm, thus promoting maturation of outer membrane porins (see references 28 and 50 and references therein). SmpA is part of the YaeT outer-membrane-protein assembly complex (55), and mutations in some of these components have been shown to cause increased sensitivity to bacitracin, novobiocin, and RIF (51). Although many of the strong susceptibilities do, in fact, result from defects in genes involved in cell wall or outer membrane synthesis or integrity (e.g., *pal*, *ompF*, *rfaC*, *htrB*), other genes are involved in intracellular functions, including protein synthesis (*tusC*, *tusD*, *rimK*), perhaps indicating this latter process as a secondary target. These results suggest that VAN does indeed get into *E. coli*, although clearly at subinhibitory concentrations. This is supported by our findings that VAN partially induces the SOS system in *E. coli* at the concentrations used here (Miller, Kim, Liu, and Tamae, unpublished).

Sensitivity to RIF. RIF binds to the β-subunit of RNA polymerase in virtually all prokaryotes, blocking transcription. Extensive work has been carried out on RIF-resistant mutants in many different microorganisms (see references 18 and 45 and references therein). We screened for mutants more susceptible to RIF, using concentrations of 7.5 μg/ml and 10 μg/ml. (The MIC for RIF of strain BW25113 is 16 μg/ml.) Here, the pattern of sensitive mutants is more varied. The primary target cannot be eliminated by a gene knockout in a viable cell, but we do see mutants with defects in replication, recombination, and repair (e.g., *uvrA* mutants) and in protein synthesis (*rpmE*, *rpmF*, and *rplA* mutants), processes affected secondarily by blocking transcription. More general mutants are affected in the cell wall, cell membrane, or transport systems, and in some cases, these are the same mutants that are more sensitive to VAN (see below).

Sensitivity to AMP. We screened for mutants sensitive to 2 μg/ml AMP. (Strain BW25113 displays a MIC for AMP of 5 μg/ml with Etests and 6 μg/ml by plating.) The primary target of AMP, an aminopenicillin, is DD-transpeptidase (59), which is involved in generating peptidoglycan cross-links in the cell wall. This and other enzymes (e.g., carboxypeptidase and endopeptidase) are part of a set of "penicillin binding proteins." Several of the strongly sensitive mutants, *mrcB* and *dacA* mutants, have defects in penicillin binding proteins, and are found only in the AMP profile. Some mutants, however, have defects in protein synthesis (e.g., *rpmF* mutants). Again, we see that many of the strongly susceptible mutants have defects in the cell wall, cell membrane, or transport systems, and some of these are involved in susceptibilities to other antibiotics.

Sensitivity to SFX. SFX blocks the fifth step in the de novo synthesis of folic acid by inhibiting dihydropteroate synthetase (52), leading to defects in the synthesis of DNA since the synthesis of purines and thymine is interrupted. Thus, a set of susceptible mutants is involved in functions related to this target, including aspects of DNA recombination and repair (e.g., *recA*, *recB*, *recC*, and *recG* mutants). One of the moderately sensitive mutants has a knockout of the *nudB* gene, which encodes dihydroneopterin triphosphate pyrophosphatase (17), which catalyzes the second step in folate biosynthesis (17; see also reference 52).

Sensitivity to GEN. We screened strains at two different concentrations of GEN, 0.3 and 0.5 μg/ml. This antibiotic is an aminoglycoside that interferes with protein synthesis by binding to the 30S subunit of bacterial ribosomes yet, as can be seen from Fig. 2, has increased effectiveness against some mutants defective in 50S ribosomal proteins or the protein elongation factor Tu, in addition to a set of mutants with altered permeability. Some of these are sensitive to other antibiotics, as exemplified by *tolC* mutants, defective in the AcrAB-TolC efflux system, but others are specific to GEN, namely *sapC* and *secG* mutants.

Sensitivity to MET. MET is used in the treatment of *Clostridium difficile*-associated disease and in therapeutic regimens for *Helicobacter pylori* (e.g., see references 49 and 57). The active form of MET, generated by pyruvate-ferredoxin oxidoreductase-mediated reduction of the nitro group, directly interacts with DNA to distort its structure. In line with this, mutants that we found to be hypersensitive to MET include those with defects related to DNA functions. Thus, from Fig. 2, we see the helicase RecG and the UvrABC repair proteins UvrA and UvrC represented. Clearly, glutathione, a thiol involved in detoxifying certain stress-inducing factors, including oxidative stress, plays a key role in preventing killing by subinhibitory concentrations of MET, since the most susceptible mutant, the *gshA* mutant (see the supplemental material and Table 5), lacks the first step in the synthesis of glutathione (γ-glutamylcysteine synthetase). (Gomez and Neyfakh [20] detected the *gshA* mutant as being involved in MET sensitivity in *Acinetobacter baylyi* but did not find sensitivity in an *E. coli gshA* knockout mutant, in contrast to the results reported here.) A mutant (the *gshB* mutant) lacking the second step is also sensitive, as is the *cysB* mutant, lacking the positive regulator of the *cys* regulon, and mutants (*gorA* and *gorB* mutants) defective in glutathione reductase. We constructed several double mutants, (*gshA fis*, *gshA recC*, *gshA recG*, and *gshA uvrD* mutants), and found that each was more sensitive than either of the two single mutants involved (Table 5).

Perspective. Small molecules with antimicrobial properties can be characterized in any one of a number of ways, such as by their spectrum of activity on different bacterial strains, their effects on transcription at subinhibitory or inhibitory concentrations (19, 62, 63), or the type of synergistic interactions displayed with other antibiotics (30, 36, 60, 61). One can also examine antibiotics by their mechanism of action of stopping cellular growth or provoking cellular death, and recently, Collins and coworkers have argued that bactericidal antibiotics induce cellular death by a common mechanism involving the generation of deleterious hydroxy radicals (32). Here, we view antibiotics through their resulting susceptibility or "sensitivity

TABLE 5. Effect of single and double knockouts on sensitivity to MET

Strain[a]	MIC (μg/ml)
BW25113	1,000
fis mutant	500
recC mutant	500
recG mutant	300
uvrD mutant	300
gshA mutant	100
gshA fis mutant	50
gshA uvrD mutant	50
gshA recG mutant	25
gshA recC mutant	25

[a] See footnote for Table 2.

profiles," namely which mutants among the 4,000 *E. coli* knockout mutants in the Keio collection (3) are strongly susceptible to a particular antibiotic. Looking at the overall picture displayed in Fig. 2, several trends emerge. We see that among the 4,000 gene knockouts, only a subset of these generate phenotypes hypersusceptible to even one of the seven different antibiotics that we examined in this study. In total, 140 gene knockouts, approximately 3.5% of all the viable knockouts, are involved in generating strongly increased sensitivities to even one of the seven antibiotics. (This number should increase as a larger number of antibiotics are examined.) These data define potential targets for small-molecule inhibitors that might potentiate one or multiple antibiotics, as has been done in several cases (7, 37, 46; see the introduction). On the one hand, each antibiotic (or at this stage, class of antibiotic) is associated with a specific set of gene knockouts that result in strong susceptibilities, for instance, *gshA*, *gshB*, and *gor* in the case of MET and *mrcB* and *dacA* in the case of AMP. These are related to primary targets of the respective antibiotics, although pinpointing these is not always simple, as the mechanisms and targets of antibiotics are more complicated than previously thought (23; see also reference 6). On the other hand, there is a distinct group of gene knockouts that appear in the sensitivity profiles of more than one antibiotic (Fig. 2). Many of these involve efflux systems, chaperones, and genes involved in cell wall and cell membrane synthesis and integrity. Thus, *tolC* mutants, lacking a key efflux system, are more sensitive to six of the seven antibiotics tested and *tatC* mutants to four of the seven. Interestingly, some mutants in this category, such as *deoT* and *dksA* mutants, are transcriptional regulators. DeoT, a member of the DeoR family of global regulators, is involved in the control of multiple unrelated genes (16), while the DksA transcription factor is also involved in many processes (48), including double-stranded-break repair (43). Taken together, all of these data allow us to envision the cell as having points of vulnerability in each of the essential processes that are favored targets of antibiotics. Specific proteins play a crucial role in providing intrinsic resistance by, in some way, protecting these points of vulnerability. Thus, we can imagine the bacterial "intrinsic resistome," a conceptual structure that is defined by the effects that we observe as we systematically dismantle it, as seen in the increased effects of double-gene knockouts (Tables 2 and 5). The intrinsic resistome involves not only multidrug efflux systems and proteins that maintain the integrity of the cell wall and outer membrane but also those that shield DNA from access to certain damaging agents and processes and those that repair DNA, the ribosomal proteins that, while not absolutely essential for cell growth, give the ribosome some resistance to certain agents.

How do "sensitivity profiles" correlate with microarray studies? Does one see an increase in expression for the genes that are pinpointed by the increased sensitivity resulting from their inactivation? The best comparison would be with *E. coli* studies using subinhibitory concentrations of the same antibiotics, such as those carried out by Shaw and coworkers (54), who used AMP, RIF, and also norfloxacin (NOR), a fluoroquinolone closely related to CPR. There is little correlation between their results and those reported here. Thus, none of the eight genes upregulated by subinhibitory concentrations of AMP and none of the five scorable genes upregulated by RIF appear in the profiles reported here. Of the eight genes upregulated by NOR, two cause increased susceptibility to CPR when inactivated, these being among the SOS genes (*recA*, *recN*) induced by this agent. At concentrations at and above the MIC, the lack of correlation is pronounced (see also reference 29). Only 2 of 55 genes upregulated by NOR, 0 of 14 scorable genes upregulated by RIF, and 1 of 11 upregulated by AMP appear in the CPR sensitivity profile. Microarray analyses are being employed to characterize the mechanism of action of antibiotics (e.g., see reference 6 and 27). Sensitivity profiles might offer a way of further characterizing mechanisms of action (see below).

Future experiments. Extensions and applications of this work are aimed at the following: (i) high-throughput screening of small-molecule libraries for codrugs that work at the subinhibitory concentration shown here and that are specific inhibitors of some of the enzymes pinpointed as being involved in preventing hypersensitivity to various antibiotics (recall that the targets revealed by the study reported here do not, in most cases, result in growth inhibition or cell death when inactivated in the absence of antibiotics); (ii) design of specific inhibitors of some of this latter group of enzymes whose three-dimensional structure is known; (iii) using the most-sensitive single and double mutants to screen for additional antibiotics in soil communities and other microbial communities (there is precedent for this, as a number of groups have utilized engineered strains to detect antimicrobial activity [e.g., see references 13 and 26]); (iv) determining the mechanism of increased sensitivity in a number of cases revealed here, for instance, those involving transcriptional regulators; and (v) characterizing additional antibiotics, both in the exact manner described here and also by using the reduced set of strains determined by this work, to rapidly detect the sensitivity profiles of large numbers of characterized and uncharacterized antibiotics after a more complete catalog of these profiles for known antibiotics is compiled.

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