Limited Evolutionary Conservation of the Phenotypic Effects of Antibiotic Resistance Mutations

Gábor Apjok,1 Gábor Boross,‡,1 Ákos Nyerges,1 Gergely Fekete,1 Viktória Lázár,‡,1 Balázs Papp,1 Csaba Pál,*,1 and Bálint Csörgő*,§,1

1Synthetic and Systems Biology Unit, Institute of Biochemistry, Biological Research Centre of the Hungarian Academy of Sciences, Szeged, Hungary
‡Present address: Department of Biology, Stanford University, Stanford, CA
§Present address: Technion – Israel Institute of Technology, Faculty of Biology, Haifa, Israel
*Corresponding authors: E-mails: csorgo.balint@brc.mta.hu; pal.csaba@brc.mta.hu.
Associate editor: Miriam Barlow

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 underline 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; Rodríguez 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 antimicrobics (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...
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).

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 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 |
| Tetracycline    | TET          | Protein synthesis, 30S | Bacteriostatic |
| Chloramphenicol | CHL          | Protein synthesis, 50S | Bacteriostatic |
| Erythromycin    | ERY          | Protein synthesis, 50S | Bacteriostatic |
| Nalidixic acid  | NAL          |                           |                                |
| Ciprofloxacin   | CPR          | GyrA            | Bactericidal                   |
| Nitrofurantoin  | NIT          | Multiple mechanisms | Bactericidal                   |
| Kanamycin       | KAN          | Aminoglycoside, 30S | Bactericidal                   |
| Tobramycin      | TOB          | Aminoglycoside, 30S | Bactericidal                   |
| Streptomycin    | STR          | Aminoglycoside, 50S | Bactericidal                   |

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.

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

|        | acrR | envZ | fis | gyrA | mprA | ompC | phoQ | ssoA | 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    | 1    |
| FOX E. coli | 1.73 | 1.73 | 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    | 2.97 | 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    | 0.69 | 2.07 | 0.65 | 0.83 |
| STR E. coli | 0.4  | 0.28 | 1.2  | 1.44 | 0.69 | 0.4  | 1    | 0.33 | 4.3  | 0.58 |
| S. enterica | 1   | 1    | >1.2 | 0.83 | 0.69 | 0.83 | 1    | 0.48 | 1.73 | 1.2  |
| CHL E. coli | 0.58 | 0.83 | 1.2  | 0.69 | 0.69 | 1.14 | 1    | 0.6  | 0.58 |      |
| S. enterica | 1   | 1    | 1    | 1    | 1    | 0.83 | 1    | 0.58 | 1.73 | 1.23 |
| 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    | 1    | 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 | 0.58 | 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    | 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.
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).
Fis has been shown to modulate the transcription of global regulatory factors evolve rapidly in bacteria and, as a result, 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 genome (intergenic epistasis). Alternatively, the difference in mutational effect could be due to genetic changes elsewhere in the genome (intragenic 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 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 E. coli and S. enterica. 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. Moreover, 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
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,
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. 5B). For example, the YcbZ*, AcrR* decreased or increased susceptibility to cefoxitin depending on the genetic background (fig. 5A and 8), 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. 5B 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 (Podneky 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 Podneky 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 *fls* 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 multi-drug treatments against bacterial pathogens.
Materials and Methods

Media

Unless otherwise noted, bacterial cell cultures were grown in Lysogeny-Broth-Lennox (LB) 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 K2HPO4, and 2 g KH2PO4 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 media was added and incubation at 30°C continued overnight. Cultures were then plated onto solid LB 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-Fidelity 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 ~ 10³ 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 OD600 values were measured using a Biotek Synergy 2 microplate reader. The MIC value was determined using a cutoff OD600 value, defined as the mean + 2 standard deviations of OD600 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\(^t\) media. Cultures of the studied mutants were incubated at 30 °C until early stationary phase, followed by the transfer of \(\sim 10^5\) cells from each into 96-well shallow plates containing 100-μl LB\(^t\) 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.

Acknowledgments
We thank Donald L. Court (National Cancer Institute, Frederick, MD) for providing the *E. coli* T-SACK strain, and Andrea Tóth for technical assistance. This work was supported by grants from the European Research Council H2020-ERC-2014-CoG 648364 - Resistance Evolution (to C.P.) and the “Lendület” Program of the Hungarian Academy of Sciences (C.P. and B.P.), and GINOP-2.3.2-15-2016-00026 (GINOP) grants (MolMedEx TUMORDNS) GINOP-2.3.2-15-2016-00002, GINOP (EVOMER) GINOP-2.3.2-15-2016-00014 (to C.P. and B.P.), and GINOP-2.3.2-15-2016-00026 (iChamber) (to B.P.); the “Lendület” Program of the Hungarian Academy of Sciences (C.P. and B.P.); Hungarian Scientific Research Fund grant OTKA PD 109572 (to B.C.); The Hungarian New National Excellence Program (UNKP) (to A.N.); and a PhD fellowship from the Boehringer Ingelheim (to A.N.).

References
Aleksish MN, Levy SB. 2007. Molecular mechanisms of antibacterial multidrug resistance. *Cell* 128(6):1037–1050.
Andersson DI, Hughes D. 2010. Antibiotic resistance and its cost: is it possible to reverse resistance? *Nat Rev Microbiol.* 8(4):260–271.
Bagel S, Hüllen V, Wiedemann B, Heisig P. 1999. Impact of gyrA and parC mutations on quinolone resistance, doubling time, and supercoiling degree of *Escherichia coli*. *Antimicrob Agents Chemother.* 43(4):868–875.
Barbosa C, Trebesch V, Kemmer C, Rosenstiel P, Beardmore R, Schulenburg H, Jansen G. 2017. Alternative evolutionary paths to bacterial antibiotic resistance cause distinct collateral effects. *Mol Biol Evol.* 34(9):2229–2244.
Baym M, Stone LK, Kishony R. 2016. Multidrug evolutionary strategies to reverse antibiotic resistance. *Science* 351(6268):aad3292.
Bonde MT, Klausen MS, Anderson MV, Wallin AI, Wang HH, Sommer M. 2014. MODEST: a web-based design tool for oligonucleotide-mediated genome engineering and recombineering. *Nucleic Acids Res.* 42(Web Server issue): W408–W415.
Bush K, Courvalin P, Dantas G, Davies J, Eisenstein B, Huovinen P, Jacoby GA, Kishony R, Kreiswirth BN, Kutter E. 2011. Tackling antibiotic resistance. *Nat Rev Microbiol.* 9(12):894–896.

Cho B-K, Knight EM, Barrett CL, Palsson BØ. 2008. Genome-wide analysis of Fis binding in *Escherichia coli* indicates a causal role for A-AT-tracts. *Genome Res.* 18(6):900–910.
Datta S, Costantino N, Court DL. 2006. A set of recombineering plasmids for gram-negative bacteria. *Gene* 379:109–115.
de Visser JAGM, Cooper TF, Elena SF. 2011. The causes of epistasis. *Proc Biol Sci.* 278(1725):3617–3624.
Dhawan A, Nicola D, Kinoze F, Abazede ME, Marusyk A, Haura EB, Scott JG. 2017. Collaborative sensitivity networks reveal evolutionary instability and novel treatment strategies in AKT mutated non-small cell lung cancer. *Sci Rep.* 7(1):1232.
Gagarinova A, Stewart G, Samanfar B, Phanse S, White CA, Aoki H, Deineko V, Belogolova N, Yakunin AF, Golshani A, et al. 2016. Systematic genetic screens reveal the dynamic global functional organization of the bacterial translation machinery. *Cell Rep.* 17(3):904–916.
Girgis HS, Hottes AK, Tavaoiz S. 2009. Genetic architecture of intrinsic antibiotic susceptibility. *PLoS One* 4(5): e5629.
Gonzales PR, Pesesky MW, Bouley R, Ballard A, Biddly BA, Suckow MA, Wolter VR, Schroeder VA, Burnham CA-A, Mobashery S, et al. 2015. Synergistic, collaboratively sensitive β-lactam combinations suppress resistance in MRSA. *Nat Chem Biol.* 11(11):855–861.
Grenier F, Matteau D, Bely V, Rodrigue S. 2014. Complete genome sequence of *Escherichia coli* BW25113. *Genome Announc.* 2(5): e01038–14.
Imamovic L, Ellabaa MMH, Dantas Machado AM, Citterio L, Wulf T, Molin S, Krogh Johansen H, Sommer M. 2018. Drug-driven phenotypic convergence supports rational treatment strategies of chronic infections. *Cell* 172(1-2):121–134.e14.
Imamovic L, Sommer M. 2013. Use of collateral sensitivity networks to design drug cycling protocols that avoid resistance development. *Sci Transl Med.* 5(204):204ra132.
Jensen PB, Holm B, Sorensen M, Christensen IJ, Sehested M. 1997. In vitro cross-resistance and collateral sensitivity in seven resistant small-cell lung cancer cell lines: preclinical identification of suitable drug partners to taxotere, taxol, topotecan and gemcitabin. *Br J Cancer* 75(6):869–877.
Karcagi I, Drakosvits G, Umenhoiffer K, Fekeger G, Kovács K, Méhe O, Balóki G, Szappanos G, Győrői Z, Fehér T, et al. 2016. Indispensability of horizontally transferred genes and its impact on bacterial genome structure. *Mol Biol Evol.* 33(5):1257–1269.
Kim S, Liebermann TD, Kishony R. 2014. Alternating antibiotic treatments constrain evolutionary paths to multidrug resistance. *Proc Natl Acad Sci U S A.* 111(40):14494–14499.
Knopp M, Andersson DI. 2018. Predictable phenotypes of antibiotic resistance mutations. *mbio* 9.e00770–18.
Kohanski MA, Dwyer DJ, Collins JJ. 2010. How antibiotics kill bacteria: from targets to networks. *Nat Rev Microbiol.* 8(6):423–435.
Laxmanrayan R. 2014. Antibiotic effectiveness: balancing conservation against innovation. *Science* 345(6202):1299–1301.
Lázár V, Nagy I, Spohn C, Cso¨rgo B, Györiky A, Nyerges Á, Horváth B, Vörös A, Busa-Fekete R, Hrtyan M, et al. 2014. Genome-wide analysis captures the determinants of the antibiotic cross-resistance interaction network. *Nat Commun.* 5:4352.
Lázár V, Pal Singh G, Spohn R, Nagy I, Horváth B, Hrtyan M, Busa-Fekete R, Bogos B, Méhe O, Cso¨rgo B, et al. 2013. Bacterial evolution of antibiotic hypersensitivity. *Mol Syst Biol.* 9:700.
Lehner B. 2011. Molecular mechanisms of epistasis within and between genes. *Trends Genet.* 27(8):323–331.
Li X, Thomason LC, Sawai J, Costantino N, Court DL. 2013. Positive and negative selection using the tetA-sacB cassette recombineering and PT insertion in *Escherichia coli*. *Nucleic Acids Res.* 41(22):e204.
Madan Babu M, Teichmann SA, Aravind L. 2006. Evolutionary dynamics of prokaryotic transcriptional regulatory networks. *J Mol Biol.* 358(2):614–633.
McClelland M, Florea L, Sanderson K, Clifton SW, Parkhill J, Churcher C, Dougan G, Wilson RK, Miller W. 2000. Comparison of the *Escherichia coli* K-12 genome with sampled genomes of a *Klebsiella pneumoniae*
and three Salmonella enterica serovars, Typhimurium, Typhi and Paratyphi. Nucleic Acids Res. 28(24):4974–4986.

McClelland M, Sanderson KE, Spieth J, Clifton SW, Latreille P, Courtney L, Porwollik S, Ali J, Dante M, Du F, et al. 2001. Complete genome sequence of Salmonella enterica serovar Typhimurium LT2. Nature 413(6858):852–856.

Munck C, Gumpert HK, Wallin AIN, Wang HH, Sommer M. 2014. Prediction of resistance development against drug combinations by collateral responses to component drugs. Sci Transl Med. 6(262):262ra156.

Nichol D, Rutter J, Bryant C, Hujer AM, Lek S, Adams MD, Jeavons P, Anderson ARA, Piddock L, Gumpert H. 2014. Antibiotic collateral sensitivity is contingent on the repeatability of evolution. Nat Commun. 10:334.

Nyerges A, Csörgő B, Nagy I, Bálint B, Bíhari P, Lázár V, Apjok G, Umenhoffer K, Bogos B, Pósfai G, et al. 2016. A highly precise and portable genome engineering method allows comparison of mutational effects across bacterial species. Proc Natl Acad Sci U S A. 113(9):2502–2507.

Okusu H, Ma D, Nikaido H. 1996. AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of Escherichia coli multiple-antibiotic-resistance (Mar) mutants. J Bacteriol. 178(1):306–308.

Oz T, Güvenek A, Yıldız S, Karaboga E, Tamer YT, Mumcuyan N, Ozan VB, Senturk GH, Cokol M, Yeh P, et al. 2014. Strength of selection pressure is an important parameter contributing to the complexity of antibiotic resistance evolution. Mol Biol Evol. 31(9):2387–2401.

Pál C, Papp B, Lázár V. 2015. Collateral sensitivity of antibiotic-resistant microbes. Trends Microbiol. 23(7):401–407.

Piddock L. 2006. Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. Clin Microbiol Rev. 19(2):382–402.

Pluchino KM, Hall MD, Goldsborough AS, Callaghan R, Gottesman MM. 2012. Collateral sensitivity as a strategy against cancer multidrug resistance. Drug Resist Updates 15:98–105.

Podnecky NL, Fredheim EGA, Kloos J, Serum V, Primicerio R, Roberts AP, Rozen DE, Samuelsen Ø, Johnsen PI. 2018. Conserved collateral antibiotic susceptibility networks in diverse clinical strains of Escherichia coli. Nat Commun. 9(1):3673.

Rodríguez de Evgrafov M, Gumpert H, Munck C, Thomsen TT, Sommer M. 2015. Collateral resistance and sensitivity modulate evolution of high-level resistance to drug combination treatment in Staphylococcus aureus. Mol Biol Evol. 32(5):1175–1185.

Rogers GB, Hoffman LR, Whiteley M, Daniels TVV, Carroll MP, Bruce KD. 2010. Revealing the dynamics of polymicrobial infections: implications for antibiotic therapy. Trends Microbiol. 18(8):357–364.

Schlösser A, Meldorf M, Stumpe S, Bakker EP, Epstein W. 1995. TrkH and its homolog, TrkG, determine the specificity and kinetics of cation transport by the Trk system of Escherichia coli. J Bacteriol. 177(7):1908–1910.

Schneider R, Lurz R, Lüder G, Tolksdorf C, Travers A, Muskhelishvili G. 2001. An architectural role of the Escherichia coli chromatin protein FIS in organising DNA. Nucleic Acids Res. 29(24):5107–5114.

Szybalski W, Bryson V. 1952. Genetic studies on microbial cross resistance to toxic agents. I. Cross resistance of Escherichia coli to fifteen antibiotics. J Bacteriol. 64(4):489–499.

Thomason LC, Sawitzke JA, Li X, Costantino N, Court DL. 2014. Recombineering: genetic engineering in bacteria using homologous recombination: recombineering. In: Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K, editors. Current protocols in molecular biology. Hoboken (NJ): John Wiley & Sons, Inc. p. 1.16.1–1.16.39.

Wang L, de Oliveira RL, Huijberts S, Pencheva N, Brunen D, Bosma A, Song J-Y, Zievenhoven J, Vries GTL. 2018. An acquired vulnerability of drug-resistant melanoma with therapeutic potential. Cell 173(6):1413–1425.

Warringer J, Blomberg A. 2003. Automated screening in environmental arrays allows analysis of quantitative phenotypic profiles in Saccharomyces cerevisiae. Yeast 20(1):53–67.

Wiegand I, Hilpert K, Hancock R. 2008. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc. 3(2):163–175.

Wong A. 2017. Epistasis and the evolution of antimicrobial resistance. Front Microbiol. 8:246.

Yeh P, Tschumi AJ, Kishony R. 2006. Functional classification of drugs by properties of their pairwise interactions. Nat Genet. 38(4):489–494.

Zhao B, Sedlak JC, Srivivas R, Creixell P, Pritchard JR, Tidor B, Lauffenburger DA, Hemann MT. 2016. Exploiting temporal collateral sensitivity in tumor clonal evolution. Cell 165(1):234–246.