Conserved collateral susceptibility networks in diverse clinical strains of *Escherichia coli*.

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Abstract

There is urgent need to develop novel treatment strategies to reduce antimicrobial resistance. Collateral sensitivity (CS), where resistance to one antimicrobial increases susceptibility to other drugs, is a uniquely promising strategy that enables selection against resistance during treatment. However, using CS-informed therapy depends on conserved CS networks across genetically diverse bacterial strains. We examined CS conservation in 10 clinical strains of *E. coli* resistant to four clinically relevant antibiotics. Collateral susceptibilities of these 40 resistant mutants were then determined against a panel of 16 antibiotics. Multivariate statistical analyses demonstrate that resistance mechanisms, in particular efflux-related mutations, as well as relative fitness were principal contributors to collateral changes. Moreover, collateral responses shifted the mutant selection window suggesting that CS-informed therapies could affect evolutionary trajectories of antimicrobial resistance. Our data allow optimism for CS-informed therapy and further suggest that early detection of resistance mechanisms is important to accurately predict collateral antimicrobial responses.

Keywords

Collateral sensitivity, cross-resistance, antimicrobial resistance, fitness cost of resistance, selection inversion

The evolution and increasing frequency of antimicrobial resistance (AMR) in bacterial populations is driven by the consumption and misuse of antimicrobials in human medicine, agriculture, and the environment (1-3). Historically, the threat of AMR was overcome by using novel antimicrobials with unique drug targets. However, the discovery rate of new antimicrobial agents has dwindled (4-6) and severely lags behind the rate of AMR evolution. While concerted scientific, corporate and political focus is needed to recover antimicrobial pipelines (7-9), there is an urgent need for alternative strategies that prolong the efficacy of existing antimicrobials and prevent or slow the emergence, spread and persistence of AMR. Current global efforts to improve antimicrobial stewardship largely focus on awareness and reducing overall consumption (8, 10-12). While these efforts will affect the evolution and spread of AMR, mounting evidence suggests that these changes alone will not lead to large-scale reductions in the occurrence of AMR (13-17).

Several recent studies have examined novel treatment strategies using multiple antimicrobials that could reduce the rate of resistance emergence and even reverse pre-existing AMR. These approaches, collectively termed “selection inversion” strategies, refer to cases where resistance becomes costly in the presence of other antimicrobial agents (18). Among the most promising of these strategies are those based on a phenomenon first reported in 1952 termed “collateral sensitivity” (CS), where resistance to one antimicrobial simultaneously increases the susceptibility to another (19). CS and its inverse, cross-resistance (CR), have been demonstrated for several bacterial species and across different classes of antimicrobials (20-26). These results have formed the

basis of proposed CS-informed antimicrobial strategies that mix drug pairs (21, 27) or alter temporal administration, e.g. drug cycling (20, 28). The notion behind these strategies is that they would force bacteria to evolve AMR along a predictable trajectory that results in CS; this predictability can then be exploited to ultimately reverse resistance and prevent the fixation of AMR and multi-drug resistance development at the population level of bacterial communities.

Initial *in vitro* experiments support using CS-based strategies to re-sensitize resistant strains (20) and reduce rates of resistance development (28); however, the broader application of this principle depends on predictable bacterial responses during antimicrobial therapy. This predictability must be general for a given drug class and should not vary across strains of the same species. To date, most studies of CS and CR have focused on describing these responses as collateral networks using resistant mutants derived from single laboratory adapted strains and few exemplary clinical isolates. Two studies on *Pseudomonas aeruginosa* have further investigated CS in collections of clinical isolates (29, 30). However, these studies lack either proper baseline controls (29) or sufficient genetic diversity (30). As valuable as previous studies have been, the responses of single strains (laboratory or clinical) may not be representative of CS and CR evolution in other strains. To address this limitation, we focused on understanding collateral networks in clinical urinary tract infection isolates of *Escherichia coli* with selected resistance to drugs widely used for the treatment of urinary tract infections; ciprofloxacin (CIP), trimethoprim (TMP), nitrofurantoin (NIT), or mecillinam (MEC).

We investigated the collateral networks to 16 antimicrobials from diverse drug classes in 10 genetically diverse clinical strains (corresponding to 40 laboratory-generated AMR mutants) to assess the factors contributing to collateral responses (both CS and CR). This approach allowed us to identify variation in the sign and magnitude of collateral responses and identify mechanisms of CS and CR that are preserved in different genetic backgrounds. By using multivariate statistical modeling of experimental data, we show that resistance mutations, particularly those affecting efflux pumps, and the relative fitness of resistant isolates are more important determinants of the structure of collateral networks than genetic background. Our results support the idea that collateral responses may be predictable.

Results

Collateral responses are frequent and vary both between and across AMR groups. Starting from 10 pan-susceptible urinary tract clinical *E. coli* isolates (**Fig. S1**) (31), a single AMR mutant was generated to each of four individual antimicrobials used for urinary tract infections treatment, CIP, TMP, NIT, and MEC. Here we define 'AMR group' as the collection of mutants from the 10 different genetic backgrounds that were selected for resistance to the same antimicrobial. In total 40 AMR mutants were generated that had resistance levels above clinical breakpoints, as determined by antimicrobial susceptibility testing using both gradient strip diffusion (**Table S1**) and inhibitory concentration 90% (IC_{90}; (20)) testing (**Table 1**). Changes in the IC_{90} of AMR mutants from each respective wild-type (WT) strain (**Fig. S2**) were compared for 16 antimicrobials (**Table 2**). The two methods are correlated but IC_{90} measurements allow

for more robust detections of small relative differences in susceptibility (32, 33). Overall, collateral responses were observed in 40.5% (243/600) of possible instances (**Table S2**); of these, roughly half, 47% (115/243), were associated with only a 1.5 and 2-fold change in IC_{90}. Such small changes would not be observed by typical 2-fold antimicrobial susceptibility testing methods frequently used in clinical laboratories.

Overall CR was more frequent than CS, 151 versus 92 instances (**Table S2**), and collateral networks varied considerably between AMR groups. We observed 20 cases of conserved collateral responses (**Fig. 1**), where CR or CS to a specific antimicrobial was found in $\geq$ 50% of the mutants within an AMR group, defined as CR_{50} or CS_{50} respectively. This indicates that some collateral responses are likely general, irrespective of genetic background. For each CS_{50} and CR_{50} observation, IC_{90} results were further assessed by generating dose response curves of representative strain:drug combinations (**Fig. S3**). Inhibition of growth was shown to vary across antimicrobial concentrations between AMR mutants and respective WT strains, confirming the changes in antimicrobial susceptibility determined by the IC_{90} assays. While conserved collateral responses were observed, there was variability across the remaining genetic backgrounds.

During the selection of AMR mutants, we often observed conspicuous changes to colony size for all AMR groups, suggesting that resistant mutants had reduced bacterial fitness. To test this, we measured the growth rates of AMR mutants and compared these to the respective WT strains (**Fig. S4**). In general, CIP^R and MEC^R mutants displayed severely reduced growth rates suggesting high costs of resistance. The cost of CIP^R and MEC^R

mutants, measured as relative growth rate, was significantly different from 1 and varied between 0.34 and 0.75 with a mean of 0.53 for all CIP^R mutants, whereas the cost of MEC^R mutants varied between 0.49 and 0.79 with a mean of 0.64. NIT^R and TMP^R mutants displayed lower levels of reduced fitness and several resistant mutants harbored apparent cost-free resistance mutations (**Fig. S4**). Only two of ten NIT^R mutants and four of ten TMP^R mutants displayed a significant cost of resistance. Relative growth rates varied between 0.93 and 1.05 and 0.68 and 1.07 with averages of 0.99 and 0.94 for NIT^R and TMP^R mutants, respectively.

Conserved collateral responses were primarily found in CIP^R mutants. Nearly half (108/243, 44%) of the observed collateral responses were in CIP^R mutants, while the remaining 135 were distributed between the other three AMR groups (**Table S2**). Within the CIP^R group, the majority of collateral responses were CR (70/108, 64.8%). Additionally, CS responses found for the CIP^R mutants were the most conserved in our dataset, with CS to GEN occurring in 8 of 10 strains and CS to FOS in 7 of 10 strains. GEN and other aminoglycosides are important for the treatment of a wide range of hospital infections (34), while FOS is primarily used for treatment of uncomplicated urinary tract infections (35, 36). The CIP^R mutants were also unique in the magnitude of observed changes, with cases of CR close to 30-fold and CS as high as 6-fold changes in IC_{90} (**Fig. S2**). Only CIP^R isolates displayed potential clinically relevant CR, where six strains had IC_{90} values above the clinical breakpoint for resistance to CHL and a single strain (K56-68 CIP^R) was above the AMX breakpoint.

Characterization of AMR mutants. We hypothesized that CS and CR variation in and between the AMR groups could be attributed to mutations causing resistance in each strain. Using whole genome sequencing we identified a total of 150 mutations in the AMR mutants (**Table S3**). Of these, 88 mutations affect previously described or putative AMR associated genes, gene regions, or pathways (**Table S3**). The remaining mutations were found in other cellular processes with no known association to antimicrobial resistance (*e.g.* metabolic pathways and virulence factors), such as mutation to the FimE regulator of FimA that was frequently observed in MEC^R mutants (**Table S3**). Aside from FimE, we did not observe similar mutations in non-AMR associated regions across strains of the same AMR group (parallel evolution), suggesting that such mutations had limited, if any effect on collateral responses in this study. For each of the 40 AMR mutants at least one putative resistance mechanism was identified, including mutations to previously described antimicrobial drug targets and promoters of drug targets, drug modifying (activating) enzymes, regulators of efflux pumps, RNA polymerases and mutations to other metabolic and biochemical processes that may contribute to resistance (**Table 3**). Briefly, all but one CIP^R mutant contained mutations in *gyrA* and efflux regulatory genes and gene-regions likely affecting efflux expression (*acrAB* and/or *mdtK*), while one strain had only drug target mutations and displayed the well-described GyrA (S83L) and ParC (G78D) mutation combination. Both efflux and drug target mutations are frequently found in surveys of clinical isolates (37-40). NIT^R mutants had mutations in one or both nitro-reductases (*nfsA, nfsB*) and the majority of strains had additional mutations in *mprA* that encodes an efflux regulator of EmrAB-TolC pump expression. TMP^R mutants contained mutations either in *folA* and/or its promoter or

183 genetic amplification of a large genetic region containing *folA*. MEC^R mutants are unique
184 in that they were evolved as single step mutants, where a single mutation could confer
185 clinically relevant resistance to MEC. Resistance development for the remaining three
186 drugs required several steps, as multiple mutations were required for resistance above
187 clinical breakpoints. In total 12 different mutations in genes and/or cellular processes
188 previously linked to MEC^R were identified in the MEC^R group (41).
189
190 Our data suggest that the few collateral responses in TMP^R mutants are attributable to a
191 specific mechanism of resistance affecting a single unique drug target (*i.e.*
192 overexpression/alteration of FolA). Similarly, the CIP^R group displayed a clear trend
193 where conserved CR responses were strongly linked to mutations in efflux regulatory
194 regions. In the remaining AMR groups we observed heterogeneous collateral responses.
195
196 The WT genomes were evaluated for the presence of genetic elements linked to
197 AMR. We detected only one acquired genetic element, *sul*2 (linked to sulfonamide
198 resistance) in K56-44, and two point mutations, PmrB V161G in K56-50 and K56-70
199 and ParE D475E in K56-78, that are linked to COL and quinolone (CIP) resistance,
200 respectively (**Table S3E**). It is unclear what, if any, effect these resistance determinants
201 have in these WT strains since all were phenotypically pan-susceptible (**Fig. S1**).
202 However, the ParE mutation could contribute to CIP^R levels in K56-78, as the K56-78
203 WT was among the higher WT values for CIP (0.015 µg/mL) (**Fig. S1**) and K56-78 CIP^R
204 was among the highest IC_{90} values of the CIP^R mutants (**Table 1**).
205

Multivariate statistical analyses suggest that efflux-related mutations and relative fitness are significant contributors to collateral responses. Multivariate statistical approaches were used to investigate the extent to which genetic (strain) background, AMR group, the putative mechanism of resistance in particular efflux-related mutations (**Table S3**), growth rate and the fitness cost of resistance explain the total variation in collateral responses. All the above factors were investigated individually and the related models are found in the supplementary material (**Fig. S5A**). Throughout the remaining analyses we focus mainly on efflux-related mutations rather than AMR group to explicitly address putative mechanisms of resistance and on relative fitness rather than the growth rate. We estimated several models with individual, or a combination of, factors to assess their effect size and significance given some level of co-linearity between fitness and efflux type (**Fig 2, Fig. S5C-F**). A model including strain background, relative fitness and efflux-related mutations as factors explained 62.53% of the total variation in IC_{90} values (**Fig. 2A, Table S4**). In this three-factor model there was clear separation of the mutants by AMR group. The CIP^R mutants showed strong CR towards TEM, CHL, CAZ and AMX separating this AMR group from the others along the first ordination axis. Along the second ordination axis, MEC^R isolates were distinct, had CR to TEM, and were more likely to have CS towards drugs such as AZT and CHL (**Fig. 2A**). Both efflux type and relative fitness were significant predictors when tested alone and in combination (**Table S4**). The model (**Fig. 2A**) also revealed that strain background had a limited, non-significant contribution. Even when modeled alone (**Fig. S5A**), strain background only accounted for 6.53% of the variation and was non-significant (**Table S4**).

229

230 We initially hypothesized that genetic background would significantly affect collateral
231 responses. Our data suggest that it does not. Arguably, the inclusion of IC_{90} data from the
232 drugs to which primary resistance was selected for could confound the analysis, despite
233 our efforts to minimize these effects using log transformed data. We used the same
234 approaches to assess a subset of collateral responses, excluding data for all of the 40
235 AMR mutants to five antimicrobials containing the drugs used for selection (CIP, MEC,
236 NIT, TMP) and SXT. Within the subset model, patterns consistent with the full model
237 were observed, but with a lower degree of clustering by AMR group (**Fig. 2B**). For
238 example, the K56-2 CIP^R mutant is now co-localized with the MEC^R isolates, indicating
239 that this isolate is distinct from other CIP^R mutants, which still showed strong tendencies
240 of CR to TEM, CHL, CAZ and AMX. Despite these changes, efflux type and fitness
241 were still significant predictors of collateral networks, whereas strain background
242 remained non-significant in models, both in combination and alone (**Fig. S5B**, **Table S4**).
243 Mutations in efflux-related genes and gene regulators were the strongest predictor of
244 collateral responses tested, explaining over 33% of the variation in the subset. Fitness
245 alone also had significant predictive value, but to a lesser extent (17% variation
246 explained). It is important to note that we observed correlation between efflux mutations
247 and relative fitness that is likely explained by reduced fitness resulting from the cost of
248 overexpression of efflux pump(s) (38).

249

250 To investigate the influence of resistance mechanism on IC_{90} variation at a higher
251 resolution, we modeled each AMR group separately relating the putative resistance

252 mechanism and fitness separately and in combination (**Fig. S5**). However potentially due
253 to a lower number of samples within AMR groups and more detailed classification of the
254 resistance mechanism, these factors had varying degrees of contribution. For TMP^R and
255 CIP^R AMR groups, resistance mechanism was non-significant, but it was a significant
256 factor for NIT^R and MEC^R mutants. Fitness was a significant factor of variation only for
257 the MEC^R group and similarly, combination models using both resistance mechanism and
258 fitness were non-significant for all AMR groups, with the exception of the MEC^R group.
259
260 **Collateral responses shift the mutation selection window.** The mutant selection
261 window (MSW) can be defined as the concentration space between the lowest
262 antimicrobial concentration that selects for and enriches resistant mutants (42) and the
263 concentration that prevents the emergence of first step resistant mutants, the mutation
264 prevention concentration (MPC) (43, 44). In theory, if drug concentrations remain above
265 the MPC during treatment AMR is less likely to evolve (43, 44). It was recently
266 demonstrated in *E. coli* MG1655 that changes in MPC correlated with collateral
267 responses in AMR mutants (20). We determined the MPC for 17 strain:drug
268 combinations that exemplified the conserved collateral responses (**Fig. 1**). The MPC for
269 each AMR mutant and its respective WT were compared (**Fig. 3**). In 12/17 (70.6%) the
270 change in MPC was consistent with the sign of collateral responses in IC_{90}. This
271 demonstrates that even small CS/CR changes can affect the MSW, correspondingly
272 shifting it down or up. However, in 4/17 (23.5%) the MPC displayed no change between
273 the WT and AMR mutant. This was observed when testing the MPC for MEC, TMP and
274 AZT, though we speculate that increasing the precision of the MPC assay (as was done

with IC_{90} testing) might negate these discrepancies. Changes in MPC results with AZT were inconsistent with the change in IC_{90} for a CIP^R mutant and the MEC^R and NIT^R mutants, which displayed a decreased MPC instead of an expected increase or no change, respectively.

Discussion

Here we present, to our knowledge, the first in depth study to identify conserved collateral responses in antimicrobial susceptibility across genetically diverse clinical *E. coli* strains following antimicrobial resistance development. Our findings are relevant beyond urinary tract infections because uropathogenic *E. coli* are shown to also stably colonize the bladder and gut (45), and to cause bloodstream infections (46). Our data show that CS and CR are pervasive in clinical *E. coli* strains, consistent with earlier results based on laboratory-adapted strains of various species (20-22, 24, 29, 47) and a limited number of clinical isolates (20, 29). Resistance to CIP resulted in a greater number of collateral responses than resistance to MEC, NIT, or TMP. CR was much more prevalent than CS, and the magnitude of the collateral responses were most often small, consistent with other reports (20-22). Overall we observed that collateral responses varied substantially by AMR group, but variation was also observed within AMR groups.

Using CS_{50} and CR_{50} thresholds to identify conserved responses, we found that conserved CR was more than twice as common as conserved CS. Whereas many of the conserved collateral responses identified in this study support the findings in previous work using

298 single laboratory-adapted strains, we observed several clinically relevant differences. For
299 example, our finding of conserved CS in CIP^R mutants to GEN was previously reported
300 in *E. coli* K12 (21) but not in MG1655 (20). In the CIP^R mutants we also observed
301 conserved CR towards CHL, as reported in (20), but not in (22). We identified conserved
302 CR of NIT^R mutants to AMX, and this was not reported in MG1655 (20). These
303 observations underscore the importance of exploring collateral networks in multiple
304 mutants of different clinical strain backgrounds and with different resistance mechanisms
305 to assess their potential clinical application. Visual inspection of the data revealed a few
306 clinically relevant examples of CS phenotypes that appeared independent of putative
307 mechanism of resistance. We show that CIP^R *E. coli* strains displayed CS towards GEN,
308 FOS, ETP and COL, and these phenotypes were conserved across multiple mechanisms
309 of resistance. These results parallel those of a recent study on *P. aeruginosa* clinical
310 isolates from cystic fibrosis patients, where CIP^R was associated with CS to GEN, FOS
311 and COL. Taken together these data support the presence of general conserved collateral
312 networks that may both affect the population dynamics of AMR during treatment and
313 counter-select for resistance as recently indicated (30).
314
315 We assumed *a priori* that genetic background, AMR group, resistance mechanism, and
316 the fitness cost of resistance could potentially affect the generality, sign and magnitude of
317 collateral networks in clinical *E. coli* strains. Despite the fact that some collateral
318 responses are conserved across different strains and mechanisms of resistance, our
319 multivariate statistical approaches show overall that mechanism of resistance is the key
320 predictor of CS and CR variability. This is primarily the case for efflux related mutations.

However, mechanism of resistance also significantly contributed to the observed CS and CR variation in the MEC mutants where no efflux mutations were found. The presented data are consistent with earlier reports based on multiple AMR mutants derived from single strains with different resistance mechanisms towards specific antimicrobials (21, 22, 48). Our finding that genetic background did not significantly contribute to collateral responses is an important addition to these earlier studies. Finally, we found that the fitness cost of resistance also contributed significantly to the observed variation in CS and CR, despite some overlap in explanatory power due to the observed correlation between efflux-related mutations and reduced fitness. Taken together, our data and previous reports indicate that applied use of collateral networks in future treatment strategies may be dependent on rapid identification of specific resistance mechanisms. Moreover, clinical application of CS as a selection inversion strategy warrants further investigations to ideally explore CS in isogenic backgrounds, representing several diverse strains, with permutations of all known AMR-associated resistance traits. Such extensive studies would likely provide valuable information on the mechanisms of CS. Other confounding factors such as mobile genetic elements with heterogeneous resistance determinants should also be investigated as they would likely influence and reduce the predictability of collateral networks.

Selection inversion, as described by (20), depends on the cycling of drug pairs that display reciprocal CS. We did not observe reciprocal CS between any of the four drugs studied here that are widely used for treatment of urinary tract infections. However, we asked if modest reductions and increases in antimicrobial susceptibilities would affect the

MSW (43) for the most prevalent CS and CR phenotypes. We subjected conserved CS and CR phenotypes to MPC assays and revealed that even a small 1.5-fold change in IC_{90} could equally alter the MPC, resulting in a shift of the MSW. These results suggest that antimicrobial treatment strategies informed by collateral networks could affect AMR evolutionary trajectories. Sequential treatment using drug pairs that display CR would, following resistance development, shift the MSW towards higher antimicrobial concentrations and increase the likelihood for resistance development to subsequent treatment options. Conversely, sequential treatment based on drug pairs that display CS can shift the MSW down and reduce the window of opportunity for high-level resistance development. This result suggests that the initial choice of antimicrobial may set the stage for later resistance development (**Fig. 4**). Based on our *in vitro* findings, TMP and NIT are attractive from a clinical perspective, as resistance to these resulted in few collateral responses, preserving the innate sensitivity to available secondary antimicrobials. However, MEC could be even more attractive, as CS largely dominates the observed collateral responses in MEC^R mutations. Additionally MEC^R isolates, especially those evolved *in vivo*, are associated with high cost of resistance (41). In contrast, CIP exposure was more likely to cause dramatic collateral responses that depended on the mechanism of resistance and could potentially negatively impact future therapeutic options. These observations align with antimicrobial treatment recommendations in Norway, where MEC, NIT and TMP are recommended for first line therapy of uncomplicated UTI, and CIP is reserved for otherwise complicated infections (49). Similarly, in the United States NIT, SXT (TMP-SMX) and MEC are recommended before fluoroquinolones such as CIP, ofloxacin, and levofloxacin (50).

Our conclusions are not without limitations. First, we acknowledge that including more clinical isolates from different infection foci, more diverse genetic backgrounds, as well as other selective agents, could change the outcome of our statistical analyses. This would allow increased sensitivity for the assessment of the different factors controlling collateral responses. A more targeted approach to assess the impact of specific resistance mechanisms on CS and CR across genetically diverse clinical strains is lacking in the field. Our analyses suggest that the fitness cost of resistance explains some variability in the collateral networks reported here. While it is known that growth rates affect antibiotic action (51), the underlying mechanism is currently unknown. It is also unclear if collateral networks will be perturbed by compensatory evolution, which eliminates the fitness costs of primary resistance (52-54). Finally, this and previous studies focus on AMR development to a single drug and there is a complete lack of data on how multidrug resistance, including resistance genes on mobile genetic elements, will affect collateral networks. We are currently investigating these and other questions that will aid in our understanding of collateral networks and their potential therapeutic application.

Methods

Bacterial strains. We used ten clinical urinary tract infection isolates of *E. coli* from the ECO-SENS collections (55, 56) originating from countries across Europe between 2000 and 2008 (**Table 1**). The isolates were chosen to represent pan-susceptible strains with diverse genetic backgrounds and were reported plasmid-free (31). Subsequent analysis based on whole-genome sequencing discovered two changes to previously reported

ST's and the presence of plasmid replicons in three strains (**Table S5E**). *E. coli* ATCC 25922 was used for reference and quality control purposes. For general growth, bacterial strains were grown in either Miller Difco Luria-Bertani (LB) broth (Becton, Dickinson and Co., Sparks MD, USA) or on LB agar; LB broth supplemented with select agar (Sigma-Aldrich) at 15 g/L. All strains were incubated at 37°C.

Selection of AMR mutants. Single AMR mutants were selected at MICs above the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints (57) for CIP, NIT, TMP and MEC (**Table 1**). Resistant mutants were selected on Mueller Hinton II agar (MHA-SA; Sigma-Aldrich) for CIP, NIT and TMP using a step-wise static selection. MEC^R mutants were selected as single-step mutants on LB agar. For more details, see **Text S1**. AMR mutants were confirmed as *E. coli* using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) analysis with MALDI BioTyper software (Bruker, MA, USA).

Antimicrobial susceptibility testing. AMR mutants were screened for resistance above EUCAST breakpoints (**Table S1**, (57)) with gradient diffusion strips following manufacturers guidelines (Liofilchem, Italy), on Mueller Hinton II agar (MHA-BD; Becton, Dickinson and Company) after 18 hours incubation. Plates with insufficient growth were incubated an additional 24 hours.

Collateral changes to 16 antimicrobials (**Table 2**) were determined by IC_{90} testing (20), with some modifications. Standard 2-fold concentrations and median values between

413 them were used as a "1.5-fold" testing scale. IC_{90} values were read as the first
414 concentration tested that resulted in ≥ 90% inhibition of growth (optical density at
415 600nm, OD_{600}) following 18 hours incubation at 700 rpm (3 mm stroke) in Mueller
416 Hinton Broth (MHB, Becton, Dickinson and Company). Percent inhibition was
417 calculated as previously described (20). IC_{90} results were determined in at least three
418 biological replicates on separate days always including the control strain ATCC 25922.
419 The final result reflects the average of a minimum of three replicates that met quality
420 control standards (**Text S1**). Fold change in IC_{90} was calculated as the ratio between the
421 AMR mutant and its respective WT.
422
423 Dose response curves were generated with averages of OD_{600} values (background
424 subtracted) for each concentration tested during the IC_{90} experiments. Averages were
425 plotted for AMR mutants and respective WT strains.
426
427 **Mutation prevention concentration testing.** CS and CR trends to single drugs present
428 in ≥ 50% of the isolates (CS_{50} or CR_{50}), were confirmed by determining the mutation
429 prevention concentrations (MPCs), essentially as described previously (58). Briefly, 10
430 mL aliquots of an overnight culture were pelleted and re-suspended in 1 mL MHB,
431 estimated to contain ≥ 10^{10} CFU (actual values were 1.4 x 10^{10} - 7 x 10^{10} CFU). The
432 inoculum was split and spread onto four large (14 cm diameter) MHA-SA agar plates for
433 each antimicrobial concentration tested (4 - 6 concentrations of a 2-fold dilution series).
434 The MPC was the lowest concentration with no visible growth after 48 h (**Text S1**).

435 AMR mutants and WTs were tested in parallel and the results represent the average of a
436 minimum of two biological replicates.
437
438 **Growth rate measurements.** Growth curves of WT and AMR mutants were generated
439 in a Versamax plate reader (Molecular Devices Corporation, California, USA) with
440 constant shaking overnight. The OD_{600} was measured every 10 minutes and growth rates
441 were estimated using GrowthRates v.2.1 software (59) (**Text S1**).
442
443 **Identification of genetic AMR determinants.** Genomic DNA was isolated using the
444 GenElute Bacterial Genomic DNA kit (Sigma-Aldrich) following guidelines for Gram-
445 positive DNA extraction. Purity and quantification of genomic DNA was determined
446 with Nanodrop (Thermo Scientific) and Qubit High Sensitivity DNA assay (Life
447 Technologies), respectively and used to prepare libraries using the DNA Ultra II Library
448 Preparation Kit (New England Biolabs, E7645) according to manufacturers description
449 (**Text S1**). Libraries were then quantified by Qubit High Sensitivity DNA assay and
450 distributions assessed by Bioanalyser DNA 1000 Chip (Agilent, 5067-1504) before
451 normalizing and pooling. The final library pool was sequenced on the MiSeq (Illumina,
452 San Diego) using 250 bp paired end reads and V2 chemistry.
453
454 WT genomes were assembled as described in **Text S1**. WT genomes were annotated
455 with Rapid Annotation using Subsystem Technology server (RAST, version 2.0) for *E.*
456 *coli* (60). SeqMan NGen (DNASTAR, Madison, WI) was used for comparative analysis
457 of raw AMR mutant Illumina reads, using standard settings. AMR mutant reads were

458 aligned to the corresponding annotated WT genome assembly. Reported SNPs had ≥ 10x
459 coverage depth and ≥ 90% variant base calls. SNPs present in the WT assembly or in at
460 least two AMR mutants of the same strain background were excluded. Genetic deletions
461 and rearrangements were identified in the structural variation report generated in SeqMan
462 Pro (DNASTAR) and were manually inspected and annotated using Gene Construction
463 Kit (Textco Biosoftware Inc., Raleigh, NC) and NCBI BLAST searches, respectively.
464
465 **Multivariate Statistical Analyses.** The fold changes of mean IC_{90} values (collateral
466 responses) were determined relative to the parental WT strain and log transformed.
467 Statistical analyses were performed on the complete data set, as well as a subset of the
468 data excluding five antimicrobials, those to which primary AMR was evolved (CIP,
469 MEC, NIT, and TMP) and SXT. To estimate and test the effects of strain background,
470 AMR group, AMR mechanism, growth rate and relative fitness we relied on multivariate
471 modeling, via redundancy analysis, to address the co-variation in IC_{90} across
472 antimicrobials. The linear constraint scores were plotted for each AMR mutant. The
473 response variables were overlaid with independent scaling to illustrate the direction of
474 "steepest ascent" (increasing CR) from the origin for each antimicrobial. The
475 significance of multivariate models and of their factors was assessed by permutation tests
476 (1000 permutations) where $p < 0.05$ was considered significant. These analyses were
477 done in R (61) using the Vegan work package (62).
478

Data availability. Whole-genome sequencing data are available within the NCBI BioProject PRJNA419689. All other relevant data are available within this article, its Supplementary Information, or from the corresponding author upon request.

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Author Contributions

P.J.J. and Ø.S. conceived the project; N.L.P., E.G.A.F., J.K., and V.S. designed and performed experiments; R.P. designed and R.P and N.L.P. performed the multivariate statistical modeling; all authors analyzed, interpreted and discussed the data; and N.L.P., E.G.A.F., A.P.R., D.E.R., and P.J.J. wrote the manuscript with contributions from the other authors.

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Supplementary Information List

Text S1

Figure S1: The distribution of average IC_{90} values for wild-type *Escherichia coli* clinical isolates.

Figure S2: Collateral changes in susceptibility of 40 AMR mutants to 16 antimicrobials.

Figure S3: Dose response curves of representative strain:drug combinations that demonstrate frequently observed collateral responses (CS_{50} and CR_{50}).

Figure S4: Relative growth rate of AMR mutants compared to their respective WT ancestors.

Figure S5: Multivariate models displaying the contribution of individual and combinations of factors to collateral networks.

Table S1: Gradient strip diffusion MIC values of AMR mutants.

Table S2: Tabulation of collateral responses detected in 40 AMR mutants.

Table S3: Genomic analyses of whole genome sequencing data in WT strains and AMR mutants.

Table S4: Summary of the output from multivariate models.

Table 1: Description of *Escherichia coli* strains used in the study and susceptibility changes following antimicrobial selection *in vitro*.

			Average Inhibition Concentration 90% (IC_{90}, µg/mL)[1]							
			Ciprofloxacin		**Mecillinam**		**Nitrofurantoin**		**Trimethoprim**	
Strain	**ST[2]**	**Origin**	**WT**	**CIP^R**	**WT**	**MEC^R**	**WT**	**NIT^R**	**WT**	**TMP^R**
K56-2	73	Greece	0.014	16	0.146	> 30	8	> 64	0.225	> 28
K56-12	104	Portugal	0.016	1.67	0.273	28	7.33	> 64	0.563	> 32
K56-16[3]	127	Portugal	0.009	3	0.167	18.7	4	> 64	0.25	> 30
K56-41	73	Greece	0.016	2.33	0.104	13.3	6	> 64	0.25	6.67
K56-44[3]	12	Greece	0.013	1.67	0.141	16	6.67	> 64	0.375	6
K56-50	100	Greece	0.012	3	0.141	10.7	12	> 64	0.172	18
K56-68	95	Sweden	0.014	4	0.141	30	6.67	> 64	0.208	18.7
K56-70	537	Sweden	0.007	2.67	0.083	> 32	4.67	> 64	0.25	14.7
K56-75[4]	69	UK	0.008	1.17	0.063	13	6	> 64	0.167	5.33
K56-78	1235	UK	0.015	6	0.141	16	8	> 64	0.5	7.33
Resistance Breakpoint[5]			> 0.5		> 8		> 64		> 4	

[1] The average IC_{90} values of three or more biological replicates. Individual results above detection limits (MEC = 32 µg/mL, NIT = 64 µg/mL, TMP = 32 µg/mL) were analyzed as those values, yielding final results with uncertainty (> average).

[2] Multi-locus sequence type

[3, 4] strains containing the Col156 or Col(MP18) replicon, respectively.

[5] EUCAST Clinical Breakpoints v 7.1 for Enterobacteriaceae (56).

Table 2: List of antimicrobials used in this study.

Antimicrobial[1]	Abbreviation	Drug Class	Drug Target(s)	Solvent
Amoxicillin	AMX	β-lactam (Penicillin)	Cell wall synthesis	Phosphate buffer[3]
Azithromycin	AZT	Macrolide	Protein synthesis (50S)	≥ 95% Ethanol
Ceftazidime	CAZ	β-lactam (Cephalosporin)	Cell wall synthesis	Water + 10% (w/w) Na_2CO_3
Chloramphenicol	CHL	Amphenicol	Protein synthesis (50S)	≥ 95% Ethanol
Ciprofloxacin	CIP	Fluoroquinolone	DNA replication, cell division	0.1N HCl
Colistin	COL	Polymyxin	Cell wall & cell membrane	Water
Ertapenem	ETP	β-lactam (Carbapenem)	Cell wall synthesis	Water
Fosfomycin	FOS	Phosphonic	Cell wall synthesis (MurA)	Water
Gentamicin	GEN	Aminoglycoside	Protein synthesis (30S)	Water
Mecillinam	MEC	β-lactam (Penicillin)	Cell wall synthesis (PBP2)	Water
Nitrofurantoin	NIT	Nitrofuran	Multiple[4]	Dimethyl sulfoxide
Trimethoprim	TMP	Antifolate	Folate synthesis (FolA)	Dimethyl sulfoxide
Sulfamethoxazole	SMX	Antifolate	Folate synthesis (FolP)	Dimethyl sulfoxide
TMP+SMX (1:19)	SXT	Antifolate	Folate synthesis (FolA+FolP)	Dimethyl sulfoxide
Temocillin	TEM	β-lactam (Penicillin)	Cell wall synthesis	Water
Tetracycline	TET	Tetracycline	Protein synthesis (30S)	Water
Tigecycline	TGC	Tetracycline	Protein synthesis (30S)	Water

[1] When available final antimicrobial concentration was determined using manufacturer-provided or calculated drug potencies, otherwise potency was assumed to be 100%. Aliquots were stored at -20°C or -80°C in single use vials. All antimicrobials and chemical solvents were obtained from Sigma-Aldrich (St. Louis, MO, USA) with the exception of CIP (Biochemika, now Sigma-Aldrich) and TEM (Negaban®).
[3] 0.1 mol/L, pH 6.0 phosphate buffer supplemented with 6.5% (v/v) 1M NaOH (sodium hydroxide).
[4] Nitrofurantoin is thought to target macromolecules including DNA and ribosomal proteins, affecting multiple cellular processes including protein, DNA, RNA and cell wall synthesis.

Table 3: The number of AMR mutants with resistance-associated mutations.

AMR Mechanism		CIP^R	MEC^R	NIT^R	TMP^R
Drug Target	Modification	10[1]			6
	Overproduction				6
Drug Activation	Nitroreductase disruption			10	
Drug Uptake	Porin mutation	1			
Efflux	AcrAB-TolC	7		1	
	MdtK	9		1	
	MdfA			1	
	EmrAB-TolC			7	
	ABC transport		1		
ppGpp synthesis (stringent response activation)	Stringent Response		4		
	tRNA synthesis		4		
	tRNA processing		1		
	Cellular metabolism		3		

[1] All CIP^R mutants contained one mutation in the *gyrA* gene, except K56-2 CIP^R mutant that contained two mutations in *gyrA* and a mutation in *parC*.

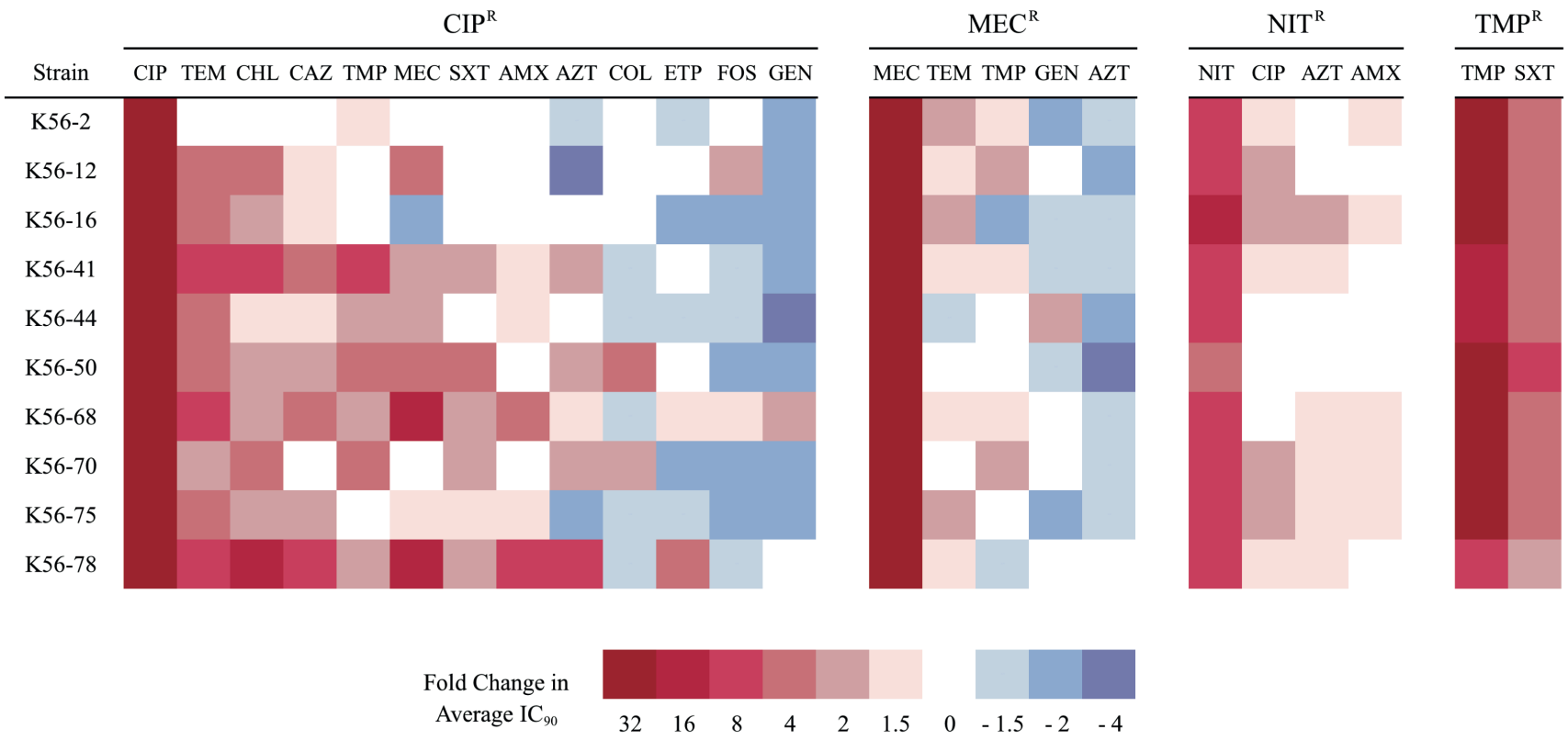

Figure 1. Conserved collateral effects observed in AMR mutants of different AMR groups. Relative change in antimicrobial susceptibility was determined by comparing average IC_{90} values of AMR mutants to their respective WT strain. Collateral changes that were found in ≥ 50% of the strains are displayed. Antimicrobials are ordered by most frequent CR (left) to most frequent CS (right) for each AMR group. The only instances where CR was present in 100% of the strains of an AMR group were linked to the drug used for selection, including TMP^R strains with CR to SXT (a combination of TMP and SMX).

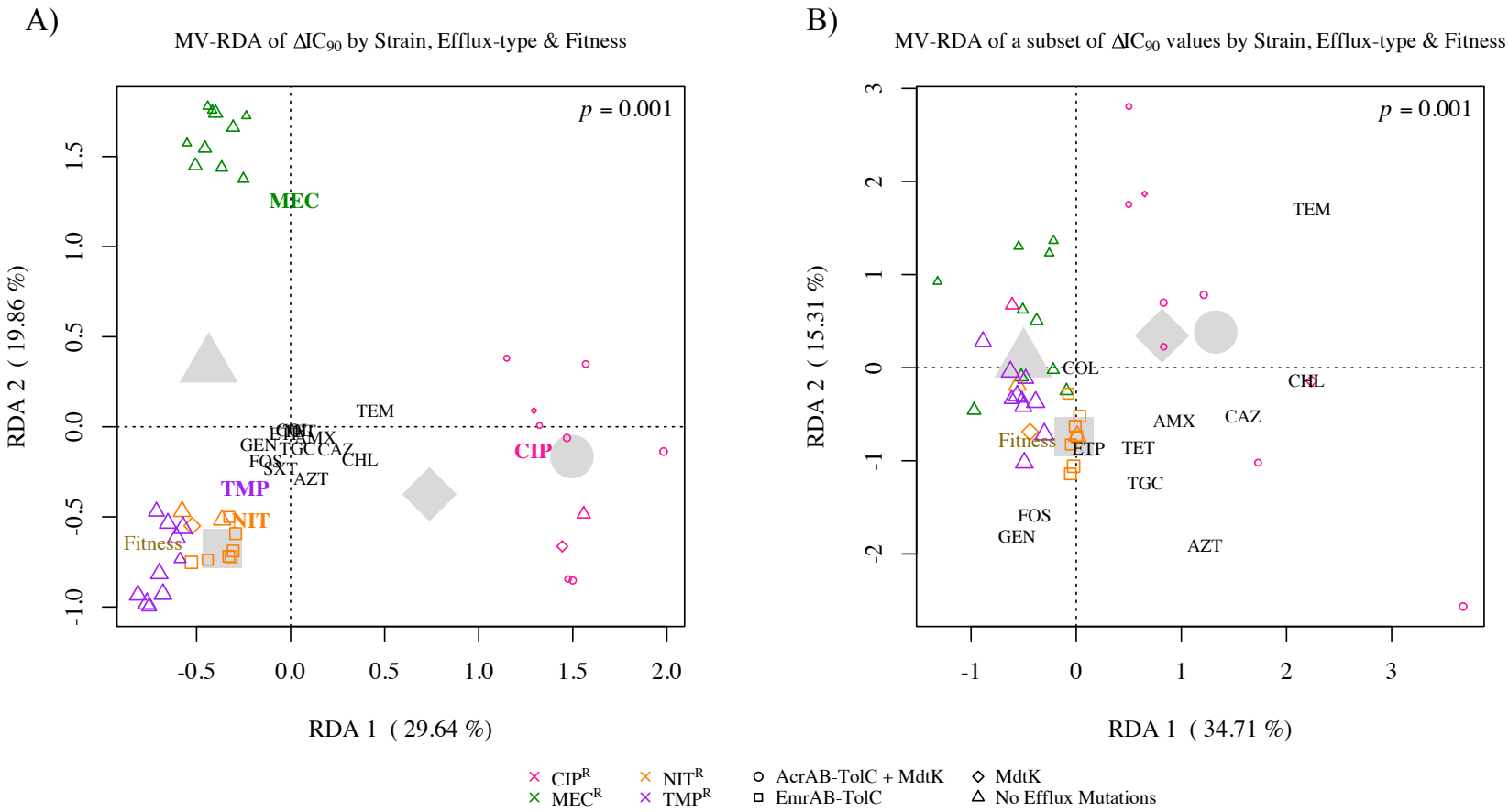

Figure 2. **Results of multivariate statistical modeling**. Graphical representations of redundancy analysis (RDA, triplot) results relating strain background, presence of efflux-related mutations and relative fitness to the observed changes in IC_{90} between AMR mutants and the respective WT strain for 16 antimicrobials tested (**A**) and a subset of the antimicrobials excluding CIP, MEC, NIT, TMP and SXT (**B**). The first and second RDA axes shown display most of the explained variation in IC_{90} changes. The weighted average of each AMR mutant is plotted as a single colored symbol, where color indicates the AMR group (CIP^R – pink, MEC^R –

green, NIT^R – gold, TMP^R – purple), shape the assigned efflux type (circle – AcrAB-TolC + MdtK, square – EmrAB-TolC, diamond – MdtK, triangle – no efflux-related mutations), and symbol size is proportional to relative fitness, where smaller size indicates a greater reduction in growth rate compared to the WT. Antimicrobial drug names indicate the tip of vectors that pass through the origin in the direction of increasing IC_{90} fold change or CR (direction of steepest ascent). These vectors can be used to interpret the change in IC_{90} for the antimicrobials shown, e.g. there was little change in the IC_{90} of drugs centered near the origin, such as COL in **B**. The vector tip of relative fitness (brown) is also shown. Large grey symbols show the centroids (average effect) for all AMR mutants within a given efflux group (shape). The majority of explained variation is driven by primary resistances (**A**), where CIP^R mutants (pink) cluster away from the other three AMR groups along the CIP vector, indicating higher resistance to CIP. CIP^R mutants are likely to show CR to CHL, CAZ, TEM and AZT, but sensitivity to GEN, FOS and TMP. MEC^R isolates primarily display low-level CS to most antimicrobials tested. TMP^R and NIT^R groups cluster together with relatively few collateral effects. The analysis of the subset (**B**) shows patterns consistent with the full model, but with less clustering by AMR group. However, in the redundancy analysis on a subset of the IC_{90} fold changes (**B**) there is far less clustering of the AMR mutants by AMR group (color).

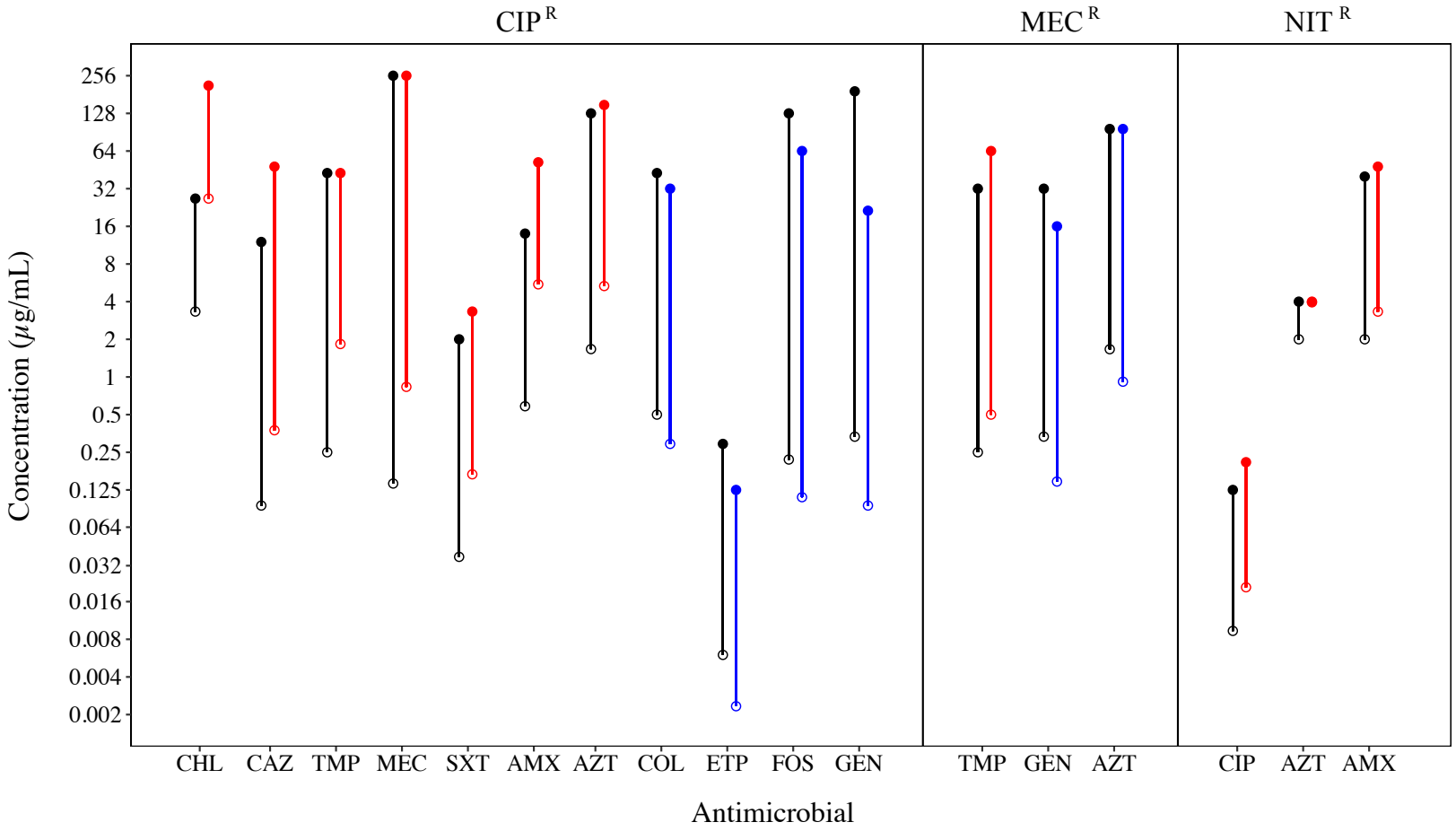

Figure 3. Summary of changes in IC_{90} and MPC results for representative strain:drug combinations. The average IC_{90} (open circles) and average mutation prevention concentration (MPC; filled circles) were determined and compared between AMR mutants (colored) with collateral responses, either CS (blue) or CR (red), and their respective wild-type strain (WT; black) in strain:drug

combinations representing conserved collateral responses. The range between the IC_{90} and MPC was considered the mutation selection window (MSW; lines). K56-16 NIT^R had equivalent IC_{90} and MPC values for AZT, thus no MSW was reported. Generally, changes in MPC values reflected observed IC_{90} changes, shifting the MSW upwards or downwards accordingly. In 8/10 tested combinations an increase in IC_{90} value (CR) from WT to AMR mutant correlated with at least a small increased MPC, with the remaining combinations showing no change in MPC value between the WT and AMR mutant. Similarly, decreased IC_{90} values (CS) correlated with decreased MPCs (5/7).

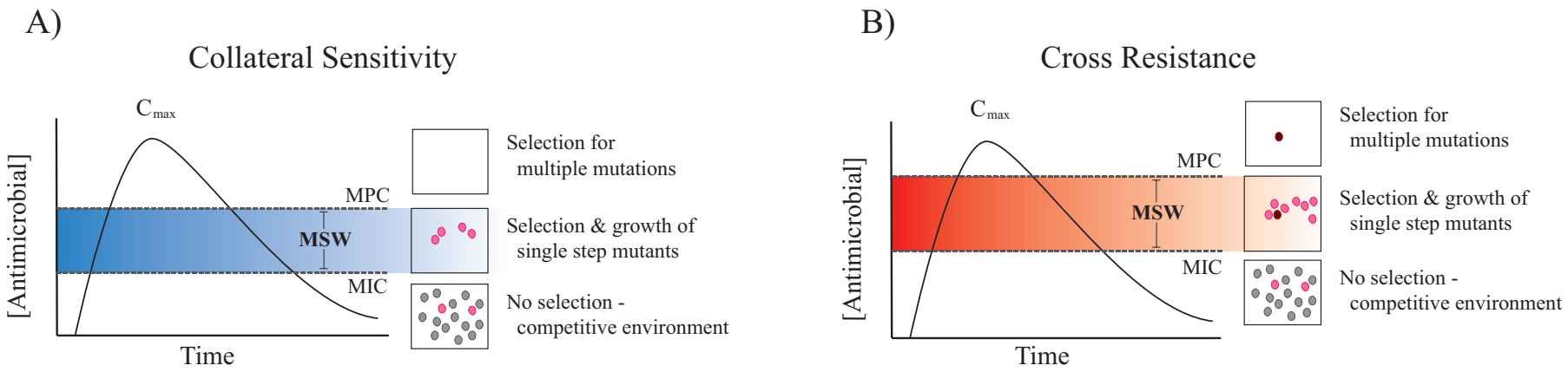

Figure 4: Graphical presentation of the potential effects of CS and CR on the MSW. Sequential drug administration informed by CS could potentially narrow or shift the MSW downwards in concentration space (left panel) whereas CR results in a widened or shifted upwards MSW (right panel). This would affect the probability of acquiring second step mutations leading to high-level resistance. Consequently, CS informed secondary therapies could reduce selection and thus propagation of first step mutants resulting in a reduced window of opportunity for second step mutations to occur. Dots represent bacteria resistant to a primary antibiotic (grey), spontaneous mutants with reduced susceptibility to a secondary drug (pink), or those with high-level resistance to the secondary drug (dark red).