Strength of Selection Pressure Is an Important Parameter Contributing to the Complexity of Antibiotic Resistance Evolution

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Abstract

Revealing the genetic changes responsible for antibiotic resistance can be critical for developing novel antibiotic therapies. However, systematic studies correlating genotype to phenotype in the context of antibiotic resistance have been missing. In order to fill in this gap, we evolved 88 isogenic *Escherichia coli* populations against 22 antibiotics for 3 weeks. For every drug, two populations were evolved under strong selection and two populations were evolved under mild selection. By quantifying evolved populations' resistances against all 22 drugs, we constructed two separate cross-resistance networks for strongly and mildly selected populations. Subsequently, we sequenced representative colonies isolated from evolved populations for revealing the genetic basis for novel phenotypes. Bacterial populations that evolved resistance against antibiotics under strong selection acquired high levels of cross-resistance against several antibiotics, whereas other bacterial populations evolved under milder selection acquired relatively weaker cross-resistance. In addition, we found that strongly selected strains against aminoglycosides became more susceptible to five other drug classes compared with their wild-type ancestor as a result of a point mutation on TrkH, an ion transporter protein. Our findings suggest that selection strength is an important parameter contributing to the complexity of antibiotic resistance problem and use of high doses of antibiotics to clear infections has the potential to promote increase of cross-resistance in clinics.

Key words: antibiotic resistance, evolution, cross-resistance, genotyping, whole-genome sequencing, antibiotic susceptibility.

Introduction

Antibiotic resistance is a growing public health problem that was initially reported only a few years after the discovery of penicillin (Levy and Marshall 2004; Taubes 2008). Many antimicrobial drugs that were once effective against bacterial infections are now ineffective as a result of the evolution of antibiotic resistance through horizontal gene transfer and/or spontaneous mutations (Martinez et al. 2009; Davies and Davies 2010). These genetic changes render drugs ineffective by several mechanisms such as pumping out drug molecules by efflux pumps, altering drugs' target enzymes, and degrading or modifying drug molecules (Alekshun and Levy 2007). There have been numerous efforts at finding novel drug therapies that can stop or slow down the evolution of resistance (Lipsitch et al. 2000). These studies either focus on finding novel drugs or novel strategies using existing drugs that are already in clinical use (Chait et al. 2007; Cokol et al. 2011). Finding novel drugs is certainly an important and challenging task to combat infectious diseases and antibiotic resistance; however, any novel drug is prone to failure if not used

appropriately. Therefore, finding novel strategies to slow down the spread of resistance by using existing drugs is a very important task that is in fact as challenging and critical as finding novel drugs.

One strategy that has been used with varying degrees of success has been to prescribe drug combinations. Although drug combinations have been used primarily to increase efficacy against pathogens, it has also been hypothesized that they can slow down the evolution of resistance, based on a naive expectation that the likelihood of developing simultaneous resistance against two or more drugs would be several orders of magnitude lower than the likelihood of developing resistance against a single drug. Several systematic studies have been conducted to characterize interactions between drug pairs and biological mechanisms behind drug–drug interactions (Yeh et al. 2006, 2009; Bollenbach et al. 2009; Cokol et al. 2011). Use of synergistic drug pairs, which have higher inhibitory effects compared with each drug's individual effect, has been particularly important and useful for antibiotic therapies as this combination type allows treating infectious

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diseases with lower drug doses (Cokol et al. 2011). For example, trimethoprim and sulfamethoxazole, which are both folic acid biosynthesis inhibitors, have long been used together for treating bacterial infections due to their synergistic interactions. Although use of synergistic drug pairs has great benefits for short-term clinical applications, recent studies have shown that use of synergistic drug pairs may promote faster evolution of resistance (Chait et al. 2007; Hegreness and Kishony 2007; Michel et al. 2008). Furthermore, it was reported that use of antagonistic drug pairs for differentially selecting against antibiotic-resistant bacteria was possible for certain drug combinations and concentrations (Chait et al. 2007). These intriguing observations raise the question of whether it would be possible to use antagonistic drug pairs to slow down the evolution of resistance in clinics. However, clinical use of antagonistic drug pairs for treating bacterial infections is currently not an option as such therapies would require high doses of individual drugs that can be toxic to patients (Torella et al. 2010).

Since antibiotic resistance problems continue to persist to the present day despite the important recent findings summarized above, other novel strategies need to be developed and explored. Recent advances in next-generation sequencing technologies and high-throughput experimental tools have enabled researchers to revisit the antibiotic resistance problem and obtain a detailed understanding of both genotypic and phenotypic changes associated with antibiotic resistance (Lee et al. 2010; Nichols et al. 2011; Toprak et al. 2012). In several recent studies addressing evolution of resistance, bacterial populations were evolved against single drugs and resulting phenotypic and genotypic changes were characterized in detail (Lee et al. 2010; Comas et al. 2012; Hartkoorn et al. 2012; Toprak et al. 2012). One interesting observation was the common theme of developing cross-resistance against antimicrobial drugs to which bacteria have never been exposed before (Kohanski, DePristo, et al. 2010). Although efflux pumps that are encoded by multidrug resistance genes are often considered to be responsible for the emergence of cross-resistance, other genetic mechanisms can cause cross-resistance. For instance, cross-resistance may be caused by multiple pathway-specific mutations that confer cross-resistance to multiple drugs or extra mutations compensating for bacterial fitness acquired throughout evolution of the population, as many drugs inhibit bacterial growth by blocking more than one cellular pathway (Kohanski, DePristo, et al. 2010; Kohanski, Dwyer, et al. 2010).

Because cross-resistance can pose urgent dilemmas for clinicians treating highly resistant bacteria, we focus here on understanding and developing strategies to combat cross-resistance. Specifically, to better understand how selection strength potentiates the evolution of cross-resistance, we carried out a large-scale experiment where we evolved 88 isogenic *Escherichia coli* populations for 21 days against 22 clinically relevant antibiotics using strong selection for two populations evolving in parallel and mild selection for two populations evolving in parallel (Materials and Methods). We characterized evolved populations by quantifying their resistance levels against all 22 drugs at the end of 21 days and

constructed separate cross-resistance networks for populations evolved under strong and mild selection. Our analysis showed that evolution of cross-resistance was dependent on the selection strength experienced by the populations: Cross-resistance was weaker in populations that evolved under mild selection compared with populations that evolved under stronger selection. Interestingly, we also found that populations that evolved resistance against aminoglycosides under strong selection developed increased susceptibility against several other antibiotic classes. In order to find the genetic changes responsible for cross-resistance and increased susceptibility, we performed whole-genome sequencing (WGS) for 96 strains isolated from evolved populations. We identified several mutations that are responsible for cross-resistance within antibiotic classes and across different antibiotic classes. We found that mutations in TrkH, an ion transporter, were responsible for increased antibiotic susceptibility found in aminoglycoside-resistant populations. For the majority of the drugs, we found that strongly selected populations acquired higher number of mutations compared with mildly selected populations although they acquired similar levels of resistance (final resistance levels were not different within our experimental resolution) to the drug they were evolved against. Furthermore, mutations found in strongly selected and mildly selected populations were often found in diverse sets of genes, reflecting the plasticity of bacteria for developing antibiotic resistance. Strikingly, strongly selected populations acquired higher numbers of mutations in genes that were specific to the target pathways of the drugs used for selection compared with the mildly selected populations. The diversity of pathway-specific gene mutations was also larger for the strongly selected populations. Our results demonstrate that selection strength is an important parameter for evolution of cross-resistance.

Results

We evolved 88 isogenic *E. coli* populations against 22 different antibiotic compounds selected from seven drug classes (table 1 and supplementary fig. S1, Supplementary Material online) following two strategies. For each of the drugs, two isogenic populations were evolved in parallel under strong selection and two isogenic populations were evolved in parallel under mild selection yielding a total of 88 populations (fig. 1A and B and supplementary fig. S1, Supplementary Material online). The selection process was continued under both strong and mild selection for 21 days and for the majority of the drugs (15 out of 22), all populations reached to similar levels of resistance regardless of the selection strength (supplementary fig. S1 and table S1, Supplementary Material online). Figure 1C–E shows three representative trajectories showing the evolutionary dynamics of resistance under strong (red lines) and mild (black lines) selection strengths. At the end of 21 days, we plated cells from evolved populations and picked representative colonies for phenotypic and genotypic characterization (Materials and Methods). We name these strains based on the drugs used for selection and the selection strength (Materials and Methods). We quantified resistance levels of all representative

Table 1. List of All Drugs Used in Laboratory Evolution Experiments, Drug Abbreviation, Drug Solvent, MIC for the Wild-Type MG1655 *Escherichia coli* Strain, Commonly Used Clinical Dose (assuming an average body fluid volume of 40 l, daily drug doses are taken from http://www.globalrph.com, last accessed June 25, 2014), Highest MIC Reported in The European Committee on Antimicrobial Susceptibility Testing—EUCAST Database, Mechanism of Action, and Phenotypic Effects.

Drug	Solvent	MIC (µg/ml) for Wild-Type E. coli	Maximum Dose Used for Selection	Clinical Dose (µg/ml/day)	Highest MIC Reported in Literature (µg/ml)	Mechanism of Action	Phenotypic Effect
Chloramphenicol (CHL)	Ethanol	6.1	256	100	512	Protein synthesis, 50S	Bacteriostatic/bactericidal
Clindamycin (CLI)	DMSO	97.5	1,280	120	NA	Protein synthesis, 50S	Bacteriostatic/bactericidal
Erythromycin (ERY)	DMSO	65.80	1,280	100	NA	Protein synthesis, 50S	Bacteriostatic/bactericidal
Spiramycin (SPR)	Ethanol	260	2,560	75	NA	Protein synthesis, 50S	Bacteriostatic
Fusidic acid (FUS)	Water	647	3,200	37.5	NA	Protein synthesis, 50S	Bacteriostatic
Amikacin (AMK)	Water	14.36	640	37.5	32	Protein synthesis, 30S (aminoglycoside)	Bactericidal
Tobramycin (TOB)	Water	1.14	512	0.1875	32	Protein synthesis, 30S (aminoglycoside)	Bactericidal
Streptomycin (STR)	Water	15.2	16,3840	25	512	Protein synthesis, 30S (aminoglycoside)	Bactericidal
Kanamycin (KAN)	Water	11.70	1,280	37.5	512	Protein synthesis, 30S (aminoglycoside)	Bactericidal
Tetracycline (TET)	Ethanol	1.23	4.8	5	512	Protein synthesis, 30S	Bacteriostatic
Doxycycline (DOX)	Water	1.70	16	5	128	Protein synthesis, 30S	Bacteriostatic
Spectinomycin (SPT)	Water	61	40,960	50	512	Protein synthesis, 30S	Bactericidal
Piperacillin (PIP)	Water	1.88	128	375	≥512	β-Lactam, cell wall	Bactericidal
Ampicillin (AMP)	Water	4.4	40	50	512	β-Lactam, cell wall	Bactericidal
Cefoxitin (CEF)	Water	1.9	2,048	300	128	β-Lactam, cell wall	Bactericidal
Nalidixic acid (NAL)	Chloroform	7.9	300	100	512	DNA gyrase	Bactericidal
Lomefloxacin (LOM)	Water	0.3	6.4	10	NA	DNA gyrase	Bactericidal
Ciprofloxacin (CIP)	HCL	0.015	2.56	37.5	128	DNA gyrase	Bactericidal
Sulfamonomethoxine (SMO)	Acetone	1.40	410	NA	NA	Folic acid synthesis	Bacteriostatic
Trimethoprim (TMP)	DMSO	4.83	614	5	≥512	Folic acid synthesis	Bacteriostatic/bactericidal
Sulfamethaxozole (SUL)	Acetone	2.45	640	20	≥512	Folic acid synthesis	Bacteriostatic
Nitrofurantoin (NIT)	DMSO	4.75	320	10	128	Multiple mechanisms	Bacteriostatic/bactericidal

NOTE.—NA denotes the unavailability of information. The phenotypic effects of these drugs can be bacteriostatic or bactericidal depending on the dose.
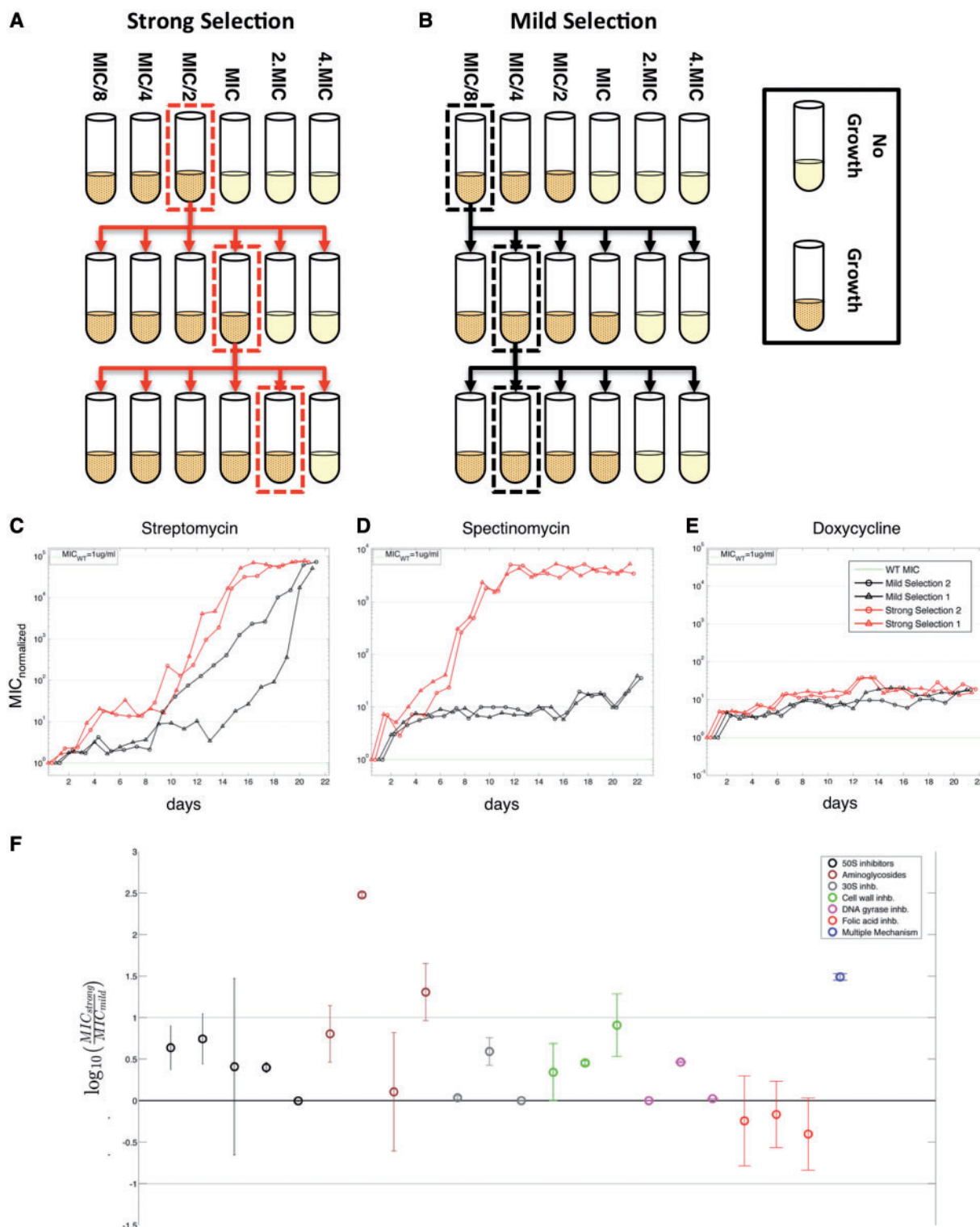
Fig. 1. Bacteria develop high antibiotic resistance under both strong and mild selection at the end of 21 days. (A) Schematic illustration of bacterial evolution experiments in liquid culture under strong selection. Bacterial populations were grown in several tubes with increased drug concentrations that span the expected MIC of the population. Populations were grown for approximately 22 h and the populations surviving in the highest drug concentration were transferred to new culture vials (yielding $60\times$ dilution, 6–7 generations per day if new mutants do not appear) with increasing drug concentrations. (B) Schematic illustration of bacterial evolution experiments in liquid culture under mild selection. (C–E) Resistance levels (MIC) recorded on a daily basis for parallel populations evolving under strong selection (red triangle and red circle) and mild selection (black triangle and black circle) against streptomycin (C), spectinomycin (D), and doxycycline (E). See supplementary table S1, Supplementary Material online, for daily-recorded MIC values for all evolved populations. (F) For every drug used in evolution experiments, average final resistance and standard deviation were calculated for the two populations evolving in parallel under strong selection and two populations evolving in parallel under mild selection. Logarithm of the ratios between final resistance of strongly selected strains and mildly selected strains are shown with colored circles. Mean value for this ratio is 0.47 ± 0.65 (mean $\pm$ standard deviation).

colonies against the drug used throughout evolution experiments (we term resistance of a strain against the drug used for selection as "direct-resistance") and compared the final resistance levels of all strongly selected and mildly selected strains as shown in figure 1F. For every drug, we calculated the mean value for the minimum inhibitory concentrations (MICs) of strongly selected and mildly selected populations and calculated the logarithm of the ratio between them ($\log_{10}$ (MIC_{strong}/MIC_{weak}), fig. 1F). Median value for this ratio was 0.43 ± 0.65 (mean $\pm$ standard deviation) indicating that strongly selected strains were on average approximately 2.7 times more resistant ($P = 0.01$, sign test) than the mildly selected strains.

Antibacterial drugs often inhibit bacterial growth by targeting more than one cellular pathway (Kohanski, Dwyer, et al. 2010). Therefore, we hypothesized that both strongly selected and mildly selected populations were likely to pleiotropically develop cross-resistance against several other drugs (Toprak et al. 2012). In order to test our hypothesis, we measured the resistance levels of all 88 representative strains we evolved against the 22 drugs used (Materials and Methods and supplementary table S2, Supplementary Material online). These measurements revealed drug-class-specific phenotypic signatures associated with elevated cross-resistance. Figure 2A demonstrates some of the measurements we performed for quantifying evolved strains' resistance levels against chloramphenicol, a protein synthesis inhibitor (Yeh et al. 2006). MIC for the ancestor wild-type strain was measured as approximately 6 µg/ml (fig. 2A, green line with circles) whereas CHL-S-2, one of the strains evolved against chloramphenicol under strong selection (fig. 2A, red line with circles) had about 40 times higher resistance against chloramphenicol compared with the wild-type ancestor strains. As we hypothesized, we found that many strains developed cross-resistance against drugs that they had never previously been exposed to. For instance, DOX-S-2, a strain evolved against doxycycline under strong selection (fig. 2A, orange line with triangles) was found to be approximately ten times more resistant against chloramphenicol than the wild-type ancestor strain (Toprak et al. 2012). However, to our surprise, we found that some of the evolved strains developed increased susceptibility (or negative cross-resistance) to several other drugs. TOB-S-2 is one such strain that is approximately ten times less resistant against chloramphenicol than its wild-type ancestor (fig. 2A, blue line with circles).

To determine whether there was a general trend for elevated antibiotic susceptibility and cross-resistance, we plotted cross-resistance matrices of the strains evolved under strong and mild selection (fig. 2B and C, respectively) where drugs are grouped according to the class they belong (table 1). All MIC values were normalized with the maximum MIC value measured for a particular drug such that elevated resistance against a drug appears as a red pixel whereas elevated susceptibility appears as a blue pixel (Materials and Methods). When we compared cross-resistance matrices for strongly selected and mildly selected strains, we found that frequency of elevated cross-resistance phenotypes was slightly higher across strongly selected strains than the mildly selected strains

(27% and 23%, respectively). Similarly, frequency of elevated antibiotic susceptibility was higher for strongly selected strains compared with mildly selected strains (10% and 6%, respectively). Elevated susceptibility was common among strains evolved under strong selection against aminoglycosides (fig. 2B); a relatively smaller portion of the strains evolved against aminoglycosides under mild selection had altered susceptibility against other drugs such as lomefloxacin (fig. 2C).

We constructed two separate cross-resistance networks for the strains evolved under strong selection (fig. 3A) and mild selection (fig. 3B) to determine phenotypic signatures associated with cross-resistance and susceptibility (Materials and Methods, supplementary fig. S2, Supplementary Material online). Increased resistance and susceptibility against a drug or drug class are shown using red and blue lines, respectively, with arrows at their ends. Thickness of a line represents the frequency of increased cross-resistance or antibiotic susceptibility against a drug or drug class. Color weight of a line represents the strength of cross-resistance or susceptibility against a drug class. For instance, a thick line with dark red color means that strong cross-resistance is commonly observed. All of the evolved phenotypes within drug classes are separately shown in these networks; however, only statistically significant cross-resistance and susceptibility across different antibiotic classes are represented for clarity (supplementary fig. S2, Supplementary Material online). Elevated cross-resistance within drug classes was quite common in both strongly selected and mildly selected strains, yet there were two exceptions for this observation where cross-resistance interactions within folic acid synthesis inhibitors and ribosomal inhibitors (30S) were rarely observed. Cross-resistance and susceptibility across different drug classes were more frequently observed among the strongly selected strains compared with mildly selected strains. There were nine cases where strains evolved against one drug class under strong selection pleiotropically evolved resistance against different drug classes, whereas this number was only four among the mildly selected strains (fig. 3 and supplementary fig. S2, Supplementary Material online). Similarly, strongly selected strains resistant to aminoglycosides were more susceptible to six other drug classes compared with their wild-type ancestor, whereas mildly selected strains against aminoglycosides were more susceptible to only two drug classes. Another intriguing observation was that none of the strains that acquired resistance against other drug classes developed resistance against aminoglycosides (fig. 3) suggesting that aminoglycoside resistance was idiosyncratic. Finally, strains that evolved resistance against folic acid synthesis inhibitors did not develop detectable resistance or susceptibility against other drugs suggesting that resistance against folic acid synthesis inhibitors was independent from other resistance mechanisms (Toprak et al. 2012).

Average cross-resistance per strain was higher for strongly selected strains compared with mildly selected strains (supplementary fig. S3, Supplementary Material online). For every evolved strain, we calculated mean cross-resistance values, and compared mean cross-resistance values of strongly selected and mildly selected strains. We found that
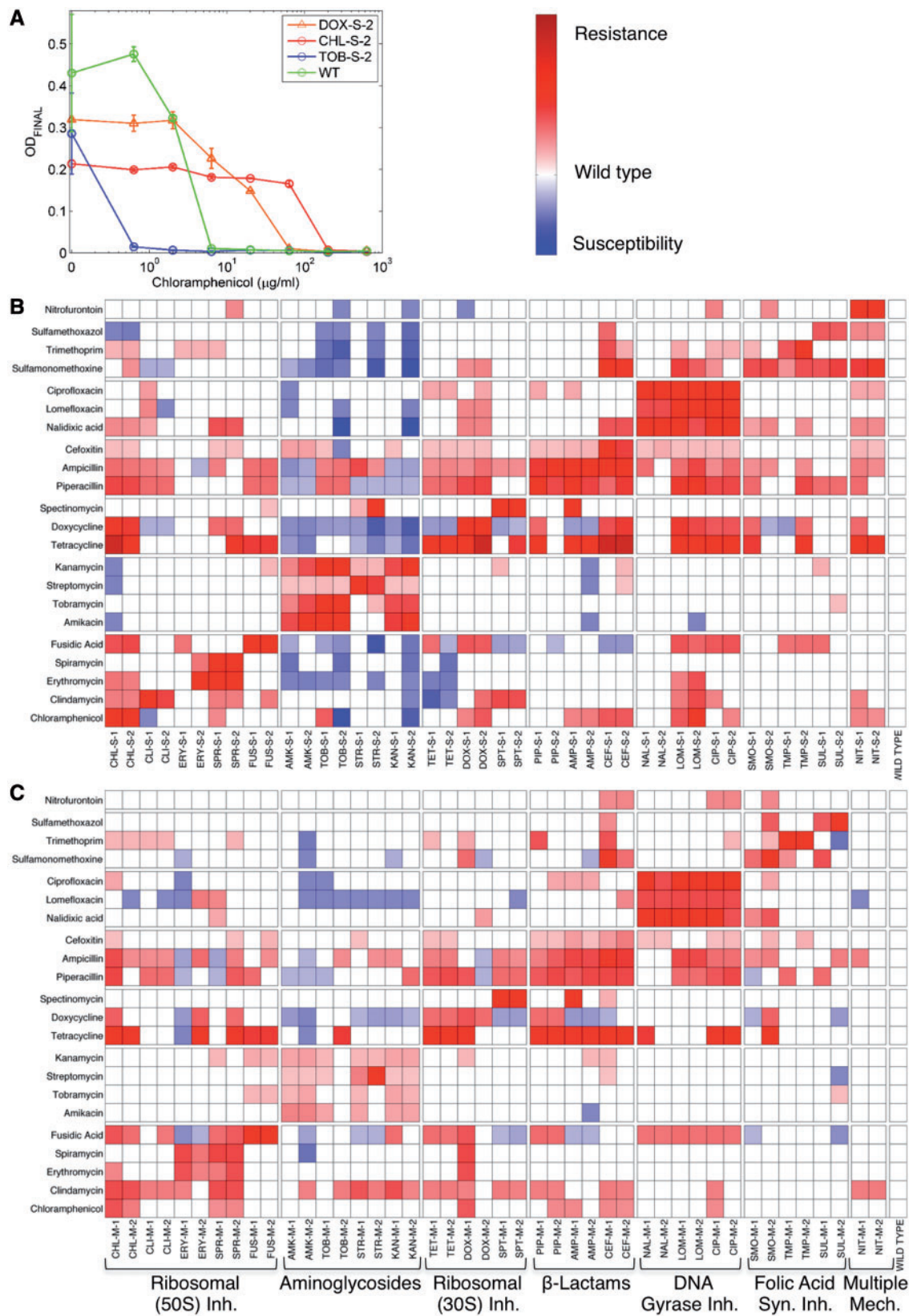
FIG. 2. Cross-resistance measurements of drug-resistant strains. (*A*) Representative strains selected from evolved populations were grown for approximately 22 h in duplicates in increasing drug concentrations and mean value of final OD reads was used to plot dose–response curves. MIC values were calculated by interpolating the drug concentrations corresponding to OD_{final} reads at 0.03. Chloramphenicol resistance of wild-type ancestor strain (green circles), a strain evolved against doxycycline (DOX-S-2, orange triangles), a strain evolved against chloramphenicol (CHL-S-2, red circles), and a strain against kanamycin (TOB-S-2, blue circles) were measured. (*B*) Cross-resistance measurements of strains evolved under strong selection and (*C*) mild selection. For every drug, we measured each strain's MIC and used the maximum direct-resistance (resistance evolved against the drug used for

(continued)

cross-resistance was stronger within the strains evolved under strong selection compared with the strains evolved under mild selection ($P = 1.4 \times 10^{-3}$, sign test). Next, we compared all of the cross-resistance and antibiotic susceptibility levels for strongly selected and mildly selected strains (fig. 2A and B, red and blue pixels, respectively). The cross-resistance levels of strongly selected strains were higher than cross-resistance levels of mildly selected strains ($P = 3 \times 10^{-2}$, Wilcoxon rank-sum test). Similarly, antibiotic susceptibility levels found in strongly selected strains were higher (more susceptible) than antibiotic susceptibility levels of mildly selected strains (2×10^{-5}, Wilcoxon rank-sum test). Finally, we tested whether relatively higher cross-resistance and antibiotic susceptibility of strongly selected strains was due to their higher direct-resistance (resistance evolved against the drug used for selection). We calculated Pearson's linear correlation coefficient between direct-resistance and cross-resistance levels of evolved strains (supplementary fig. S4, Supplementary Material online), and found that there was weak correlation ($R = 0.23$; $P = 3.3 \times 10^{-2}$). Therefore, we conclude that cross-resistance level cannot be explained simply by the strength of directly acquired antibiotic resistance.

In order to determine genetic changes responsible for elevated direct-resistance, cross-resistance, and susceptibility, we performed WGS for 88 evolved strains and the wild-type ancestor strain (supplementary table S3, Supplementary Material online). Furthermore, we sequenced two strains that were propagated for 28 days in the absence of any antibiotics in minimal media and found that there were deletions of 82 bp in the *pyrE-rph* operon in both strains. This deletion was previously reported as a genetic change related to adaptation in lactate minimal media (Conrad et al. 2009). In order to test whether *rph-pyrE* mutations had any effect on growth of resistant strains, we measured growth rates of all evolved strains (supplementary fig. S5, Supplementary Material online) and strains propagated in minimal media in the absence of drugs (for 21 and 28 days). The doubling time for the ancestor wild-type *E. coli* strain was 70 ± 4 min (mean $\pm$ standard deviation) whereas the strains propagated in minimal media without antibiotics were doubling every 48 ± 3 min proving that the reported deletion was indeed increasing the growth rate in minimal media (Materials and Methods). Mutations in the *rph-pyrE* operon were observed in 29 of the antibiotic-resistant strains and majority of these strains (24 out of 29) were growing significantly faster (supplementary fig. S5, Supplementary Material online; $P < 0.05$, Wilcoxon rank-sum test) than the wild-type ancestor strain. Of the faster growing strains, 20 were evolved under mild selection and 4 were evolved under strong selection explaining. Finally, 17 (12 strongly selected and 5 mildly selected) of

the resistant strains had significantly ($P < 0.05$, Wilcoxon rank-sum test) slower growth rates compared with the wild-type ancestor strain. Consistent with these observations, the average growth rate for the strains evolved under strong selection was 71 ± 16 min whereas the average growth rate for the strains evolved under mild selection was 59 ± 12 min.

In figure 4A and B, we show the genetic changes found in strains evolved against two of the drug classes by radially distributing mutations on circular plots according to mutations' locations on *E. coli* reference genome. Genetic changes found in strains evolved against rest of the drug classes are shown in supplementary figure S6, Supplementary Material online. Predicting the phenotypic effects of genetic changes such as single-nucleotide polymorphisms (SNPs) and insertions–deletions (indels) was relatively easier for us as gene amplifications were detected in only two strains, AMK-M-1 and SPR-S-2, in a region spanning *acrAB* multidrug resistance gene operon (Okusu et al. 1996). Gene amplifications were not very common in our evolved strains probably because of our experimental settings that promote gradual adaptation (Yona et al. 2012). About half of the genetic changes we detected were SNPs (113 SNPs; fig. 4, filled red and black circles) and the other half were indels (102 indels; fig. 4, filled red and black triangles). Strongly selected strains acquired 124 mutations in total (111 in coding regions and 13 in intergenic regions) and mildly selected strains acquired 91 mutations (83 in coding regions and 8 in intergenic regions). Among these mutations, there was only one synonymous mutation in *rhsB* gene. Interestingly, two of the strains evolved against cefoxitin (CEF-S-1 and CEF-S-2, supplementary table S3, Supplementary Material online) acquired 558 mutations in total and 139 of these mutations were synonymous mutations. We excluded genetic changes we found in these two strains from our analysis as they both are mutator strains.

Benefiting from the phenotypic and genotypic signatures specific to strongly and mildly selected strains, we carried out a comparative analysis to predict mutations responsible for resistance and cross-resistance (figs. 3 and 4). For this purpose, we curated all of the mutations found in evolved strains and listed the cellular pathways of mutated genes (fig. 5A and supplementary table S3, Supplementary Material online). As demonstrated in figure 5A, we grouped all of the strains that developed resistance against a particular drug and counted the number of mutations belonging to major cellular pathways, including protein synthesis, DNA/RNA synthesis, membrane proteins, folic acid synthesis, and multidrug resistance (Baba et al. 2006; Kohanski, DePristo, et al. 2010; Kohanski, Dwyer, et al. 2010; Nichols et al. 2011; Toprak et al. 2012). Genes that belonged to pathways other than the listed major

Fig. 2. Continued

selection) we measured against that drug for normalization. We color scale every measurement after calculating a metric for cross-resistance using the following formula: $MIC_{normalized} = (\log_{10} [MIC_{measured}/MIC_{WT}]/\log_{10} [MIC_{max}/MIC_{WT}])$. Cross-resistance measurements are shown in red for increased resistance and in blue for increased susceptibility with darker colors indicating stronger phenotypic changes. Increased resistance and susceptibilities less than 90% of the dilution factor of cross-resistance measurements are shown in white. See supplementary table S2, Supplementary Material online, for numerical values for MIC measurements.
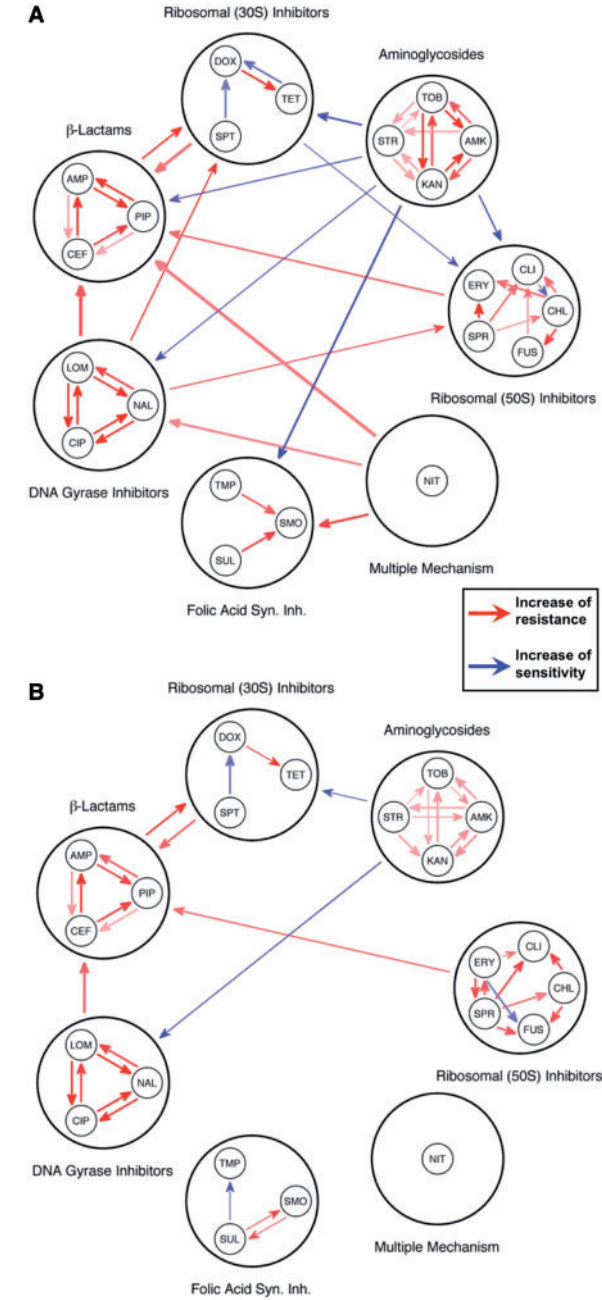
Fig. 3. Cross-resistance networks for strains evolved (*A*) under strong selection and (*B*) mild selection (Materials and Methods and supplementary fig. S2, Supplementary Material online). If a strain evolved in the presence of drug A developed resistance against drug B, a red line originating from drug A was drawn toward drug B with an arrow pointing drug B. Lines are colored in blue if increased susceptibility is observed and there are no lines when there is no detectable phenotypic change. Thicknesses of these lines represent the frequency of cross-resistance or antibiotic susceptibility, and darker colors indicate stronger phenotypic changes. All cross-resistance within drug classes are separately shown, however, only average cross-resistance and antibiotic susceptibility changes were shown (if existed) between drug classes for clarity.

Fig. 4. Mutations found in strains evolved against a drug class under strong selection (outer red circle) and mild selection (inner black circle) are shown with filled red and black markers, respectively. SNPs are shown with filled circles and indels are shown with filled triangles. Mutated genes' names are printed using standard annotations; however, mutations are printed as "unknown" if there are no annotated genes found in literature (supplementary table S3, Supplementary Material online). Pathway-specific mutations are printed in blue. (*A*) Mutations found in strains evolved against 50S ribosomal inhibitors. (*B*) Mutations found in strains evolved against aminoglycosides. The *TrkH* gene, which is mutated in five aminoglycoside-resistant strains, is shown with a magenta arrow.

pathways were grouped as "others" and genetic changes found in uncharted regions on the genome where we could not find annotated genes were grouped as "unknown." In addition, we determined whether mutated genes were
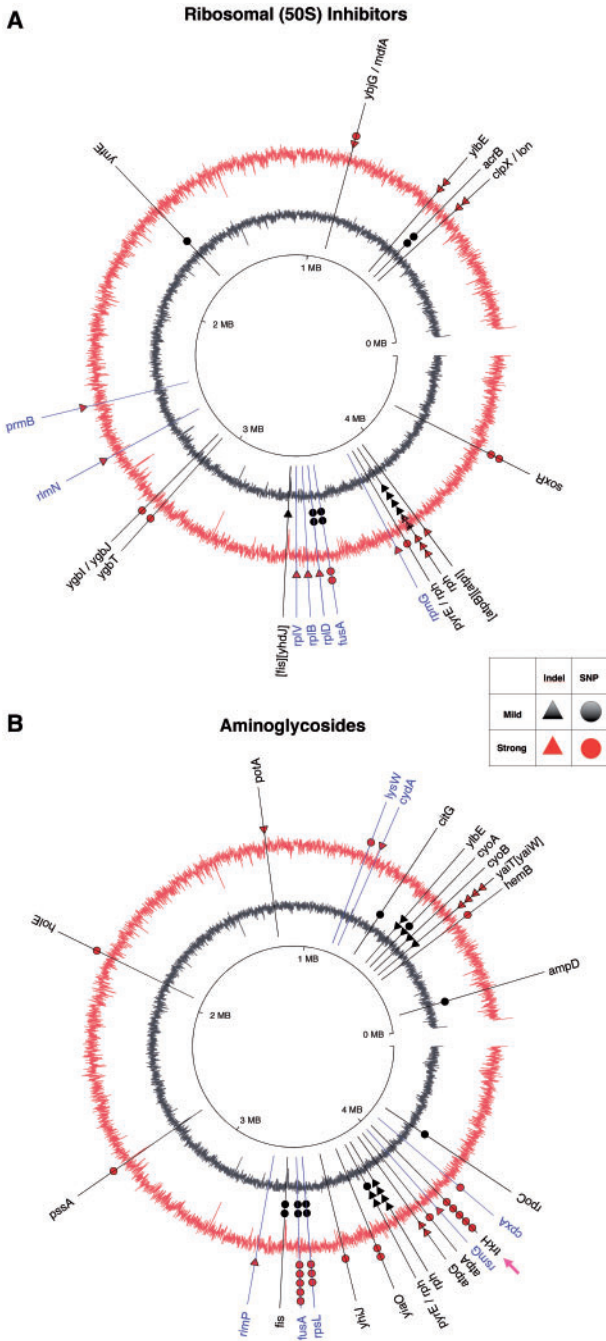
specific to the pathways inhibited by the drugs we used (Baba et al. 2006; Kohanski, DePristo, et al. 2010; Kohanski, Dwyer, et al. 2010; Nichols et al. 2011; Toprak et al. 2012). We name these genes as "pathway-specific genes" in the rest of
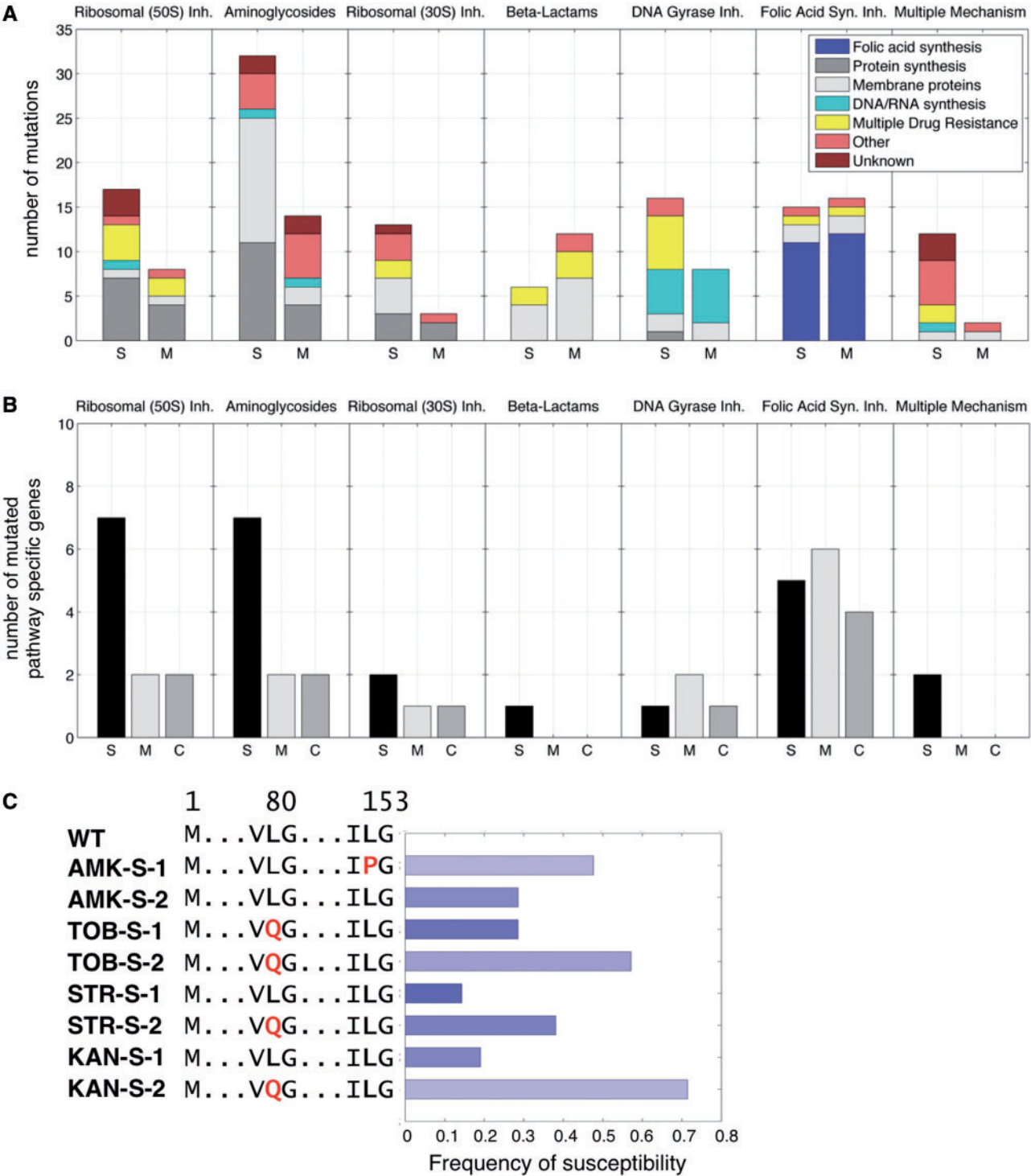
Fig. 5. (A) Diverse genetic changes found in antibiotic-resistant strains. For every drug class, numbers of mutations belonging to major cellular pathways are shown with different colors. These changes are grouped for strongly (S) selected strains and mildly (M) selected strains. (B) Number of mutated pathway-specific genes per drug class for strongly (S) selected strains, mildly (M) selected strains, and found to be common (C) in both strongly selected and mildly selected strains. (C) *TrkH* mutations and drug susceptibility of aminoglycoside-resistant strains. Color weight of the bars in the histogram indicates the strength of antibiotic susceptibility of strongly selected aminoglycoside-resistant strains.

the text. In figure 4 and supplementary figures S6–S10, Supplementary Material online, pathway-specific mutations are printed in blue. Finally, for every drug class, we counted number of mutated pathway-specific genes in the strains evolved under strong selection and mild selection (fig. 5B).

Our analysis allowed us to predict several genetic changes responsible for resistance and cross-resistance (table 2). For every drug that was used in evolution experiments, we searched for mutated genes that were pathway-specific and frequently mutated genes. For all of the drugs we used in

Table 2. Drug Classes, Drugs Used for Selection, Mutated Pathway-Specific Genes, Mutated Off-Pathway Genes.

Drug Class	Drug	Pathway-Specific Mutations	Off-Pathway Mutations
50S, Protein synthesis inhibitor	Chloramphenicol		[soxR]*; [mdfA]*
50S, Protein synthesis inhibitor	Clindamycin	[prmB]; [rplB]*; [rpmG]	
50S, Protein synthesis inhibitor	Fusidic acid	[fusA]*	[ylbE]
50S, Protein synthesis inhibitor	Spiramycin	[rplD]*; [rlmN]*	
50S, Protein synthesis inhibitor	Erythromycin	[rplV]*	[acrB]*; [fis]; [ylbE]
Aminoglycosides	Streptomycin	[lysW]; [rimP]; [rpsL]*	[trkH]
Aminoglycosides	Amikacin	[cydA]; [fusA]*	[trkH]; [ylbE]
Aminoglycosides	Kanamycin	[cpxA]*; [fusA]*	[trkH]; [fis]
Aminoglycosides	Tobramycin	[fusA]*	[trkH]; [fis]; [atpG]; [yiaO]; [ylbE]
30S, Protein synthesis inhibitor	Doxycycline		[acrR]*; [fis]; [marR]*
30S, Protein synthesis inhibitor	Spectinomycin	[rpsE]*; [rplB]*	
30S, Protein synthesis inhibitor	Tetracycline		[mlaA]
Cell-wall synthesis inhibitors	Ampicillin	[ftsI]*	[acrB]*; [envZ]*
Cell-wall synthesis inhibitors	Cefoxitin	[ftsI]*	[acrB]*; [envZ]*; [ompR]*; [ompF]*
Cell-wall synthesis inhibitors	Piperacillin	[ftsI]*	[envZ]*; [ompR]*
DNA gyrase inhibitors	Ciprofloxacin	[gyrA]*; [gyrB]*	[acrR]*; [ompF]*
DNA gyrase inhibitors	Nalidixic acid	[gyrA]*	
DNA gyrase inhibitors	Lomefloxacin	[gyrA]*	[acrR]*; [marR]*
Folic acid synthesis inhibitor	Trimethoprim	[folA]*	
Folic acid synthesis inhibitor	Sulfamethaxozole	[folP]*; [folM]*; [folX]*	
Folic acid synthesis inhibitor	Sulfamonomethoxine	[folM]*	[mprA]*; [ompR]*
Multiple mechanism	Nitrofurantoin	[nfsA]*; [rpoA]	[mprA]*; [ompR]*; [ylbE]

NOTE.—Genes that are previously reported to be related to antibiotic resistance are marked with asterisks.

evolution experiments (except chloramphenicol, doxycycline, and tetracycline), we were able to identify several pathway-specific gene mutations in evolved strains. Strongly selected strains acquired a total of 46 pathway-specific gene mutations (~1.1 pathway-specific gene mutations per strain) whereas mildly selected strains acquired 28 mutations in pathway-specific genes (~0.6 pathway-specific gene mutations per strain). Most of the pathway-specific genes (15 out of 21) were found in more than one strain. In addition, many of the mutated pathway-specific genes had the exact same amino acid replacement or nucleotide change in promoter regions, suggesting that these genetic changes were selected for (table 2). Several of the mutated pathway-specific genes (table 2, genes marked with asterisks) are genes previously reported to be involved in antibiotic resistance in studies using clinical isolates or isolates from laboratory evolution experiments. For example, fusA, a protein chain elongation factor, was mutated in 11 strains and L438Q amino acid replacement was found in three fusidic acid-resistant strains (Norstrom et al. 2007). Similarly, gyrA mutations were found to be in 10 strains resistant against DNA gyrase inhibitors and in eight cases the amino acid replacement was S83L (Heisig et al. 1993). Therefore, we conclude that in both strongly selected and mildly selected strains, pathway-specific mutations listed in table 2 are selected for throughout evolution of antibiotic resistance.

Following a similar algorithm, we searched for genes mutated more than once and did not belong to cellular pathways inhibited by the drugs we used (table 2). We term these genes "off-pathway" genes. We found that strongly selected strains

acquired a total of 71 mutations (~1.7 mutations per strain) in off-pathway genes and mildly selected strains acquired 38 mutations (~0.86 mutations per strain) in off-pathway genes (mutations in the pyrE-rph operon are excluded as they are involved in minimal media adaptation). Phenotypic effects of off-pathway mutations cannot be simply attributed to multi-drug resistance concept as only six of these off-pathway mutations were previously classified as multidrug resistance genes (acrB, acrR, marR, mdfA, mprA, and soxR; table 2) (Alekshun and Levy 2007; Kohanski, DePristo, et al. 2010). Mutations in off-pathway genes may instead elevate antibiotic resistance either independently or together with other mutations. Alternatively, mutations found on the off-pathway genes in antibiotic-resistant bacteria may also have compensatory effects as resistance-conferring mutations in essential enzymes may often come with a fitness cost (Baba et al. 2006; Lozovsky et al. 2009). Some of the off-pathway genes have the exact same mutations multiple times indicating that these mutations were selected for throughout evolution of antibiotic resistance. As an example, envZ which is a membrane protein involved in osmoregulation had a V241G amino acid replacement in five evolved strains (Jaffe et al. 1983). Strains evolved against chloramphenicol, doxycycline, and tetracycline had only off-pathway mutations in genes such as soxR and acrB whereas strains evolved against fusidic acid, nalidixic acid, trimethoprim, and sulfamethaxozole did not have any off-pathway gene mutations. The remainder of the strains acquired mutations in both pathway-specific and off-pathway genes. Consistent with their genotypes, strains evolved against fusidic acid, nalidixic acid, trimethoprim, and

sulfamethaxozole developed low levels of cross-resistance against drugs from different classes (figs. 2 and 3). For instance, nalidixic acid-resistant strains, which carried only gyrA mutations, had cross-resistance against only DNA gyrase inhibitors. On the contrary, strains carrying pathway-specific and off-pathway mutations such as ciprofloxacin-resistant strains that carried mutations in gyrA, acrR, and ompF genes developed cross-resistance against DNA gyrase inhibitors and also many other drugs from other classes. As off-pathway mutations were more frequent in strongly selected strains and cross-resistance between different drug classes were more profound in strongly selected strains compared with the mildly selected strains (fig. 3), we conclude that mutations in the off-pathway genes we identified (table 2) are involved in cross-resistance between different drug classes.

Bacterial populations developed antibiotic resistance by accumulating diverse genetic changes regardless of the selection strength throughout evolution, reflecting the plasticity of bacteria for adaptation and limiting the predictability of antibiotic resistance evolution (Weinreich et al. 2006; Conrad et al. 2009; Lozovsky et al. 2009; Palmer and Kishony 2013). On average, strongly selected strains acquired approximately three mutations (124 mutations in total) and mildly selected strains acquired approximately two mutations (91 mutations in total) where these mutations belonged to a diverse set of cellular pathways regardless of the selection strength (fig. 5, top panel). Furthermore, even mutations found in two populations evolved against the same drug in parallel were very rarely identical regardless of the selection strength. The highest reproducibility regarding the genetic changes responsible for resistance against a particular drug was observed for DNA gyrase inhibitors and folic acid synthesis inhibitors which are synthetic drugs designed to inhibit activity of very specific enzymes (Flensburg and Skold 1987; Ohmae et al. 2001; Ruiz 2003; Watson et al. 2007). However, even for these target-specific drugs, there was high diversity among evolved strains' genotypes: We found several other mutations in off-pathway genes and also different amino acid replacements on the target enzymes such as folA (table 2 and supplementary table S3, Supplementary Material online).

In our experimental settings where bacteria evolved against antibiotics under strong selection, we expected to find more genetic changes targeting pathway-specific genes compared with mildly selected strains as bacterial evolution takes place at the edge under strong selection. Indeed, average number of pathway-specific genetic changes for strains evolved under strong selection was higher than the average number of pathway-specific mutations for strains evolved under mild selection ($\sim$1.1 $\pm$ 0.9 and $\sim$0.65 $\pm$ 0.75 for strongly and mildly selected strains, respectively [mean $\pm$ standard deviation]; $P = 0.0085$, Wilcoxon rank-sum test). The number of pathway-specific mutations per strain per drug class which were observed in both strongly selected strains and mildly selected strains was 0.57 indicating that many of the pathway-specific gene mutations found in mildly selected strains were also existing in strongly selected strains. More specifically, 9 out of 11 pathway-specific mutations found in mildly selected strains were also mutated in the strongly

selected strains. In addition, when we compared the diversity of pathway-specific genes in strongly selected strains with mildly selected strains, we found that the diversity of pathway-specific genes in strongly selected strains was 2-fold larger than the diversity found in mildly selected strains (fig. 5B). For example, there were seven different pathway-specific genes that were commonly mutated in seven strains evolved against aminoglycosides whereas eight mildly selected strains against aminoglycosides accumulated mutations in only two pathway-specific genes (fig. 5B). We conclude that bacterial populations are more likely to develop cross-resistance within a drug class and across different drug classes when evolved under strong selection because of accumulating high number of diverse pathway-specific and off-pathway genetic changes.

Finally, we searched for the genetic changes responsible for increased susceptibility of aminoglycoside-resistant strains. Aminoglycoside-resistant strains that were evolved under strong selection had significant susceptibility against other antibiotic classes and this effect was more evident in six of the eight aminoglycoside-resistant strains. We looked for genetic changes specific to only these six strains and found that five of the strains had one mutation on TrkH gene (fig. 4B, magenta arrow) that codes for TrkH protein, a potassium ion transporter (Cao et al. 2011). Figure 5C shows the amino acid changes found in these strains together with frequency and strength of drug susceptibility detected for the strongly selected aminoglycoside-resistant strains. Four of these strains had an amino acid replacement at position 80 (L80Q) and one strain had an amino acid replacement at position 153 (L153P). Strains with TrkH mutations, on average, had susceptibility against six other drugs suggesting that mutations in TrkH were responsible for elevated susceptibility. This finding was consistent with a recent study by Lazar et al. (2013) where the authors reported that an amino acid change on TrkH gene (T350L) was primarily responsible for elevated antibiotic hypersensitivity in E. coli. Consistent with our observations, it was proposed that TrkH mutations antagonized the activity of proton-motive-force-dependent major efflux pumps and hence led to antibiotic hypersensitivity by increasing the effective antibiotic concentration inside bacterial cytoplasm (Lazar et al. 2013).

Discussion

In this study, we highlight selection strength as an important factor that can add to the complexity of antibiotic resistance evolution. Combining large-scale laboratory evolution experiments with high-throughput phenotyping and subsequent genotyping, we provided evidence that selection strength throughout the evolution of antibiotic resistance is a critical factor that can potentiate evolution of cross-resistance. Bacterial populations that evolved resistance against antibiotics under strong selection developed cross-resistance against several antibiotics, whereas other bacterial populations evolved in parallel under mild selection acquired relatively weaker cross-resistance. Variations between cross-resistance and antibiotic susceptibilities of strongly selected strains and mildly selected strains were largely due to the

presence of the higher number of pathway-specific and off-pathway mutations in strongly selected populations.

Strength of selection pressure in an evolving population is an important factor that affects the phenotypic and genotypic diversity. When selection pressure is mild, several subpopulations with diverse genotypes can coevolve if these subpopulations are able to tolerate growth stress. In experimental settings like ours where antibiotic dose was gradually increased to maintain mild selection strength, evolving bacterial populations may have opportunities to acquire resistance-conferring mutations with relatively lower fitness costs, such as mutations in multidrug resistance genes or transcription factors instead of more costly mutations in essential enzymes (i.e., RNA polymerases and ribosomal genes) (Bilgin et al. 1990; Baba et al. 2006; Lozovsky et al. 2009). On the other hand, for populations evolving under strong selection pressure, the options for resistance-conferring mutations may be more constrained. This is because under strong selection, survival, not fitness, becomes the immediate priority, upon first introduction of the selection pressure. Thus, populations evolving under strong selection may acquire resistance-conferring mutations for survival even if these mutations target important cellular machineries (such as ribosomes) and have large fitness costs. In such cells, costly genetic changes may appear early in evolution and emergence of further genetic changes that can compensate for fitness loss or increase the resistance even further is expected to appear later in evolution (Lieberman et al. 2011; Comas et al. 2012; Toprak et al. 2012). The diversity we observed in the genotypes of clones evolved under strong selection pressure was indeed larger; these clones had more pathway-specific mutations and off-pathway mutations compared with mildly selected clones. For example, strains evolved against 50S ribosomal inhibitors under mild selection contained mutations in only two pathway-specific genes, fusA and RplD, which are both involved in protein translation (fig. 4A). On the other hand, strongly selected strains acquired mutations in seven essential genes involved in protein translation (fusA, RplB, RplD, RplV, RpmG, prmB, and rlmN).

Cross-resistance is an obstacle for designing effective drug therapies as it limits possible antibiotic options following an unsuccessful drug treatment of a patient and also imposes problems for patients who get colonized by resistant bacteria. Nevertheless, except the early studies of Szybalski and Bryson, evolution of cross-resistance has not received much attention until very recently (Szybalski and Bryson 1952; Chao 1986; Toprak et al. 2012; Dragosits et al. 2013; Imamovic and Sommer 2013; Lazar et al. 2013). In a recent study by Imamovic and Sommer (2013), E. coli cells were grown in whole-gradient plates for several generations and resistance levels of evolved populations against 24 antibiotics were quantified to construct cross-resistance networks. In that study, cells growing at the highest drug concentrations were continuously propagated until growth was observed in all areas of the whole-gradient plates. Despite the differences between their and our experimental settings (i.e., different drugs and experimental protocols), there are substantial similarities between the cross-resistance and

antibiotic susceptibilities they observed and cross-resistance and antibiotic susceptibilities we observed in populations evolved under strong selection pressure. This similarity is in fact not surprising as the selection assay adapted by Imamovic et al. (2013) applies strong selection pressure in evolving populations. Imamovic et al. also showed that populations evolving against aminoglycosides developed increased susceptibility against many other drugs, which was consistent with our findings (figs. 2 and 3). Although they did not provide genetic data for cross-resistance and susceptibility, they elegantly demonstrated that increased susceptibility can be used to design therapies where drug pairs are deployed in cycles to minimize evolution of resistance (Imamovic and Sommer 2013). In a second independent study, Lazar et al. (2013) evolved E. coli populations against several antibiotics in liquid cultures where drug concentrations were gradually increased (comparable to the assay we used for strong selection) or kept constant at sublethal concentrations. Lazar et al. (2013) also showed that bacteria develop cross-resistance against several drugs and they also reported that populations that evolved against aminoglycosides developed resistance against several other drugs. By sequencing evolved strains and using biochemical and biophysical tools, they thoroughly showed that mutations in TrkH protein were primarily responsible for the elevated susceptibility. Although both of these studies share similar conclusions regarding the evolution of cross-resistance and susceptibility, neither addresses the contribution of selection strength pressure on the evolution of cross-resistance. Increased antibiotic susceptibility as a result of evolution of resistance against aminoglycosides will certainly open new avenues in antibiotic therapies. However, further studies are needed to address this phenomenon in detail by using clinical pathogens and also answer why elevated susceptibility develops only when bacteria evolve against aminoglycosides.

We conclude that selection strength is a hidden factor that contributes to the evolution of resistance, cross-resistance, and susceptibility. Since populations generally evolve similar levels of resistance to the drug they were evolved against regardless of the selection strength, the differences in their cross-resistance phenotypes are often overlooked, even though cross-resistance and antibiotic susceptibility can significantly change the epidemiology of resistance in clinics. In clinical use of drugs, understandably, use of the highest possible doses of antibiotics (strong selection) that are nontoxic to patients is a common practice to treat patients with persistent bacterial infections. Our study highlights how this practice has the potential to promote the increase of cross-resistance. Future studies to optimize selection pressure strength in clinical applications to minimize evolution of cross-resistance are necessary to find novel therapeutic strategies to combat the evolution and spread of infectious diseases.

Materials and Methods

Bacterial Strains, Media, and Growth Conditions

Drug-sensitive, wild-type MG1655 E. coli strain was used in all experiments. Bacterial cells were grown at 30 °C in sterile M9

minimal medium supplemented with 0.4% glucose and 0.2% amicase (Sigma).

Laboratory Evolution Experiments

A total of 88 isogenic *E. coli* populations were evolved against 22 different antibiotic compounds (table 1) following two strategies. For each of the drugs, two isogenic populations were evolved in parallel under strong selection and two isogenic populations were evolved in parallel under mild selection. On a daily basis, for every evolving population, several sterile vials were filled with 3 ml of minimal growth media containing a range of drug concentrations that spanned the expected MIC of evolving populations (fig. 1A and B). Cultures were grown for approximately 22 h and every culture was inspected for growth either visually or with the help of a spectrophotometer if growth was not clear to the eye. Average population size when cultures reached to the steady state was between 5×10^9 and 10^{10} cells. The minimum drug concentration that inhibited growth ($OD_{final} < 0.1$) was daily recorded as MIC of the population (fig. 1A and B). On a daily basis, 1 ml of cells was frozen and stored at $-80\,°C$ in 15% glycerol for further characterization. For populations evolving under strong selection, on a daily basis, a small volume (30 μl) of cells from the culture that was grown in the drug concentration equal to half of MIC was added to each of the new culture vials with increasing drug concentrations (fig. 1A). For the populations evolving under mild selection, cells growing at a drug concentration equal to one-eighth of MIC value were propagated to culture vials with increasing drug concentrations (fig. 1B). This process was continued for both strong and mild selection for 21 days (supplementary fig. S1 and table S1, Supplementary Material online). Evolved populations were named based on the drugs used for selection and the selection strength. For example, DOX-S-1 is the first population evolved in the presence of doxycycline under strong selection and TOB-M-2 is the second population evolved in the presence of tobramycin under mild selection.

Phenotyping Evolved Populations

At the end of 21 days, cells from evolved populations are plated and ten random colonies from each population are picked. All of the colonies were grown in liquid media in increasing drug concentrations and their MIC values were quantified. Resistance levels of these randomly picked colonies did not show variations in their MIC values within our experimental resolution; therefore, one colony from all evolved populations were assigned as representative colonies to carry out all future genotyping and phenotyping experiments. Representative colonies were organized in a 96-well master plate for simultaneous MIC measurements for all strains. Drug resistances of representative strains were measured using a plate reader (Tecan M200). For every drug, several 96-well plates were filled with drugs in minimal growth medium, with increasing drug concentrations. Each well in a 96-well plate was filled with 130 μl of growth medium with the same concentration. Drug concentration

of these plates ranged from drug free to the highest concentration that we can dissolve in growth medium. Drug concentrations across plates were diluted by a factor of $10^{1/2}$ ($[drug]_{n-1} = 10^{1/2} \times [drug]_n$). However, a dilution factor of $2^{1/2}$ was used for the drugs where even the most resistant strain developed only 10- to 20-fold resistance compared with the ancestor wild-type strain. Cells in master plates were transferred into 96-well plates using a 96-pinner (V&P Scientific) and were grown for approximately 22 h with rapid shaking at $30\,°C$. Final optical densities (OD_{final}) of the cells were measured using a plate reader (Tecan M200). Phenotyping experiments were performed in duplicates for every drug and the mean values of these measurements were used for MIC calculations. Background-corrected OD_{final} reads from phenotyping experiments were used to calculate the MIC values of the evolved strains. We calculated mean OD_{final} values for every strain in every drug concentration we used. The MIC values were calculated by interpolating the drug concentrations corresponding to mean OD_{final} reads corresponding to 0.03 (fig. 2A).

Constructing Cross-Resistance Networks

Resistance levels of all evolved strains against all 22 drugs are normalized with the maximum direct-resistance against each drug and a 22×88 matrix for all resistant strains is created (supplementary table S2, Supplementary Material online). These MIC values are then converted to trinary values of -1, 0, and 1, respectively, for antibiotic susceptibility, unchanged resistance, and increased resistance. For both strongly selected and mildly selected strains, evolved strains are clustered according to the drug classes they were evolved against and their cross-resistance frequencies (f_{CR}) and antibiotic susceptibility frequencies (f_{AS}) against each drug class are calculated. Furthermore, we calculate the mean cross-resistance ($0 \le CR \le 1$; 1 being the strongest possible resistance) and antibiotic susceptibility ($-1 \le AS \le 0$; -1 being 20-fold less resistance compared with the wild-type ancestor) values for every cluster. In this way, we generated a 7×7 matrix (supplementary fig. S2, Supplementary Material online) with frequency and cross-resistance (or antibiotic susceptibility) values for strongly selected (panels on the left) and mildly selected (panels on the right) strains. The 22×88 trinary matrix is then randomly shuffled for 10^5 times and the actual f_{CR} and f_{AS} values for each cluster are recorded (histograms in panels). Finally, we calculated the probability (P) of randomly getting a frequency higher than the actual f_{CR} and f_{AS} values. We consider the phenotypic changes within clusters which have P values less than 0.05 as significant and score these interactions as increased cross-resistance or increased antibiotic susceptibility. A similar algorithm is used for scoring the phenotypic changes within drug classes. For every pair of strains evolved against a particular drug, we calculated the frequency and strength of resistance or susceptibility against another drug from the same drug class. Phenotypic changes within drug classes are then represented in figure 3 using arrowed lines. Thicknesses of these lines represent the

frequency of cross-resistance or antibiotic susceptibility, and darker colors indicate stronger phenotypic changes.

Whole-Genome Sequencing

Isogenic bacterial cells were genotyped by Illumina WGS using a HiSeq platform. Agar stabs of evolved strains were submitted to Genewiz Incorporation for sequencing service. Service from Genewiz included genomic DNA extraction, library preparation, multiplexing, sequencing, and data delivery. Sequencing was performed on the Illumina HiSeq2000 platform, in a 2×100 bp paired-end configuration, with at least $100\times$ coverage for each sample. We aligned resulting reads onto the MG1655 reference chromosome (NC_000913.2) using the Bowtie 2 toolkit (Langmead and Salzberg 2012). We analyzed aligned sequences for genetic changes using SAMtools and BRESEQ software (Barrick et al. 2009; Li et al. 2009). Both algorithms had similar performances for finding SNPs; however, BRESEQ was more efficient in finding indels. For all of the genetic changes that were not detected by both algorithms, we did a visual inspection to confirm the genetic changes. All genetic changes we found are listed in supplementary table S3, Supplementary Material online. To test the accuracy of sequencing procedure, we sequenced six strains twice and found that there were no differences in the detected genetic changes between sequenced replicates. Genetic changes were detected in 86 of the 88 evolved strains. No mutations or gene amplifications were found in two strains (KAN-S-1 and ERYS-1). When we examined resistance phenotypes of these two strains, we found that ERY-S-1 was not different than the wild type although ERY-S-1 population was approximately 50-fold more resistant than the wild type, indicating a possible experimental error during the sequencing process. However, KAN-S-1 was resistant to kanamycin as well as several other drugs. The reason why no genetic changes were detected in this strain is unknown to us. In order to see whether there were any contaminations in our sequenced strains, we compared all of the genetic changes we found for sequenced strains. Among all sequenced strains, only TMP-S-2 and TMP-M-1 had exact same mutations but all three mutations found in the genomes of TMP-S-2 and TMP-M-1 are well-known and frequently found mutations in folA gene that confer resistance against trimethoprim (Toprak et al. 2012). Finally, both of the strains evolved against cefoxitin under strong selection became mutator strains and had more than 250 genetic changes per strain although both strains evolved against cefoxitin under mild selection had 4–5 mutations per strain. Therefore, we excluded genotypic data coming from cefoxitin-resistant population from our analysis we used for figures 4 and 5. Genetic changes found in these strains are still listed in supplementary table S3, Supplementary Material online, as a separate sheet. WGS data from the evolved strains have been deposited in the NCBI Sequence Read Archive (SRP042989).

Functional Classification of Mutated Genes

Functions of all mutated genes and the cellular pathways mutated genes belonged to were found using the EcoCyc gene database for the bacterium E. coli K-12 MG1655 (see URLs in supplementary table S3, Supplementary Material online) (Keseler et al. 2013). For all the strains evolved against a drug class, we identified pathway-specific genes by asking whether mutated genes found in these strains were found in the pathways targeted by that particular drug class. We classified such genes as "pathway-specific genes" and the rest of the genes were classified as "off-pathway genes."

Supplementary Material

Supplementary figures S1–S6 and tables S1–S3 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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