Factors affecting the sign, magnitude, and generality of collateral susceptibility networks in clinical *Escherichia coli* strains.

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INTRODUCTION

Antimicrobial Resistance (AMR)

From an evolutionary perspective, the emergence of AMR is an inevitable outcome due to our use and high consumption of antimicrobials. Development of new antimicrobials, improved antimicrobial stewardship, and novel approaches to reduce AMR emergence and spread are essential to maintain effective treatments.

Cross-Resistance (CR) and Collateral Sensitivity (CS)

AMR to one drug can cause CR to others, often of the same class and with similar drug target(s), or to drugs of diverse classes due to general AMR mechanisms such as efflux. However, AMR can result in CS, where an AMR strain is more susceptible to other antimicrobials [1]. Previous studies suggest that treatment strategies based on CS profiles, specifically using mutually-exclusive AMR traits [1] or drug cycling regimens [1,2], could be beneficial.

Knowledge Gaps

While CS is described in some clinical isolates [2], it is unclear how robust CS/CR networks are across large collections of clinical isolates and isolates with different resistance determinants. Additionally, the contribution of AMR mechanism to CS networks is not well-described.

Approaches

We generated AMR to common antimicrobials for treatment of urinary tract infections in pan-susceptible clinical *Escherichia coli* isolates. The resulting collateral networks were compared to assess the generality within AMR groups. Whole genome sequencing was used to identify the AMR mutations and to investigate correlations between CS and resistance mechanisms.

MATERIALS AND METHODS

Selection for mutations conferring clinical resistance

10 pan-susceptible *E. coli* isolates from urinary tract infections, representing different MLST sequence types (ECOSENS collection [3]) were selected on MH II or LB agar with mecillinam (MEC), ciprofloxacin (CIP), nitrofurantoin (NIT), or trimethoprim (TMP) to achieve AMR above clinical breakpoints (www.eucast.org).

Determination of CS/CR networks

The concentration inhibiting ≥ 90% of growth (IC90 [2]) was determined for 16 antimicrobials in MH II broth using a 2-fold dilution series with half steps. Fold changes in IC90 for all 10 AMR mutants to their respective WT were calculated. A redundancy analysis of the data was performed to the CS/CR network. This analysis was done in R [5] using the Vegan work package [6].

Assessing changes in the Mutation Prevention Concentration (MPC)

MPCs were determined for drugs displaying CS_{50} or CR_{50}, with the exception of temocillin. One mutant-WT pair was tested per drug, and for each concentration tested, $\geq 10^{10}$ CFUs were spread onto four large MHII agar plates. Drug concentrations increased in a 2-fold dilution series, pin-pointing the lowest drug concentration that inhibits growth of single step mutants in a large population after 48 hours.

Identification of AMR mechanism(s) conferring CS changes

DNA was isolated using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich). Paired-end libraries were prepared using the TruSeq DNA Sample preparation kit (Illumina, USA). Libraries were sequenced to a read length of 300 bp on a Hi-Seq. The DNASTAR SeqMan NGen software (Madison, WI) was used to analyze Illumina data, using standard settings, to each isolate's WT parental strain.

Figure 1. Pervasive collateral susceptibility changes in resistant mutants. The frequency of CS (*blue bars*) and CR (*red bars*) changes in IC90 are summarized for each set of 10 AMR mutants. General trends were identified using CR_{50} and CS_{50} cutoffs, where 50% or more of an AMR group (5 of 10 mutants) displayed CR or CS, respectively, to a given drug. Ciprofloxacin resistant mutants displayed the majority of conserved collateral susceptibility changes.

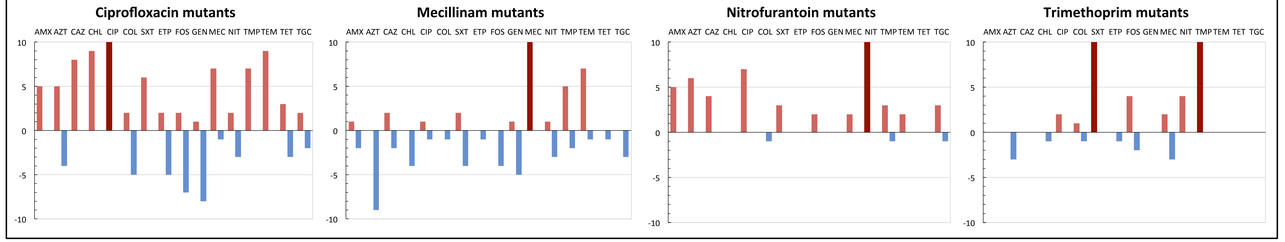


Figure 2. Collateral susceptibility changes correlate with changes in the MPC. Below is a comparison of the average IC90 (*lower-limit*) and MPC (*upper limit*) values for representative strain-drug combinations where conserved CR_{50} (*red bars*) and CS_{50} (*blue bars*) trends were found. These results are compared to the WT (*black bars*). In the majority of cases collateral changes in the MPC and IC90 correlated well, with few exceptions. These findings suggest that small changes in antimicrobial susceptibility can affect the mutation selection window and thus the evolutionary trajectory of single AMR mutants toward multidrug resistance.

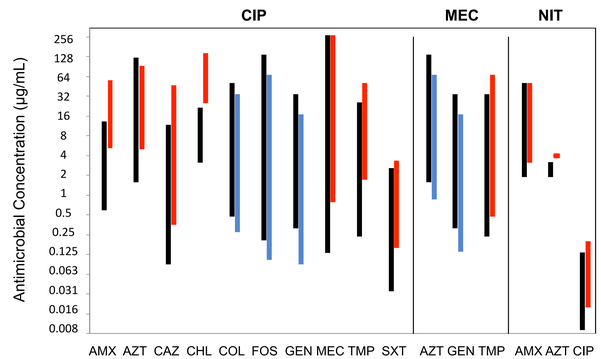


Figure 3. Variation in relative susceptibility changes may be explained by the selected AMR rather than genetic variation between mutants. A tri-plot of a redundancy analysis representing 66.2% of the variation of IC90 changes shows clustering of the AMR mutants (*open circles*), near the population average (*large filled circle*), based on their selected AMR (*colors*). Antimicrobial abbreviations (*black text*) are placed at the tip of vectors passing through the origin, that point in the direction of increasing MIC change relative to the WT. Ciprofloxacin resistant mutants (*pink circles*) cluster at higher values of several antimicrobial vectors, including TMC and CHL, indicating CR to those drugs. Mecillinam mutants (*green circles*) have distinct clustering towards low values of some antimicrobial vectors, including SXT and AZM, showing CS to those.

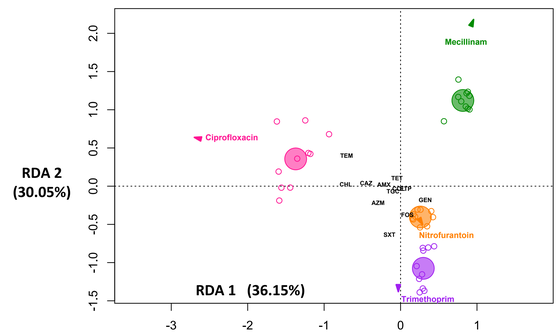
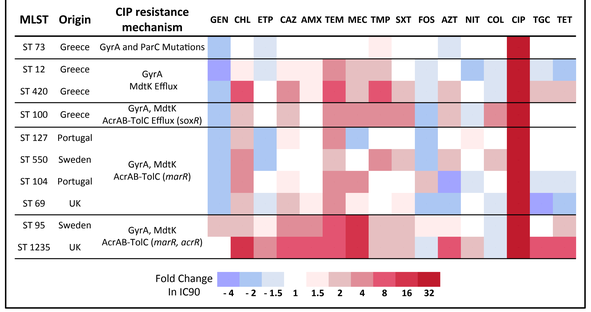


Table 2. Identification of putative AMR mechanisms. Whole genome sequencing identified putative resistance mechanisms in all 40 AMR mutants. All CIP mutants had mutation(s) in *gyrA* but only 1 strain also had a mutation in the second drug target, *parC*. The remaining 9 CIP mutants had one or more mutations to genes known to effect expression of either the AcrAB-TolC or Mdtk-mediated efflux pumps. MEC mutants had mutations in a number of genes, some previously unreported, that likely affect the activation of the stringent response. All NIT mutants had inactivation of at least one of two oxygen-insensitive nitroreductases, and the majority had mutations to regulation of the EmrAB-TolC efflux pump. Finally TMP resistant isolates all had either modification of FolA or mutations in the promoter regions of FolA likely leading to overexpression, one isolate had genomic amplification of a large region including the *folA* gene.

AMR Mechanism		CIP	MEC	NIT	TMP
Drug Target	Modification	10 (1)	–	–	6
	Overproduction	–	–	–	6
Reduced Drug Activation	Nitroreductase disruption	–	–	10	–
Reduced Drug Uptake	Porin mutation	2	–	–	–
Regulation of Efflux	AcrAB-TolC	7	–	1	–
	MdtK	9	–	1	–
	mdfA	–	–	1	–
	EmrAB-TolC	–	–	7	–
	ABC transport	–	1	–	–
ppGpp synthesis (activation of stringent response)	Strigent response	–	4	–	–
	tRNA synthesis	–	4	–	–
	tRNA processing	–	1	–	–
	Cellular metabolism	–	3	–	–

Table 2. AMR mechanism likely contributes to variation in CS/CR patterns. Ciprofloxacin resistant mutants show the greatest variety of AMR mechanisms and initial comparisons suggest that AMR mechanism plays a critical role in CS/CR patterns. The IC90 fold changes of the CIP resistant mutants compared to their cognate WT strains are shown in the heat map below, where CS (*blue boxes*) and CR (*red boxes*) changes appear to vary by AMR mechanism. Isolates are ordered by AMR mutation, from drug target mutations alone to combinations of efflux pumps and multiple efflux pump regulator mutations. CR was frequent in isolates with efflux-related mutations and was greatest in those with mutations to multiple regulators of the AcrAB-TolC efflux pump.



CONCLUSIONS

Collateral changes in susceptibility were found in 47% of cases

- Ciprofloxacin resistance greatly alters the susceptibility to many diverse antimicrobials, and CR is more frequent than CS.
- Ertapenem, fosfomycin and gentamicin susceptibilities were largely maintained or increased across all AMR groups.
- Collateral changes in antimicrobial susceptibly affect the span of the mutation selection window.

Collateral changes in susceptibility are dependent on the genetic mechanism of resistance

- Collateral effects appear independent of the genetic background but instead are likely related the AMR mechanism.
- CR is frequent in isolates with increased efflux expression.

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