Limited Evolutionary Conservation of the Phenotypic Effects of Antibiotic Resistance Mutations

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Abstract

Multidrug-resistant clinical isolates are common in certain pathogens, but rare in others. This pattern may be due to the fact that mutations shaping resistance have species-specific effects. To investigate this issue, we transferred a range of resistance-conferring mutations and a full resistance gene into *Escherichia coli* and closely related bacteria. We found that resistance mutations in one bacterial species frequently provide no resistance, in fact even yielding drug hypersensitivity in close relatives. In depth analysis of a key gene involved in aminoglycoside resistance (*trkH*) indicated that preexisting mutations in other genes—intergenic epistasis—underlie such extreme differences in mutational effects between species. Finally, reconstruction of adaptive landscapes under multiple antibiotic stresses revealed that mutations frequently provide multidrug resistance or elevated drug susceptibility (i.e., collateral sensitivity) only with certain combinations of other resistance mutations. We conclude that resistance and collateral sensitivity are contingent upon the genetic makeup of the bacterial population, and such contingency could shape the long-term fate of resistant bacteria. These results underlie the importance of species-specific treatment strategies.

Key words: **antibiotic resistance, collateral sensitivity, multidrug resistance, evolution of mutational effects.**

Introduction

The rapid emergence and spread of antibiotic resistance mechanisms in bacteria may soon lead to a world-wide health crisis of grave proportions (Bush et al. 2011). As the development and discovery of novel drugs has slowed significantly over recent decades, alternative avenues to combat resistant microbes are required (Laxminarayan 2014). Multidrug strategies aim to exploit the evolutionary trade-offs that resistance mutations present by identifying pairs of drugs where adaptation to one antibiotic simultaneously induces susceptibility to another (Baym et al. 2016). This phenomenon, known as collateral sensitivity (Pál et al. 2015), was first observed in a pioneering study 65 years ago (Szybalski and Bryson 1952). Recent years have seen a renewed focus on the characterization and potential exploitation of collateral sensitivity phenomena (Pál et al. 2015; Baym et al. 2016) with several in vitro, system-wide studies revealing general patterns (Imamovic and Sommer 2013; Lázár et al. 2013, 2014; Munck et al. 2014; Oz et al. 2014; Rodriguez de Evgrafov et al. 2015; Imamovic et al. 2018) and specific molecular mechanisms (Lázár et al. 2013; Imamovic et al. 2018) underlying these interactions. Rational treatment approaches such as alternating collaterally sensitive drug pairs (Imamovic and Sommer 2013; Imamovic et al. 2018; Kim et al. 2014), or the simultaneous utilization of multiple collaterally sensitive antibiotics (Gonzales et al. 2015) may be viable clinical strategies against multidrug-resistant pathogens. Additionally, the clinical implications of collateral sensitivity are not limited to the antimicrobial treatment of pathogenic bacteria but include therapeutic approaches against cancer as well (Pluchino et al. 2012). In fact, several studies have revealed vulnerabilities of resistant tumor cell lines against other anticancer drugs (Jensen et al. 1997; Zhao et al. 2016; Dhawan et al. 2017; Wang et al. 2018), indicating the therapeutic potential of collaterally sensitive drug pairs.

Despite this significant progress in the study of collateral sensitivity over the recent years, there are several key questions that have remained unanswered (Pál et al. 2015). Most importantly, little is known regarding the extent of evolutionary conservation of multidrug resistance and collateral sensitivity (fig. 1). The limited number of studies aiming to map the networks of cross-resistance and collateral sensitivity interactions has mostly focused on single model organisms (Pál et al. 2015; Barbosa et al. 2017) or different strains of the same species (Podnecky et al. 2018), leaving the question of the universality of these findings unexplored. Recent work has shown selected instances of conserved collateral responses of different, antibiotic-resistant clinical isolates of *Escherichia coli*

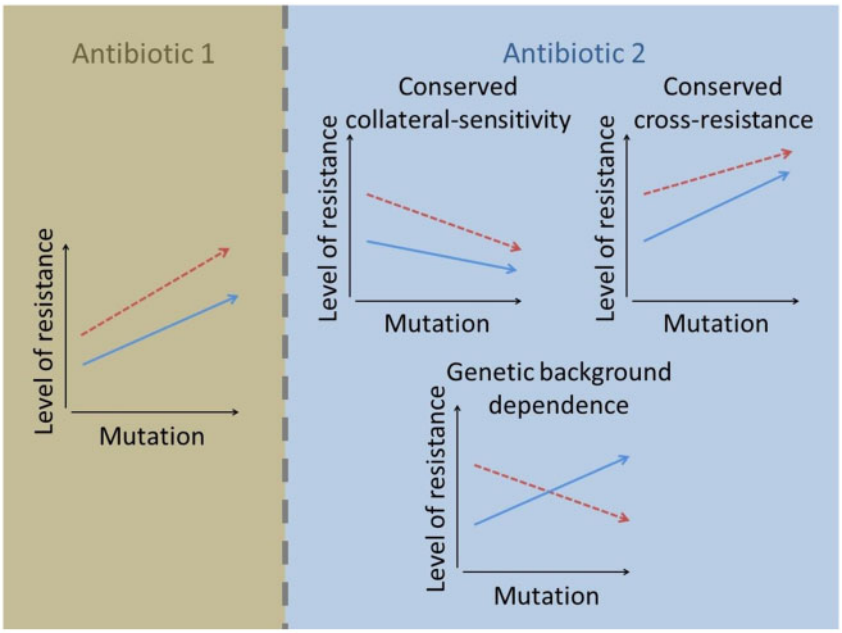

Fig. 1. Evolutionary conservation of cross-resistance and collateral sensitivity. The conceptual figure displays the impact of a hypothetical mutation on antibiotic 1 and antibiotic 2 susceptibilities. Resistance changes in the two hypothetical bacterial genetic backgrounds are indicated by blue solid and red dashed arrows. The mutation increases resistance level to antibiotic 1 in both genetic backgrounds. It may increase resistance to antibiotic 2 in both genetic backgrounds (conserved cross-resistance), increase susceptibility to antibiotic 2 in both genetic backgrounds (conserved collateral sensitivity), or change susceptibility to antibiotic 2 in a different manner in the two genetic backgrounds (intergenic epistasis dependent effect).

regardless of the strain (Podnecky et al. 2018). In another recent study, in addition to conserved instances of collateral responses, major differences were also observed between parallel samples of the same strain of *Pseudomonas aeruginosa* adapted to the same antibiotic (Barbosa et al. 2017). Overall, the impact of genetic background on such interactions remains mostly unknown. More specifically, to what extent does genetic epistasis influence the collateral response networks of antibiotic-resistant bacteria? It is well known that the phenotypic effects of a mutation frequently depend on its interactions with the specific genetic background; mutations beneficial in one genetic context are often neutral or even detrimental in another (Lehner 2011; de Visser et al. 2011). This could be true for antimicrobial resistance mechanisms, as mutations can have substantially different fitness effects across species (Wong 2017).

In this work, we elucidate the evolutionary conservation of resistance and collateral sensitivity (see fig. 1 for a conceptual overview). By constructing a set of previously identified resistance mutations in two related bacteria, *E. coli* and *Salmonella enterica*, we directly compared the impact of resistance mutations in two species. By transplanting an entire resistance-conferring mutated gene and its wild-type counterpart from one species to the other, we elucidated the relative roles of intragenic epistasis (epistatic effects owing to mutational differences within the resistance gene) and intergenic epistasis (epistatic effects derived from mutations elsewhere in the genome). Finally, by constructing isogenic strains carrying different combinations of known resistance mutations affecting multiple genes, we observed that mutations that provide cross-resistance in one genetic background frequently yield collateral sensitivity in another. These results point to the variable nature of multidrug resistance and collateral sensitivity and highlight the importance of genetic epistasis as an underlying driving force.

Results

Mutations Can Have Opposite Effects on Susceptibility in Different Species

Our first aim was to investigate the level of conservation of mutational effects in two related bacterial species. Based on results of a prior study (Lázár et al. 2014), we selected ten point mutations that influence susceptibility to multiple antibiotics in *E. coli* strain BW25113. The corresponding genes encode proteins involved in various cellular processes including regulation of efflux pumps, maintenance of membrane permeability, or transcriptional control of general stress-defense mechanisms (supplementary table 1, Supplementary Material online). Importantly, many of these genes have been implicated in cases of clinical resistance (Okusu et al. 1996; Piddock 2006; Alekshun and Levy 2007).

Using the pORTMAGE recombineering method, applicable to a range of bacterial species (Nyerges et al. 2016), we constructed these ten mutations individually in both *E. coli* BW25113 (Grenier et al. 2014) and *S. enterica* subsp. serovar Typhimurium strain LT2 (McClelland et al. 2001). This technique is especially suitable for such purposes, as it allows the genomic incorporation of specific point mutations without detectable off-target mutations (Nyerges et al. 2016). The selected genes are generally highly conserved between the two species with amino acid sequence similarities ranging

from 79% to 100%, with an average of 91.2%. Importantly, the specific mutated amino acid residues are also conserved in all ten cases. Next, we compared the impact of these mutations on resistance against a panel of 12 antibiotics with different modes of action (table 1). Using an established liquid culture-based standard broth dilution technique (Lázár et al. 2013), the minimum inhibitory concentration (MIC) values of each mutant strain against a panel of clinically relevant antibiotics were determined in *E. coli* BW25113 and *S. enterica* LT2 (table 2 and supplementary File 1, Supplementary Material online, for raw data). Previously, we have demonstrated that the protocol can reliably detect as small as 10% differences in MICs across mutant strains (Lázár et al. 2014). The relative difference in MIC values between the wild-type and the specific mutant gives an estimate on the effect of a particular mutation on antibiotic susceptibility in a given species. Finally, these relative MIC values were compared in the two species (table 2).

Table 1. List of Employed Antibiotics.

Antibiotic Name	Abbreviation	Mode of Action	Bactericidal or Bacteriostatic
Ampicillin	AMP	Cell wall	Bactericidal
Cefoxitin	FOX	Cell wall	Bactericidal
Doxycycline	DOX	Protein synthesis, 30S	Bacteriostatic
Tertracycline	TET	Protein synthesis, 30S	Bacteriostatic
Chloramphenicol	CHL	Protein synthesis, 50S	Bacteriostatic
Erythromycin	ERY	Protein synthesis, 50S	Bacteriostatic
Nalidxic acid	NAL	Gyrase	Bactericidal
Ciprofloxacin	CPR	Gyrase	Bactericidal
Nitrofurantoin	NIT	Multiple mechanisms	Bactericidal
Kanamycin	KAN	Aminoglycoside, 30S	Bactericidal
Tobramycin	TOB	Aminoglycoside, 30S, 50S	Bactericidal
Streptomycin	STR	Aminoglycoside, 30S	Bactericidal

NOTE.—Functional classification is based on previous studies (Yeh et al. 2006; Girgis et al. 2009). These antibiotics are well characterized, cover a wide variety in modes of action, and are frequently deployed in the clinic.

Analysis of all possible 120 combinations of antibiotic–mutation pairs revealed major differences in mutational effects between the two species. Overall, in 33% of these 120 cases, a mutation that either increased or lowered the MIC in one species had no effect in the other. Moreover, over 13% of the total mutation–antibiotic combinations displayed antagonistic mutational effects, that is, introduction of the mutation increased susceptibility in one species but elevated resistance in the other. For example, a mutation in *ycbZ*, a putative protease, elevated sensitivity in *E. coli* to the studied aminoglycosides but had an antagonistic effect when present in *S. enterica*, resulting in increased levels of resistance against these antibiotics (fig. 2). Similarly, a mutation in *acrR*, a multidrug efflux pump regulator, caused increased sensitivity to nalidixic acid when present in *E. coli* but led to increased resistance to the same drug when present in *S. enterica* (fig. 2). Even in cases where a mutation had the same phenotypic effect in both organisms (leading either to increased resistance or sensitivity), the extent of the effect often varied

Table 2. Ten Different Antibiotic Resistance Mutations Identified in *Escherichia coli* Were Constructed in *Salmonella enterica*.

		acrR	*envZ*	*fis*	*gyrA*	*mprA*	*ompC*	*phoQ*	*soxR*	*trkH*	*ycbZ*
AMP	*E. coli*	1.73	2.07	0.83	1	1.2	0.83	2	2.07	0.49	1
	S. enterica	1.2	1.73	1.2	1.244	0.83	1.2	1.44	2.49	1.2	1
FOX	*E. coli*	1.73	3	0.83	1	1	0.69	1.75	2.07	0.8	1
	S. enterica	1.3	1.96	1	1.44	1.44	1	2.07	3.58	0.69	1
DOX	*E. coli*	0.58	0.83	0.375	1	1	1	1	1.73	0.5	1
	S. enterica	1	1.2	1	0.69	0.69	0.69	1	2.07	0.23	1
TET	*E. coli*	1.44	1.2	1	1	1	1	1.25	1.44	0.5	1
	S. enterica	1	1.44	1	1	1	1.2	0.69	2.07	0.65	0.83
STR	*E. coli*	0.4	0.28	1.2	1.44	0.69	0.4	1.2	0.33	4.3	0.58
	S. enterica	1	1	>1.2	0.83	0.69	0.83	1.2	0.48	1.73	1.2
CHL	*E. coli*	0.58	0.83	1.2	1	0.69	0.69	1.14	1	0.6	0.58
	S. enterica	1.2	1	1	1	1	0.83	0.58	2.07	1	1
ERY	*E. coli*	1.2	1.73	2.07	1	1.2	0.83	1.39	1.73	0.9	2.49
	S. enterica	1	1	1.44	0.83	1	1	0.58	1.73	1.23	>1.51
NAL	*E. coli*	0.4	1	0.33	97.66	>3	1	0.71	>2.49	0.3	1
	S. enterica	1.2	1.44	<0.58	610	2.07	0.83	0.69	2.49	<0.58	1
CPR	*E. coli*	1.73	2.98	<0.18	7.69	1	0.58	1.33	4.3	0.6	1
	S. enterica	1.2	1.73	0.58	16	1	0.83	1	2.49	1	1
NIT	*E. coli*	1.2	0.58	0.33	1	1.2	1	1.2	1	0.8	0.58
	S. enterica	1	1	0.69	1	1.73	1.2	0.69	1.44	1	0.83
KAN	*E. coli*	0.58	0.18	1	1	0.58	0.28	1.39	0.4	2.01	0.4
	S. enterica	1	0.83	1.44	1	0.69	0.83	1.73	0.58	2.07	1.2
TOB	*E. coli*	0.58	<0.22	1.2	1	0.58	0.69	1.41	0.4	3.4	0.58
	S. enterica	0.83	1	2.07	0.83	0.69	1	2.98	0.58	1.73	1.44

NOTE.—The effect of these mutations on the susceptibility to 12 antibiotics was measured in both organisms. Blue, orange, and white colors indicate an increase, decrease, and no change in resistance, respectively. Numbers indicate relative resistance level of the mutant compared with the corresponding wild-type, estimated by changes in MICs. For antibiotic abbreviations, see table 1.

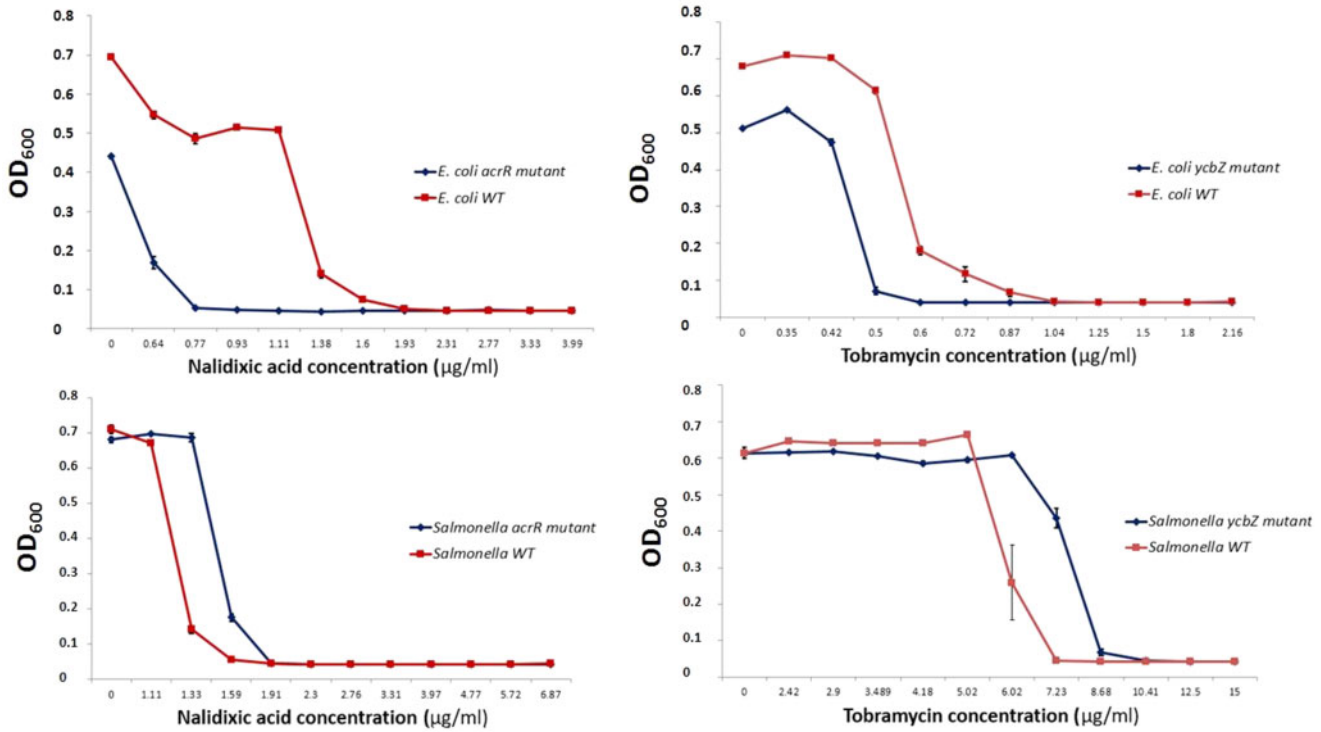

FIG. 2. Examples of dose–response curves of the generated *Escherichia coli* and *Salmonella enterica* mutants (specific mutation indicated in the chart legends) where mutations had opposite effect compared with the wild-type ancestral strain (*E. coli* BW25113 and *S. enterica* serovar Typhimurium LT2, respectively) on resistance against given antibiotics (indicated at the bottom of each chart). Each strain was measured in three replicates ($n = 3$), error bars represent standard error of the mean (SEM). OD data indicated on the *y* axis represent optical density measurements taken at 600 nm.

greatly. For instance, the common S83L mutation of the GyrA protein target identified in clinical *E. coli* isolates in response to nalidixic acid or ciprofloxacin stress (Bagel et al. 1999) conferred a >600-fold increment in resistance level against nalidixic acid in *S. enterica*, whereas the same figure was approximately 100-fold in *E. coli* BW25113 (Nyerges et al. 2016). Conversely, a mutation affecting the outer membrane porin-encoding *ompC* resulted in increased susceptibility to kanamycin in both species, however, the effect was over four times greater when present in *E. coli* compared with *S. enterica*.

Limited Evolutionary Conservation of Collateral Sensitivity across Species

Evolution of resistance toward a given antibiotic can simultaneously decrease resistance against multiple other drugs, a phenomenon termed collateral sensitivity (Pál et al. 2015). By focusing on single bacterial species, prior studies have revealed that this phenomenon is wide spread and may be clinically relevant (Imamovic and Sommer 2013; Lázár et al. 2013, 2014; Rodriguez de Evgrafov et al. 2015; Imamovic et al. 2018). However, the evolutionary conservation of collateral sensitivity remains largely unknown. This issue depends on the extent of overlap in the mechanisms underlying resistance and physiological trade-offs across related species. To investigate this issue, we first focused on *trkH*, which encodes a potassium ion transporter (Schlösser et al. 1995). By reducing the proton motive force across the inner bacterial membrane, the studied TrkH T350K mutation decreases the uptake of aminoglycoside antibiotics (Lázár et al. 2013). As a side effect, TrkH T350K diminishes the activity of protein motive force dependent major efflux pumps, leading to hypersensitivity to several other antibiotics in *E. coli* (Lázár et al. 2013). Introduction of the TrkH T350K mutation into the *S. enterica* genome leads to a significant decrease in aminoglycoside susceptibility in the mutant strain (table 2). However, increased susceptibility of the mutant *S. enterica* strain was observed to only four out of the nine nonaminoglycoside antibiotics, as opposed to all nine in the case of the mutant *E. coli*. In fact, under ampicillin and erythromycin stresses, TrkH T350K induced collateral sensitivity in *E. coli*, but cross-resistance in *S. enterica*. Other notable examples indicating the limited conservation of collateral sensitivity effects could be seen with mutants of *mprA*, a regulator of multidrug resistance pumps, and *phoQ*, a regulatory histidine kinase (table 2). Overall, 46% of the total mutation–antibiotic pairs displayed a lack of conservation of collateral sensitivity or cross-resistance between the two species.

Species-Specific Fitness Costs of Resistance Mutations

Prior studies have shown that antibiotic resistance mutations frequently reduce fitness in antibiotic-free media (Andersson and Hughes 2010). Indeed, growth analysis of the mutant and wild-type strains revealed that in antibiotic-free medium, 5 and 3 out of the 10 mutants showed significantly lower fitness than the wild-type in *E. coli* and *S. enterica*, respectively (fig. 3).

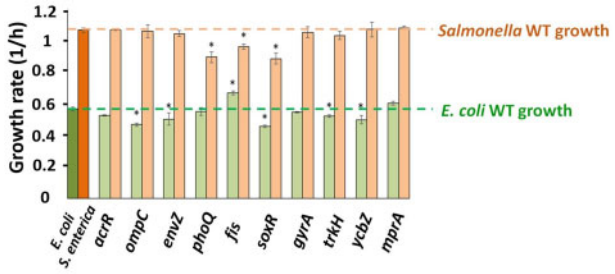

FIG. 3. Fitness cost of antibiotic resistance mutations in *Escherichia coli* and *Salmonella enterica*. The figure shows the growth rates of wild-type and individual mutant strains in *E. coli* (light green bars) and *S. enterica* (light orange bars). Error bars represent standard deviation, based on eight biological replicates for each strain. *Significant difference in growth rates between mutant and the corresponding wild-type ($P < 0.05$, two-tailed *t*-tests).

However, only the mutation in *soxR* reduced fitness in both species. In fact, a mutation in the Fis protein, a DNA-binding protein regulating the organization of nucleoid structure (Schneider et al. 2001) caused a significant fitness increase in *E. coli*, but a significant fitness decrease in *S. enterica* (fig. 3). Fis has been shown to modulate the transcription of over 20% of all *E. coli* genes (Cho et al. 2008), and target genes of global regulatory factors evolve rapidly in bacteria (Madan Babu et al. 2006). Moreover, the amino acid sequence similarity of Fis is 100% between *E. coli* and *S. enterica*. Therefore, it is plausible that the difference in the fitness effects of the Fis mutation reflect an altered set of target genes in the two species.

Intergenic Epistasis Shapes Mutational Effects in TrkH

The above data indicate that the genetic makeup of the recipient species shapes the mutational effects on antibiotic resistance, collateral sensitivity, and fitness cost alike. The effect of the investigated mutation may be dependent on the presence of one or more preexisting genetic changes in the same target gene (*intragenic epistasis*). Alternatively, the difference in mutational effect could be due to genetic changes elsewhere in the genome (*intergenic epistasis*). To tease apart the relative contributions of intragenic and intergenic epistasis, we transplanted an entire resistance gene from one species to another. We focused on the previously described *trkH*, a gene with a central role in aminoglycoside resistance and corresponding collateral sensitivity patterns to other antibiotics. TrkH is nonessential and shows a high level of amino acid sequence similarity (97.5%) between *S. enterica* and *E. coli*. As described previously, the focal TrkH T350K mutation increases resistance to aminoglycosides, but simultaneously yields hypersensitivity to several other antibiotics in *E. coli*.

Using an established recombineering method (Thomason et al. 2014), we first replaced the entire *trkH* gene in the *E. coli* chromosome with its *S. enterica* ortholog (Materials and Methods). Importantly, both the wild-type and the TrkH T350K mutation-carrying sequence of *S. enterica trkH* were introduced into *E. coli* BW25113 with the exact gene boundaries as the original *E. coli trkH* gene. In sum, we generated four strains of *E. coli* with isogenic genomic backgrounds, the only difference being whether they carried *S. enterica* or *E. coli trkH* gene with or without the TrkH T350K mutation. As the genomic backgrounds of the four strains are identical, any difference in mutational effect of TrkH T350K can be attributed to intragenic epistasis only (see fig. 4A for a conceptual overview).

We measured the antibiotic resistance in all four strains across 12 antibiotics, allowing us to compare the impact of TrkH T350K on resistance in strains carrying the *S. enterica* or the *E. coli trkH* gene (fig. 4B). It thereby allows for the assessment of intragenic epistatic effects on antibiotic susceptibility. As expected, TrkH T350K confers resistance to aminoglycosides (kanamycin and tobramycin) and streptomycin regardless of the gene of origin. Moreover, it simultaneously elevated susceptibility to a range of other antibiotics belonging to cell wall, protein synthesis, and gyrase inhibitors. The comparison revealed only one minor difference in mutational effects. TrkH T350K elevated susceptibility to erythromycin only when the strain carried the *E. coli* and not the *Salmonella trkH* gene. We conclude that the phenotypic effects of TrkH T350K are similar regardless of the origins of *trkH* itself, indicating a limited impact of intragenic epistasis on resistance. This pattern is unlikely to reflect the high sequence level of conservation of *trkH* gene only, as transplantation of the *S. enterica trkH* sequences into *E. coli* caused a significant fitness decline (supplementary fig. 1, Supplementary Material online). This indicates that despite the 97.5% amino acid sequence similarity, the differences in *trkH* sequences between *S. enterica* and *E. coli* have functional consequences. Future work should establish the extent to which the role of intragenic epistasis increases with diminishing sequence identity of the genes considered.

Next, we focused on the role of intergenic epistasis by studying the impact of the genomic background on mutational effects. For this purpose, we generated *E. coli* and *S. enterica* strains both of which carried the *Salmonella trkH* gene with or without TrkH T350K. This allows comparison of the effect of the TrkH T350K mutation in *E. coli* and *S. enterica*. This ensured that the differences in mutational effects were not due to species-specific differences in the *trkH* gene itself (fig. 4A). This comparison revealed major qualitative differences in the resistance and collateral sensitivity profiles (supplementary table 2, Supplementary Material online). Most significantly, TrkH T350K elevated susceptibility to ampicillin and erythromycin in *E. coli* BW25113 but increased resistance to the same drugs in *S. enterica*. More generally, TrkH T350K elevated susceptibility to eight antibiotics in *E. coli* BW25113, but to only four in *S. enterica* (fig. 4B). The relatively low evolutionary conservation of collateral sensitivity patterns seen with TrkH may reflect species-specific variation of drug uptake mechanisms (Kohanski et al. 2010).

Evolution of Multidrug Resistance on the Adaptive Landscape

To investigate step-by-step evolution of multidrug resistance and collateral sensitivity, we reconstructed a resistance

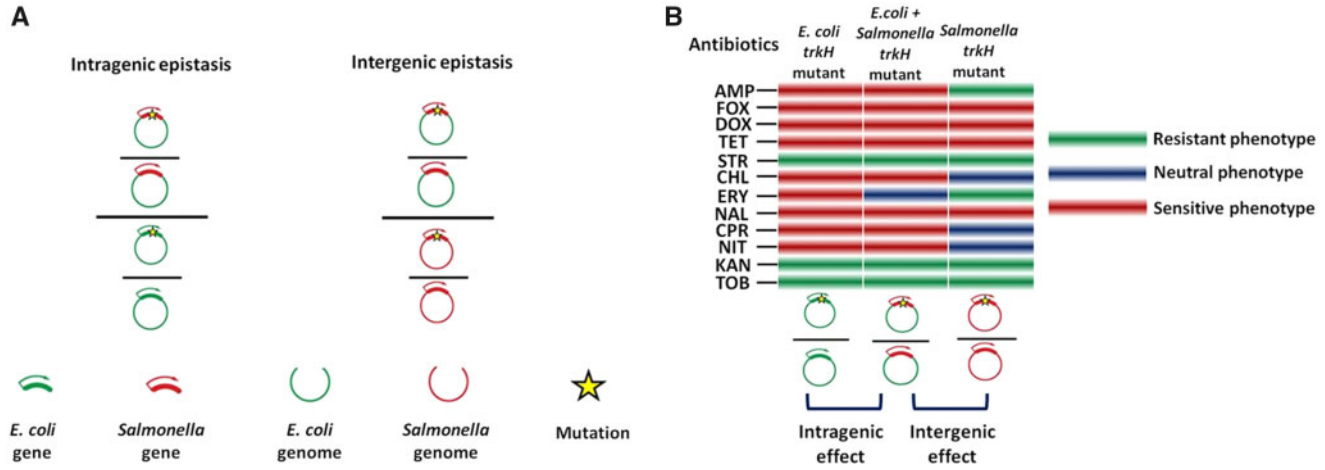

FIG. 4. Effects of intragenic and intergenic epistasis on collateral sensitivity. (*A*) Strains used for analysis. Six strains were considered: (1) wild-type *Escherichia coli*, (2) *E. coli* with trkH mutation (T350K), (3) *E. coli* with wild-type Salmonella trkH gene, (4) *E. coli* with mutant Salmonella trkH gene, (5) wild-type a *Salmonella enterica*, and finally (6) trkH mutant *S. enterica*. Comparison of strains with *E. coli* genomic background reveals intragenic epistasis, whereas comparison of strains with Salmonella trkH gene reveals intergenic epistasis. (*B*) Impact of TrkH T350K mutation on resistance level in three genetic backgrounds: (i) *E. coli*, (ii) *E. coli* carrying *S. enterica* trkH gene, and (iii) *S. enterica*. Green coloring marks instances where mutants expressed increased antibiotic resistance compared with the corresponding control, blue represents no change in antibiotic susceptibility, whereas red boxes refer to the decreased antibiotic resistance of the given mutant, compared with the reference strain.

landscape across multiple antibiotics. For this purpose, we constructed a set of *E. coli* strains carrying various, previously identified antibiotic resistance mutations in all possible combinations (see supplementary fig. 2, Supplementary Material online, for a conceptual overview of this experimental approach). We employed mutations previously identified in a laboratory-evolved *E. coli* strain (Lázár et al. 2013). Briefly, starting from a single clone of *E. coli*, ten parallel populations were previously exposed to gradually increasing concentrations of erythromycin. One isolate from each population was subsequently subjected to detailed genomic and phenotypic analyses (Lázár et al. 2014). Notably, each adapted strain had distinct combinations of mutations that provided resistance against erythromycin. Here, we focused on the ERY2 strain, as it showed an exceptionally high frequency of resistance to multiple drugs, while also showing hypersensitivity to others (Lázár et al. 2014). Therefore, the mutations in ERY2 were viable candidates to investigate how multidrug resistance changes as adaptive evolution toward erythromycin proceeds.

ERY2 had accumulated four point mutations during the course of laboratory evolution. The corresponding mutated proteins have various functions, including stress-induced regulators of multidrug transport (AcrR) and oxidative stress (SoxR), a global modulator of nucleoid structure (Fis), and a protein with an unknown function (YcbZ), implicated in translational processes and ribosome biogenesis (Gagarinova et al. 2016). As the investigated regulatory proteins control the expression of multiple genes, they are expected to have substantial pleiotropic effects when mutated, making them appealing candidates for the study of intergenic epistatic interactions. For example, nearly 900 Fis-associated regions across the *E. coli* genome have been previously identified, and as noted earlier, expression of over 20% of the genes are affected by the deletion of *fis* in the same species (Cho et al. 2008). Using pORTMAGE (Nyerges et al. 2016), we constructed all possible combinations of the four mutations, resulting in 15 total strains (supplementary table 3, Supplementary Material online). Next, the susceptibilities of all strains were measured against the 12 antibiotics used throughout this work (supplementary table 4, Supplementary Material online). The susceptibility profiles of the different combinatorial mutant strain were analyzed to determine the extent by which cross-resistance and collateral sensitivity change in response to the introduction of new resistance mutations.

Due to the pleiotropic effect of the studied mutations, they are expected to influence genetic susceptibility to many other drugs as a collateral effect. This appears to be so: Single mutants generally showed a mild, but significant increment in resistance level to a range of other antibiotics and elevated antibiotic susceptibility to others (supplementary table 4, Supplementary Material online). For example, both the *fis* and the *soxR* mutations (SoxR* and Fis*) increase erythromycin resistance when introduced individually into the wild-type genetic background (fig. 5A), but the same mutations have opposite effects on cefoxitin resistance: SoxR* increases, whereas Fis* decreases resistance (fig. 5A).

We first systematically studied the effects of each 32 mutational step on erythromycin resistance: 31 out of the 32 steps increased resistance, and one was neutral with respect to resistance (fig. 5A). This indicates that erythromycin resistance can follow multiple mutational paths. The picture was markedly different when we studied the impact of each 32 mutational step on cross-resistance to 11 other antibiotics (supplementary table 4, Supplementary Material online). Fifty-four percent of all 352 mutational step-antibiotic combinations increased, 32% combination decreased resistance, while 14% led to no change. The resistance landscape under cefoxitin acid stress highlights the general pattern. In this case,

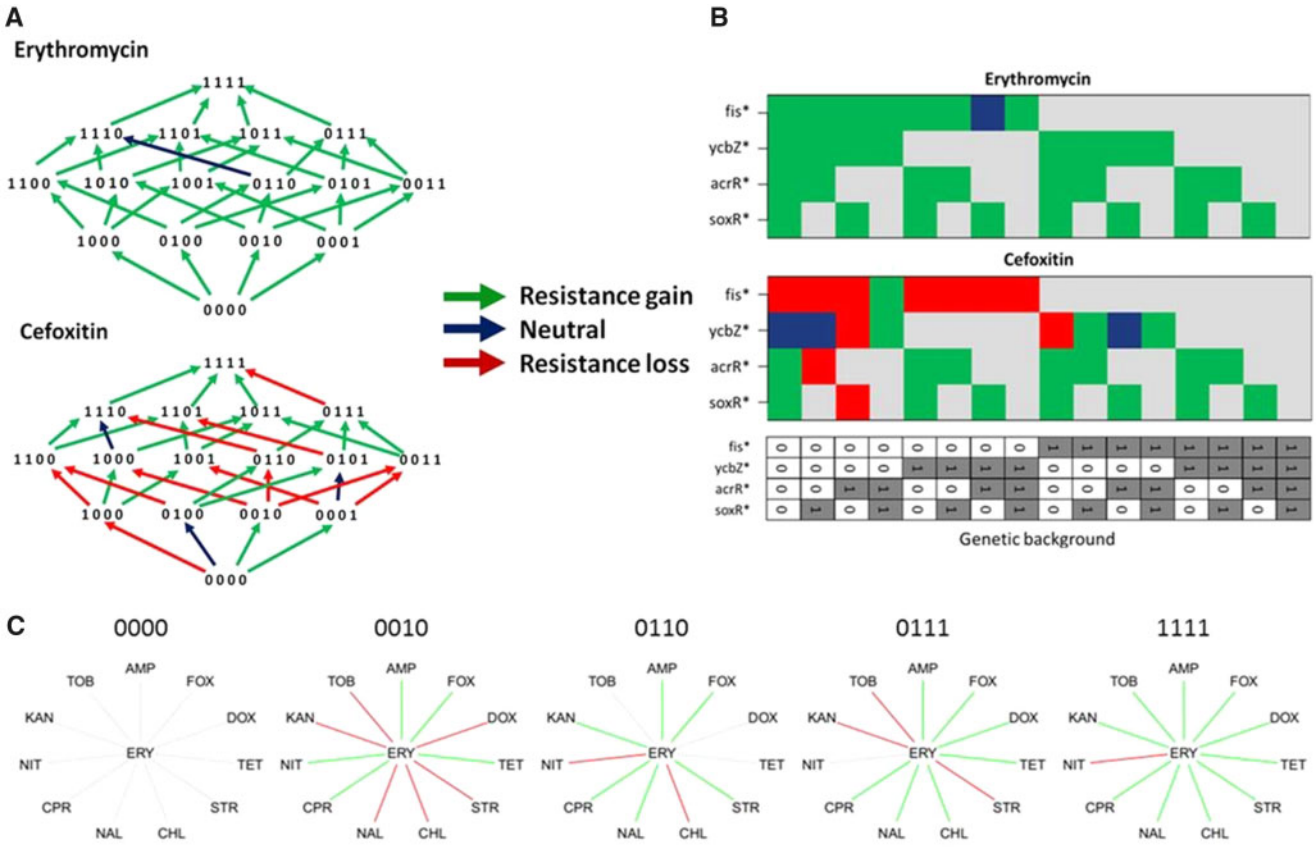

FIG. 5. Evolution of cross-resistance collateral sensitivity on the adaptive landscape. (*A*) The figure shows 32 genotypes defined by all possible combinations of four resistance mutations. The four digit codes encode a given genotype, where 0 and 1 refer to the wild-type and mutant variant of a given gene, respectively (see also *B* bottom panel). Green arrows indicate adaptive mutational steps (resistance gain), red arrows indicate maladaptive steps (resistance loss), while blue arrows are neutral steps (no change in resistance). For the raw data across all studied 12 antibiotics, see supplementary table 4, Supplementary Material online. (*B*) The figure shows the impact of each mutation on resistance level in different genotypes. Color codes are as in panel *A*. (For all antibiotics, see supplementary fig. 4, Supplementary Material online.) (*C*) Changes in cross-resistance and collateral sensitivity interactions as genetic adaptation toward erythromycin progresses. The neighboring genotypes are one mutational step away from each other, and hence they form an adaptive route from wild-type (0000) toward a genotype with high erythromicin resistance level (1111). We compared the resistance level of the mutant genotypes (0010, 0110, 0111, 1111) to the wild-type (0000) across 11 antibiotics. Erythromycin was excluded from the analysis, as all genotypes show reduced susceptibilities to this drug (*A*). Green and red lines indicate cross-resistance and collateral sensitivity, respectively. For more details and examples, see supplementary figure 3, Supplementary Material online.

(fig. 5*B*), 18 mutational steps were adaptive (increased resistance), whereas 11 and 3 were deleterious (decreased resistance) and neutral (no change in resistance), respectively.

To investigate this issue from a different angle, we analyzed how individual mutations shape resistance across different genetic backgrounds. In the case of erythromycin, the pattern was very simple: Mutations increased erythromycin resistance irrespective of the genetic background, the only exception being that the mutation in *fis* was neutral in the strain that carried *ycbZ* and *acrR* mutational combinations only. The picture was very different when other antibiotics were considered. Overall, mutations increased resistance level with certain combinations of other mutations only (fig. 5*B*). For example, the YcbZ*, AcrR* decreased or increased susceptibility to cefoxitin depending on the genetic background (fig. 5*A* and *B*), and Fis* increased cefoxitin resistance only in combination with AcrR* and SoxR* mutations.

Finally, we systematically compared the antibiotic susceptibilities of the wild-type and mutant clones. For this purpose, we estimated the corresponding changes in the sensitivities of each mutant strain to the wild-type across all antibiotics, ultimately leading to a map of cross-resistance and collateral sensitivity interactions for each mutant. Comparison of the network of these interactions across mutants revealed that collateral sensitivity frequently changes into cross-resistance and vice versa as evolution toward erythromycin resistance progresses (fig. 5*B* and *C* and supplementary fig. 3, Supplementary Material online).

These findings indicate that each adaptive mutation under erythromycin stress has variable and largely unpredictable effects on susceptibility to other drugs. It should be emphasized that other strains adapted in parallel to erythromycin (Lázár et al. 2014) gained distinct combinations of mutations that conferred resistance. Based on our results, it is highly probable that these adapted lines also have unique collateral

sensitivity networks. This underscores the notion that cross-resistance and collateral sensitivity interactions are highly variable and dependent on the genetic background.

Discussion

In this work, through the combination of complementary experimental approaches, we studied the evolutionary conservation of mutational effects on bacterial resistance against a wide range of antibiotics. First, we directly compared the phenotypic effects of specific antibiotic resistance mutations in two related species, *E. coli* and *S. enterica*, and found significant differences (fig. 2). We found that certain mutations increased resistance against an antibiotic in one species, whereas introduction of the same mutation into the other species increased sensitivity to the same antibiotic. Second, we investigated a mutation (TrkH T350K) that causes resistance to aminoglycosides, but collateral sensitivity to a range of other antibiotics in *E. coli*. By introducing the same mutation into *S. enterica*, we found that only a fraction of these collateral effects remained conserved, mainly due to intergenic epistatic effects. Finally, by reconstructing a mutational landscape, we studied how individual mutational steps change resistance to multiple antibiotics. We found that collateral sensitivity and cross-resistance effects strongly depend on the genetic background. Even a single resistance mutation can alter collateral responses to other antibiotics.

Taken together, our results point to the limited conservation of mutational effects driving collateral sensitivity and cross-resistance phenotypes of antibiotic-resistant bacteria. Several lines of evidence point to the same direction. First, evolution of resistance is frequently achieved through divergent molecular mechanisms leading to substantial differences in the collateral sensitivity patterns (Nichol et al. 2019). Second, a previous study showed that one third of the 60 studied collateral responses could be considered conserved (i.e., observed in at least 5 out of 10 different clinical isolates of *E. coli* adapted to the same antibiotic [Podnecky et al. 2018]). Our own results indicate a lower level of conservation, which is likely due to major differences in the extent of genetic distance spanned in the two studies. In particular, we compared the conservation of susceptibility phenotypes between *E. coli* and *S. enterica*, two species that differ by ~16% at the nucleotide level (McClelland et al. 2000), whereas even the most diverged *E. coli* strains from Podnecky et al. (2018) accumulated <0.04 substitutions per site. Third, by studying the antibiotic resistance profiles of clinical *P. aeruginosa* isolates, Imamovic and colleagues found that susceptibilities of individual strains isolated from the same patient in a longitudinal study varied greatly over time (Imamovic et al. 2018). These changes in resistance levels point to the dynamic nature of collateral sensitivity, most likely owing to various genomic mutations occurring over time. Fourth, there are substantial differences in the efficacy of collateral sensitivity treatment across species. For example, evolution of resistance to ciprofloxacin induces collateral sensitivity against doxycycline in *E. coli*, but cross-resistance in *Staphylococcus aureus* (Rodriguez de Evgrafov et al. 2015). Similarly, aminoglycosides and piperacillin display cross-resistance in a *P. aeruginosa* strain, but collateral sensitivity in *E. coli* (Barbosa et al. 2017). Additionally, in the same study, the authors observed instances where parallel samples of *P. aeruginosa* PA14 adapted to the same antibiotic (e.g., cefsulodin) showed opposite changes in susceptibility against other antibiotics (including gentamicin and streptomycin). This shows that various modes of adaptation to the same antibiotic entail differences in the corresponding collateral sensitivity profiles. Finally, a recent study by Knopp and Andersson examined the effects of antibiotic resistance mutations when introduced into a range of related bacterial species and strains (Knopp and Andersson 2018). Mutational effects of resistance mutations in regulatory proteins were relatively variable, whereas target mutations had relatively conserved effects across species.

Taken together, these findings support the dynamic nature of collateral response in antibiotic-resistant bacteria. This phenomenon may not be limited to prokaryotes. A recent study showed that during different intermediate stages of clonal evolution, a subpopulation of cancer cells resistant to BCR-ABL1-targeted inhibitors developed collateral sensitivity against other anticancer agents. However, the extent of collateral sensitivity was dependent on the specific genotype of the evolved clones (Zhao et al. 2016).

It is important to emphasize that there are instances where the underlying molecular mechanisms of collateral sensitivity are conserved across strains and therefore such cases may be promising starting points for clinical treatments. For example, clinical samples of *P. aeruginosa* resistant to ciprofloxacin isolated from cystic fibrosis patients were shown to have conserved collateral sensitivity against aminoglycosides, allowing this approach to be utilized for effective in vivo treatment of a chronic cystic fibrosis patient (Imamovic et al. 2018). Indeed, our own data lend further support to these examples, as antibiotic resistance mutations in *fis* and *acrR* displayed highly conserved collateral responses against ciprofloxacin, irrespective of the genetic background (supplementary table 4, Supplementary Material online). Narrowing down collateral responses to the most robust and conserved such as this example is imperative for successful clinical outcomes if bacterial collateral sensitivity responses are to be used as treatment strategies. This is all the more relevant in clinical settings where infections are frequently polymicrobial in nature (Rogers et al. 2010), further increasing the need for the identification of highly conserved collateral interactions. Finally, it should be noted that conclusions based on our findings have limitations. For example, for strains exhibiting significantly higher resistance, the possibility that collateral sensitivity interactions would be less dependent on the genetic makeup of strains cannot be ruled out. Nonetheless, we show that such factors must be taken into account, as they have the potential to greatly influence the outcome of therapies that apply combinations of antibiotics. Overall, the dynamic nature of the majority of collateral sensitivity interactions will be an important aspect to consider when designing multidrug treatments against bacterial pathogens.

Materials and Methods

Media

Unless otherwise noted, bacterial cell cultures were grown in Lysogeny-Broth-Lennox (LB^L) media (10 g tryptone, 5 g yeast extract, and 5 g sodium chloride per 1 l water) for cell manipulations and growth measurements. Terrific-broth media (24 g yeast extract, 12 g tryptone, 9.4 g K_2HPO_4, and 2 g KH_2PO_4 per 1 l of water) was used for cell recovery following electroporation.

Strain Construction, Oligonucleotides

For a full list of oligonucleotides used in the study (with brief descriptions), see supplementary File 2, Supplementary Material online. Bacterial strains used in the study were constructed on background strains *E. coli* K-12 BW25113 or *S. enterica* serovar Typhimurium LT2 using the pORTMAGE recombineering method described previously (Nyerges et al. 2016). Briefly, individual antibiotic resistance mutations were introduced via synthetic ssDNA oligonucleotides (oligos), ordered with standard purification and desalting from Integrated DNA Technologies. These oligos were designed using the MODEST online tool (Bonde et al. 2014) and were 90 nucleotides long and had complementary sequences (save for the mutations of interest) to the replicating lagging strand with a minimized secondary structure (>-8 kcal/mol). Each oligo also contained two subsequent phosphorothioate bonds at both 5′ and 3′ termini for the evasion of endogenous nuclease-mediated oligo processing. Recombineering was performed in electrocompetent cells carrying the pORTMAGE-2 plasmid (Addgene ID #72677), incubated at 42 °C for 15 min to induce expression of the λ Red recombinase enzymes, as well as a dominant negative allele of the MutL mismatch-repair enzyme for the negation of off-target mutations (Nyerges et al. 2016). The induced electrocompetent cells were then transformed with 1 μl of the 100-μM mutation-carrying oligo. Cells were recovered in 5-ml terrific-broth media after electroporation and incubated at 30 °C for 60 min, after which 5-ml LB^L media was added and incubation at 30 °C continued overnight. Cultures were then plated onto solid LB^L agar media plates from which individual colonies could be genotypically analyzed.

Colonies carrying the desired mutations were screened using either allele-specific polymerase chain reaction (PCR) or high resolution melting (HRM) analysis. Briefly, allele-specific primers were designed and tested using wild-type colonies using a gradient PCR protocol using a Bio-Rad CFX96 Touch thermocycler. PCR annealing temperatures for colony screening were set at a temperature 1 °C higher than the temperature where the last visible fragment could be detected using the wild-type colony after gradient PCR. Alternatively, HRM colony-PCR was used to screen colonies with the Luminaris HRM Master Mix (Thermo Fisher Scientific) using a Bio-Rad CFX Touch thermocycler according to the manufacturer's instructions. Candidate colonies were subsequently verified using capillary-sequencing.

Strains with genotypes carrying multiple mutations were constructed sequentially, that is each mutation-carrying oligo was transformed separately, the mutations were screened and verified and the following oligo was then transformed, etc. The pORTMAGE-2 plasmid was cured from sequence-verified colonies by growing the cells once overnight at 42 °C.

Gene Replacement

Gene replacement of the entire *trkH* sequence (along with the corresponding upstream regulatory sequence) was carried out using both the wild-type and resistance point mutation-carrying *S. enterica* sequence, into a wild-type *E. coli* background. Gene replacement was achieved using a two-step selection/counter-selection recombineering method (Thomason et al. 2014) involving a *tetA-sacB* cassette (Li et al. 2013).The *tetA-sacB* cassette was PCR amplified using Phusion High-Fidleity DNA polymerase (Thermo Scientific) using the *E. coli* T-SACK strain (Li et al. 2013) as a template (a kind gift from Donald L. Court, National Cancer Institute, Frederick, MD). The cassette was amplified using primers with 60-bp 5′ overhangs carrying homologies to the *E. coli trkH* sequence. λ-Red-mediated recombineering of the purified fragment was achieved using the pSIM-5 plasmid (Datta et al. 2006) using a well-established protocol (Thomason et al. 2014). Tetracyclin-resistant *trkH* insertants were verified by testing for sucrose sensitivity as well as *trkH* and cassette-specific PCR. Recombineering was then repeated on verified strains carrying the tetA-sacB cassette, transforming PCR-amplified *S. enterica* wild-type and mutant *trkH* cassettes. These cassettes were previously amplified using primers with 50-bp 5′ overhangs carrying homologies to the sequences directly flanking *E. coli trkH*. Cells were plated onto a special Tet/SacB counter-selection medium (Li et al. 2013) and incubated at 42 °C for 48 h. Colony-PCR was then performed on individual colonies using a forward primer specific to *S. enterica trkH* and a reverse primer specific to the *E. coli trkH* flanking region. Colonies that produced an amplified fragment (around 10% of tested colonies) were then verified for the desired outcome using Sanger sequencing.

MIC Measurement

Measurement of MIC values was performed using a standard linear broth dilution method (Wiegand et al. 2008). Twelve linear dilution steps, with a dilution rate of 1.2, were prepared in 96-well microtiter plates. Approximately $3 \sim 10^3$ cells were inoculated into volumes of 1 ml using a 96-pin replicator tool. Cultures were grown in a shaking incubator at 30 °C shaken at 300 rpm, with three replicates per strain for all antibiotic concentrations. Following 24 h of incubation, raw OD_{600} values were measured using a Biotek Synergy 2 microplate reader. The MIC value was determined using a cutoff OD_{600} value, defined as the mean + 2 standard deviations of OD_{600} values of bacteria-free wells containing only growth medium. In order to measure the effect of each individual mutation, the MIC value of each mutant was determined compared with the wild-type strain (referred to as relative MIC value). The sensitivity of each mutant was measured against a set of 12 antibiotics representing a wide variety of mode of actions (see table 1). See supplementary File 1, Supplementary

Material online, for raw data of all MIC measurements conducted in this study.

Growth Rate Measurement

Growth rate measurements of selected strains were performed by growing replicates ($n = 24$) of randomly chosen individual colonies in LB^L media. Cultures of the studied mutants were incubated at 30 °C until early stationary phase, followed by the transfer of $\sim 10^3$ cells from each into 96-well shallow plates containing 100-μl LB^L media. Growth curves were recorded by measuring OD_{600} every 7 min for 24 h at 30 °C using a Biotek Powerwave XS2 automated plate reader. Growth rate was calculated from the obtained growth curves following a previously reported procedure (Warringer and Blomberg 2003; Karcagi et al. 2016).

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

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