Systematic investigation of resistance evolution to common antibiotics reveals conserved collateral responses across common human pathogens.

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Abstract

As drug resistance continues to grow, treatment strategies that turn resistance into a disadvantage for the organism will increasingly be relied upon to treat infections and to slow the rate of multidrug resistance. The majority of work in this area has investigated how resistance evolution toward a single antibiotic effects a specific organism’s collateral response to a wide variety of antibiotics. Results of these studies have been used to identify networks of drugs which can be used to drive resistance in a particular direction. However, little is known about the extent of evolutionary conservation of these responses across species. We sought to address this knowledge gap by performing a systematic resistance evolution study of the ESKAPE pathogens (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter cloacae) under uniform growth conditions using five clinically relevant antibiotics with diverse modes of action. Evolved lineages were analyzed for collateral effects and the molecular mechanisms behind the observed phenotypes. Fourteen universal cross-resistance and two global collateral sensitivity relationships were found among the lineages. Genomic analyses revealed drug dependent divergent and conserved evolutionary trajectories amongst the pathogens. Our findings suggest that collateral responses may be preserved across species. These findings may help extend the contribution of previous collateral network studies in the development of treatment strategies to address the problem of antibiotic resistance.
Introduction

The discovery and introduction of antibiotics has been called the greatest health care advancement in history (1). In addition to reducing morbidity and mortality from bacterial infections, antibiotics have made modern medical procedures such as surgery, organ transplants and chemotherapy possible (2). Unfortunately the widespread use of these drugs has resulted in extensive resistance which threatens to reverse many of the medical advancements made in the last century and return us to a pre-antibiotic era (3). A slow-down in the discovery and development of new antibiotics, due to scientific difficulties and financial and regulatory hurdles (4, 5) means that society cannot rely upon new antimicrobials becoming available. Given this critical junction, there is an urgent need to consider treatment strategies that combat resistance and prolong the use of our current antibiotic arsenal.

Treatment strategies that turn resistance into a disadvantage for the organism are the most desirable and are referred to as collateral sensitivity informed antimicrobial strategies (6). Collateral sensitivity (CS) is when resistance to one antibiotic simultaneously results in susceptibility to another (7). Over the last decade, several studies have considered how resistance evolution toward a single antibiotic effects CS and its converse, cross resistance (CR), for a wide variety of antibiotics and bacterial species (8–20). Results of these works have been used to identify networks of drugs which can be used to drive resistance in a particular direction to take advantage of collateral sensitivity.

To date most of this work has been performed using specific laboratory-adapted strains (8–12, 15, 17, 18, 20) or a limited number of clinical isolates (6, 13, 14, 16). While these studies have been vital to our understanding of CS in a wide variety of species, little remains knowns regarding the extent of evolutionary conservation or universality of CS and CR across species (21). Such answers are important for determining the predictability of collateral responses. Recent work performed using several clinically resistant urinary tract *Escherichia coli* isolates found that collateral responses may be predictable across a species (6).
To investigate the extent of evolutionary conservation, we sought to identify commonalities in collateral networks of the ESKAPE pathogens - *E. faecium*, *S. aureus*, *K. pneumonia*, *A. baumannii*, *P. aeruginosa*, and *E. cloacae* (1). Members of the ESKAPE group are capable of causing a wide variety of illnesses, including bacteremia, skin, soft tissue, respiratory and urinary tract infections (22–27). Our networks were derived from populations systematically evolved to five antimicrobials with diverse modes of action under uniform growth conditions. Whole genome sequencing was used to characterize the molecular mechanisms involved in resistance and collateral responses. Our results suggest that collateral responses may be consistent across the species for select drugs.

Materials and Methods

**Bacteria and reagents.**

Drug sensitive *E. faecium* (DSM 2146), *S. aureus* strain Newman, *K. pneumonia* (DSM 30104), *A. baumannii* (ATCC 17978), *P. aeruginosa* (PAO1), *E. cloacae* (ATCC 13047) were evolved to five antibiotics: cefepime, ciprofloxacin, gentamicin, meropenem, and tetracycline. Drug stock solutions were prepared every 10 days and stored at -20°C until needed. Working stock solutions were prepared from frozen stocks every four days and stored at 4°C. All experiments were performed in cation adjusted Mueller Hinton broth (Sigma) supplemented with 0.5% (w/v) glucose.

**Antimicrobial susceptibility testing**

Wild type (WT) inhibitory concentrations 90% (IC90) and minimum inhibitory concentrations (MIC) were established for every organism to each antibiotic. Each WT organism was plated on nonselective media and allowed to grow overnight. Four individual colonies from each species were then randomly selected and grown in nonselective media for 4-6 hours. After the allotted growth period the optical density (OD) of each preculture was measured and a cell concentration was determined. Dilutions of approximately $10^3$ cell/µl were made for all pre-cultures.

All susceptibility tests were performed in 96-well microtiter plates containing 150µl of media. A 10-dilution step 2-fold drug gradient was used on every microtiter plate. Each plate also included positive (non-antibiotic containing media plus bacterial
inoculum) and negative (non-antibiotic containing media only) controls. All
inoculated wells received 10µl of diluted preculture. Following inoculation, the plates
were incubated at 37°C without shaking for 18 hours. After the allotted growth
period, the OD600 of each plate was measured using an Epoch Absorbance Reader
(Biotek). The average OD600 values of the negative controls were subtracted from
all remaining OD600 values. Percent inhibition was calculated according to equation
1:

\[
\text{Percent inhibition} = 1 - \frac{\text{OD600}_{\text{growth}} - \text{OD600}_{\text{negative control}}}{\text{OD600}_{\text{positive control}} - \text{OD600}_{\text{negative control}}}
\]

The drug concentration causing 90% growth inhibition was determined using a
python script.

Evolution of antibiotic resistant isolates.
All evolution experiments began at concentrations eight times lower than the
corresponding WT MIC and increased in two-fold steps. All evolution experiments
were performed in quadruplicate in two 24-well microtiter plates. Each well
contained 2mL of media. Positive (non-antibiotic containing media plus bacterial
inoculum) and negative (non-antibiotic containing media only) controls were included
in all evolution experiments. The positive control isolates are referred to as media
adapted (MA) lineages.

A single WT colony of each species was grown overnight in liquid media. This
overnight culture was used as inoculum to begin the evolution experiments. All
inoculated wells received 50µl of overnight culture. Following inoculation, the
microtiter plates were incubated at 37°C without shaking for 18 hours. After the
allotted growth period, the OD600 of each plate was measured using an Epoch
Absorbance Plate Reader (Biotek) at a wavelength of 600nm. The OD600 values of
the negative control was subtracted from all remaining OD600 values. Percent
inhibition was calculated according to equation 1. A 60% inhibition cut-off was used
to determine the starting concentration of the next experiment. This concentration
was referred to as the experimental MIC. The 60% inhibition value was selected
based on previous work (28). The well with the best growth closest to the
experimental MIC concentration was used as the inoculum for the next evolution
experiment. The evolution process was repeated for 12 (A. baumannii, P. aeruginosa, and E. cloacae) or 18 days (E. faecium, S. aureus, and K. pneumoniae). Aliquots of the selected wells were frozen in 96 well plates every three days. Media only evolved populations experienced no inhibition and were passaged daily. Aliquots of the media evolved populations were also frozen every three days. On the final day of evolution, aliquots from the most resistant wells that met the 60% inhibition cutoff were plated and grown overnight. A single colony from each agar plate was randomly picked and grown overnight in liquid media. Aliquots of these overnight cultures were saved for future analysis. A total of 144 isolates were saved.

Antimicrobial susceptibility testing post evolution.

Following resistance evolution, isolates of the drug evolved and MA populations were profiled for their individual resistance to each antibiotic. Final exposure populations were plated on nonselective media and allowed to grow overnight. Four colonies from each population were randomly selected and grown in nonselective media for 4-6 hours. These isolates were then tested against all five drugs used in the resistance evolution experiments. Susceptibility testing and data analysis were performed according to the method described in Antimicrobial susceptibility testing section. Each WT species also had its antimicrobial susceptibility reestablished. MA and WT isolates were tested on the same day.

Whole-genome sequencing.

A single isolated colony from each of the 144 evolved lineages and the six WT species was grown overnight in nonselective media. Genomic DNA was isolated from the overnight cultures using an UltraClean Microbial DNA Isolate Kit (MoBio Laboratories, Inc.). The DNA was sent to Macrogen for library construction using the TruSeq Nano (550bp) DNA Library Prep kit (Illumina) and sequencing by Illumina MiSeq 300bp paired end. Additional sequencing was performed by the Sequencing Core Facility at the Novo Nordisk Foundation Center for Biosustainability.

Analysis of genome sequences.

The following chromosomes were used to align their corresponding sequence data: A. baumannii (Accession NZ-CP018664; Version NZ-CP018664.1), P. aeruginosa
A minimum coverage of 30-fold was obtained for each isolate. All sequences were trimmed to remove low-quality reads and reads less than 75 bp. All sequences were aligned to their corresponding reference genome. Point mutations and short InDels were identified using the Quality-based variant detection option in Genomic Workbench 9.0.1. Only loci with a phred score of at least 30 and occurring with a frequency of at least 80% were used for analysis. Single nucleotide polymorphisms (SNPs) present in the WT the media evolved lineages, or in at least two mutants resistant to different antibiotics from the same strain background were excluded from further analysis as they were considered to be present in the starting culture prior to the start of the experiments.

Detection of large deletions.

Large deletions were identified in Genomic Workbench 9.0.1 using a previously described workflow (29). Briefly, all genomes were analyzed for large deletions (>300nt) using Genomic Workbench. Each genome was assembled de novo and this assembly was then used as a reference on which to map the WT genome reads. Any WT reads that could not be mapped to the de novo reference were collected and de novo assembled into contigs. The newly assembled unmapped WT reads were considered to contain deleted regions. Only regions with more than 300 nt and greater than 30X coverage were used for further analysis.

Results

Evolution leads to high resistance levels in both populations and isolates

Wild type (WT) strains of E. faecium, S. aureus strain Newman, K. pneumonia, A. baumannii, P. aeruginosa, and E. cloacae were evolved in four replicate lineages,
designated as A, B, C, and D, to increasing concentrations of five broad spectrum antibiotics (Table 1). To account for possible resistance phenotypes due to media evolution, parallel media only evolution experiments were also performed using the WT for each species. These replicate lineages were designated as MA, MB, MC, MD. Evolution of each lineage was performed by growing a single freshly restreaked colony of each WT organism overnight in liquid media (Fig. 1). An aliquot of each overnight culture was inoculated into five different antibiotic conditions with increasing antibiotic concentrations and grown for 18 hours. All evolution experiments began at antibiotic concentrations eight times less than the corresponding WT MIC values (Table 2). At the end of each growth period, optical density was measured and the most resistant culture from each replicate with an inhibition less than 60% (Materials and Methods) was used as the next inoculum. E. faecium, S. aureus, and K. pneumonia were evolved for 18 days while for A. baumannii, P. aeruginosa, and E. cloacae were evolved for 12 days. A total of 144 lineages (120 drug evolved and 24 media only) were evolved.

Overall, resistance increased steadily within each resistance group (Fig 2). A resistance group refers to all species evolved to the same antibiotic (6) – e.g. ESKAPE evolved to FEP are the FEP resistance group. Resistance improvement values less than one were occasionally observed in the early days of the evolution period and indicated that some populations were less resistant than initially determined. The first day’s resistance improvement of PAO to FEP was markedly higher than any other species and drug combination. This large gain in improvement was likely the result of inoculum effect, which is frequently observed in in vitro studies using cephalosporins (30). Plateaus in the evolution process were observed in every resistance group. Nearly all populations (94%) completed the evolution period with at least a 10-fold increase in resistance compared to their corresponding WT. E. cloacae populations evolved to ciprofloxacin and ceftazidime were able to grow in concentrations 1000X greater than their WT (Fig 2). In contrast, S. aureus populations evolved to meropenem had the smallest increase, 5-fold or less, in resistance relative to their WT.

Following resistance evolution, four isolates from each of the most evolved populations were tested for their individual resistance levels. The isolate results
were then compared with the final resistance evolution concentrations of their corresponding populations. To determine if the selected media influenced the resistance evolution of each species, isolates from all media evolved lineages were also tested for their individual resistance levels.

In general, the resistance levels of the isolates mirrored those of their respective populations (Fig 3). However, there were seven instances where the difference in the population and the isolates resistance levels were statistically significant. Approximately half these cases involved lineages evolved to the beta-lactams and in particular those evolved to cefepime (Fig 3). These isolates were markedly less resistant than their corresponding populations. We speculate that this difference may be due to storage of our populations at -80 degrees C prior to performing subsequent phenotypic testing. Loss of resistance following cryo-storage has previously been observed in *H. pylori* resistant to amoxycillin (31) and *S. aureus* resistant to methicillin (32, 33) and vancomycin (34). Alternatively, the difference may be due to inoculum effect, which is known to occur with beta-lactams, in particular cephalosporins (30, 35). In the case of PAO, inoculum effect is most likely the cause of the difference observed between the populations and their corresponding isolates.

With the exception of the meropenem resistance group, the media used in the evolution experiments does not appear to have promoted or hindered evolution (Fig 3). *E. faecium*, *S. aureus*, and *A. baumannii* MA lineages all experienced notable changes in their susceptibility to meropenem. Interestingly, the media used appears to have had a slightly negative impact on the resistance evolution of *A. baumannii*.

*Universal cross-resistance relationships emerge across the species*

We investigated the collateral responses of laboratory strains of *E. faecium*, *S. aureus*, *K. pneumonia*, *A. baumannii*, *P. aeruginosa*, and *E. cloacae* post resistance evolution. Following the evolution period, four isolates from each of the most evolved populations were tested against all five drugs – cefepime, ciprofloxacin, gentamycin, meropenem, and tetracycline – used in this study. A total of 480 isolates were inoculated into the different antibiotic conditions and allowed to grow
for 18 hours. The IC90 values of the resistant isolates were compared with those of their corresponding WT for the different antibiotics.

Overall, collateral responses were widespread and observed in 55% (254/464) of possible instances. Collateral responses were proportionately distributed across all drugs and species (Fig 4a). Cross resistance accounted for 80% (202/254) of all the collateral responses observed. Occurrences of collateral sensitivity were less frequent and comprised 20% (52/254) of the observed collateral responses. Cross-resistance or collateral sensitivity was defined as an 2-fold increase or decrease in the MIC of the antibiotic evolved strain relative to its WT (9).

A collateral response was considered universal when all species of a resistance group shared the same response toward a particular drug. A collateral response was considered broad when five out of six species of a resistance group shared the same response toward the same drug. Finally, a collateral response was considered extended when four out of six species of a resistance group shared the same collateral response toward the same drug. A collateral response was decided for each species of a resistance group when ≥50% of the lineages exhibited resistance or sensitivity.

A review of the cross-resistance instances revealed a high degree of agreement among the different species with three universal, six broad, and five extended cross resistance relationships being found (Fig 4b). The universal relationships observed were cefepime evolution resulting in tetracycline cross-resistance, ciprofloxacin evolution conferring cross-resistance to tetracycline, and meropenem evolution resulting in cefepime cross-resistance. The broad relationships found were cefepime cross-resistance in lineages evolved to ciprofloxacin or gentamycin, ciprofloxacin cross-resistance in lineages evolved to gentamycin and meropenem, and finally bidirectional cross-resistance between tetracycline and meropenem. The extended relationships observed were cefepime resistance resulting in cross-resistance to ciprofloxacin and meropenem, tetracycline resistance conferring cross-resistance to ciprofloxacin and cefepime, and cross-resistance to tetracycline resulting from gentamycin resistance. Conspicuously absent is cross-resistance to gentamycin.
Surprisingly, resistance evolution of *E. faecium* toward any single drug invariably resulted in cross resistance toward meropenem (Fig 4a).

Global collateral sensitivity responses were fewer and less robust (Fig 4b). Gentamycin collateral sensitivity was observed in half the resistance groups evolved to cefepime or ciprofloxacin. Interestingly, resistance evolution of *S. aureus* toward any single drug consistently resulted in collateral sensitivity toward ciprofloxacin (Fig 4a).

Review of the collateral response data based on the drug evolved to revealed an evenness in the number of cross-resistance and collateral sensitivity interactions observed across the drugs used in the evolution process (Fig 4c). Evaluating this same data on a per drug tested to basis revealed an inequality in the distribution of cross-resistance across the tested drugs (Fig 4d). All resistance groups tested against tetracycline or cefepime were frequently observed to be cross-resistant to these two drugs (20 and 21 instances, respectively). Indicating that these two drugs would likely be a poor second drug choice in a drug cycling plan. In contrast, resistance groups tested against gentamycin consistently displayed either a neutral response (11 instances) or a collaterally sensitive response (eight instances) to this drug. Indicating that gentamycin could be good second drug choice in a multistep treatment plan. Collateral sensitivity to gentamycin following resistance evolution has previously been observed in several single species adaptive evolution studies (6, 16, 36). Analysis of the response data on a per species basis uncovered an average of 13±4 (SD) cross-resistance, 5±2 neutral, and 2±2 collateral sensitivity responses per species. Deviating from these values considerably were the *S. aureus* populations which had only six cross-resistance, but nine neutral and five collateral sensitivity interactions (Fig 4e).

*Evolutionary responses are divergent or conserved in a drug dependent manner*

To explore the molecular basis of the drug resistances observed in our experiments we sequenced a single isolate from each of the most evolved populations (120 drug evolved isolates and 24 media only evolved) and a WT ancestral isolate for each species. The sequenced isolates were then analyzed according to their resistance
group. Mutations arising from evolution to the media were identified and removed from further analysis in the drug evolved isolates.

A total of 874 mutations was identified in the resistant isolates and of these 69% (605/874) were nonsynonymous (NS) mutations. The average number of NS mutations per drug was less than 20 with the exception of gentamycin, where a single hypermutating *E. faecium* isolate had 187 NS mutations (Fig 5a). Approximately 26% (155/605) of NS mutations were located in genes previously associated with resistance.

Analysis of the mutations revealed two categories of genomic response – a diverse response group and a conserved one (Sup File A1-5). The diverse response group was comprised of the beta-lactam and tetracycline resistance groups and was characterized by changes in a variety of pathways (Fig 5b-d, Sup File A1-3). For example, resistance groups evolved to meropenem had mutations in genes related to target modification, efflux pumps, beta-lactamases, and reduced permeability (Fig 5c, Sup File A2). *E. faecium, S. aureus,* and *P. aeruginosa* lineages all had target modification mutations, while in *K. pneumoniae, A. baumannii,* and *E. cloacae* lineages mutations were found in efflux pumps. *P. aeruginosa* lineages also had mutations in efflux pumps and a gene related to reduced permeability. The genetic response to the beta-lactams appears to have been be preserved within certain species. For example, *E. cloacae* lineages evolved to both cefepime and meropenem had mutations in *ampD* and *cpxA* (Sup File A1 and A2). Similar findings were observed in *A. baumannii* and *K. pneumoniae* lineages. Interestingly, *E. faecium* lineages evolved to FEP and MEM possessed few mutations, which could be associated with conferring beta lactam resistance. A closer examination of the *E. faecium* WT species revealed genes related to cell envelop stress response and to altering cell wall charge, but none associated with beta-lactam resistance.

In contrast, the conserved response group was composed of resistance groups evolved to drugs (ciprofloxacin and gentamycin) that elicited a limited number of changes in select pathways (Fig 6a and 6b, Sup File A4-5). Indeed, all species evolved to gentamycin had mutations in the *fusA* gene. Analysis of the *fusA* gene from all our evolved isolates revealed that the observed mutations were primarily
confined to Domain IV. This domain is situated at the A site of the 30S subunit and interacts with mRNA, tRNA and the decoding center on h44 (37). Mutations in this domain have previously been identified as responsible for kanamycin resistance (38). Alignment of the fusA gene from all six species revealed 80% of the Domain IV mutations were contained between amino acids (AAs) 510 and 610 (based on PAO1 numbering) (Fig 6c). Amino acids (AA) 516, AA595, and AA608 were mutated in more than one species. AA608, in particular, was mutated twice in K. pneumoniae, once each in A. baumannii and E. cloacae. Previous work conducted using PAO1 hypothesized that fusA mutations lead to structural changes that altered the binding of aminoglycosides to h44 thus lowering their affinity for the ribosome (39). fusA mutations in have previously been shown to confer resistance to aminoglycosides in S. aureus (28, 40), P. aeruginosa (41, 42), and E. coli (11, 43).

In general, mutations in the fusA gene correlated with a 100-fold increase in resistance to gentamycin relative to the WT strains (Fig 6d). The exceptions to this observation were the A. baumannii lineages, which had 1000-fold increase in resistance, and E. faecium lineages, which had a 100-fold increase in resistance regardless of mutations in the fusA gene. The A. baumannii lineages had additional mutations in the two-component system adeRS. This system regulates the resistance-nodulation-cell division (RND) pump AdeABC, which is known to confer high level resistance to aminoglycosides, beta-lactams, fluoroquinolones, tetracyclines, and several other antimicrobials (44). The WT E. faecium strain used possessed a wide array of antibiotic efflux mechanisms. It is likely that the gentamycin resistance observed in those lineages without fusA mutations is due to these mechanisms.

In addition to fusA mutations, the Gram-negative species had additional mutations in efflux pump regulators, parRS (P. aeruginosa) and the two-component envelope stress response system, cpx (K. pneumoniae and E. cloacae). Mutations in cpxA are known to confer aminoglycoside resistance in E. coli (11, 45, 46). The presence of cpx mutations in E. cloacae and K. pneumoniae lineages may explain the observed cross-resistance to cefepime and meropenem (K. pneumoniae only).
Another example of conserved mutations was observed in lineages evolved to ciprofloxacin. With the exception of *E. faecium*, all species had mutations in gyrA/B (DNA gyrase) and parC/E (topoisomerase IV), the primary targets of quinolone drugs. The majority of mutations observed in our study were located in the quinolone-resistance-determining region (47, 48) at two specific amino acid site (AA90 or AA94 numbered according to *S. aureus*). The genome of the *E. faecium* WT was found to contain the *pmrA* gene, which is known to confer low level ciprofloxacin resistance (49–51). It is possible that the *pmrA* gene is responsible for the observed resistance and may explain the absence of drug target gene mutations in these lineages.

Previous work has shown that large deletions are commonly observed in laboratory evolution studies (29). Large deletions were found in every species and drug condition investigated. While the majority of deleted genes were classified as hypothetical proteins with unknown functions, there were a few instances where the gene deletions were either extensive or included known resistance functions. For example, the tetracycline evolved *P. aeruginosa* isolates all had several large (>10) gene cluster losses. In one isolate in particular these deletions included the *mexR*, *mexA*, *mex B* and *oprM* genes, which are known to be involved with drug resistance (52) (Sup File A3). As neither the mutations nor the gene deletions were reintroduced into their corresponding WT organisms it is not possible to assess their contributions to the observed phenotypic response.

*Genetic basis for observed collateral sensitivity is obscure*

We sought to provide a genomic explanation for the observed collateral sensitivity in resistance groups evolved to FEP and CIP. Using Gene Ontology (GO) as a means of categorizing the observed mutations, we examined the difference between lineages with and without GEN collateral sensitivity within the same resistance group and then across resistance groups. GO provides information about the molecular function, cellular component, and biological process each gene product may carry out (53, 54). The GO characterization of each mutated gene was found in the UniProt Knowledgebase (UniProtKB) (55). Information pertaining to molecular function (MF) was the most consistently available, thus MF terms were used to
categorize the mutation data. MF terms describe the molecular activities of individual gene products. Genes where a product has not been annotated are described as having unknown activities.

In FEP evolved lineages with GEN collateral sensitivity, genes with unknown activities comprised the largest portion of mutations (31%) followed by those associated with ATP binding (17%) and DNA binding (12%). FEP evolved lineages without collateral sensitivity had a similar distribution of mutations. Genes with unknown activities accounted for one third of the mutations followed by those associated with ATP binding (18%) and DNA binding (8%). Approximately 33% of all the mutations observed in the non-sensitive lineages were also found to be present in those with sensitivity.

Analysis for CIP evolved lineages with and without GEN collateral sensitivity yielded similar results. In the collaterally sensitive lineages, genes with unknown activities once again comprised the largest group of mutations (41%) followed by those associated with ATP binding (32%) and DNA binding (14%). In the non-sensitive lineages, genes with unknown activities represented the largest category of mutations (38%) followed by those associated with ATP binding (31%). Genes with products related to DNA binding accounted for only 5% of the observed mutations. Analogous to the FEP evolved lineages, 37% of mutations found in the non-sensitive lineages were also found in the sensitive lineages.

The large fraction of genes with unknown activities found in all lineages in both resistance groups obscure the genetic mechanisms responsible for the observed collateral sensitivity. Additionally, the mutation overlap observed between lineages with and without collateral sensitivity within each resistance group makes it unclear as to why some lineages became sensitive and others did not. These similarities prevent conclusions being drawn across resistance groups.

Discussion

To date, the bulk of collateral network studies have only considered the effects of resistance evolution of a single laboratory species. Questions remain about the universality of these findings (21). Recent studies have attempted to extend these
findings by comparing the results of laboratory strains to clinical isolates (16), by considering the conservation of collateral findings across different clinical isolates of the same species (6), and by investigating the role that genetic background plays in collateral interactions (21). What has not yet been explored is the extent of conservation of collateral networks are across different species. The work presented here sought to address this question.

To this end, we performed a systematic resistance evolution study of the ESKAPE pathogens under uniform growth conditions using five clinically relevant antibiotics with diverse modes of action. Our evolved lineages were analyzed for collateral effects and the molecular mechanisms behind the observed phenotypes.

A total of fourteen universal CR and two global CS relationships were observed. Similar to previous studies (6, 8–10), the magnitude of collateral interactions observed was generally low (<10X) (Fig 4A). In contrast to an earlier study that considered several clinical isolates of the same species (6), the collateral responses observed in our work did not vary statistically across the response groups (Fig 4C). Instead, collateral responses varied by the drug tested against and species (Fig 4D and 3E). Regardless of the antimicrobial a lineage had been evolved to, if the next drug exposure was to gentamycin, there was a greater likelihood for a CS interaction to take place than a CR interaction. Conversely, if the next drug exposure was to tetracycline or cefepime, the likelihood of a CR interaction was high. Our evolved S. aureus lineages were recalcitrant to collateral effects, whereas PAO1 became multidrug resistant following resistance evolution toward one antimicrobial. This difference is likely due to PAO1 having substantially more inherent resistance mechanisms than the S. aureus strain used.

Drug dependent divergent and conserved evolutionary trajectories were revealed in genomic analyses of the most evolved lineages. Cefepime, meropenem and tetracycline elicited a diverse genomic response that involved mutations in a wide range of genes across the different species, whereas for ciprofloxacin and gentamycin the genomic response appeared to be limited to few genes across the different species. In the case of gentamycin, mutations in the fusA gene could be correlated with an increase of 100X resistance improvement in most species. The
observed diverse and converse genomic response could not be correlated with occurrence of CR or CS (Fig 4C).

To date there are several resistance evolution studies of individual members of the ESKAPE group (9, 13, 16, 56–61); however, no study has examined the commonalities that arise from resistance evolution of these species under uniform conditions toward more than one class of antibiotic. A study by Michiels et al considered the persistence of the ESKAPE pathogens toward aminoglycosides, but was performed under inconsistent environmental conditions (62). The work presented in this study is the first to consider the simultaneous resistance evolution of all ESKAPE species toward a broad suite of antibiotics under uniform conditions and to identify commonalities in their collateral and genomic responses.

The findings presented here, while addressing an existing shortcoming, do have limitations. First, our results stem from a laboratory evolution environment in which conditions, such as the absence of competition for nutrients, environmental stresses, etc., are unlike those encountered in a clinical setting. Nevertheless, some of the mutations observed in our work have also been found in clinical isolates. In particular, gyrase mutations contributing to ciprofloxacin resistance have been previously observed in clinical isolates of E. coli (63), S. epidermidis (64), S. aureus (65), A. baumannii (66), and P. aeruginosa (67). Additionally, the work presented here does not consider the role horizontal gene transfer (HGT) in multidrug resistance. HGT is an important driver of antibiotic resistance for several of the ESKAPE pathogens, in particular those from the Enterobacteriaceae and the Enterococcaceae families (68). It is important to note that the choice of adaptive evolution methodology plays a role in the evolutionary trajectory taken. In particular, the role that biofilms (61, 69) and population size play (70) in the outcomes of resistance evolution. Moreover, the mutations observed in this work were not reintroduced back into the species where they were found in therefore it cannot conclusively be said that they confer the resistance attributed to them. Further, the number of drugs used in our study was small compared with other laboratory resistance evolution studies (6, 8–11, 17, 28, 29). This was due to difficulty in finding antimicrobials to which all our species were sensitive. It is likely that we could have included more antimicrobials following the resistance evolution period. Finally, our
work only considers a single laboratory representative of each ESKAPE member. The results of this work would be strengthened by a larger study that included a larger number and wider variety strains from each ESKAPE member. Despite the aforementioned limitations, the results of our work serve as a first attempt in addressing the question of universality of collateral interactions across a wide range of species.

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**FIGURE LEGENDS**

**Figure 1. Resistance evolution of ESKAPE pathogens to individual drugs.** An illustration of the initial evolution process and an example of the decision-making process used to determine which well to passage from. (A) An overnight culture of wild type species X was used to inoculate microtiter plates containing different drugs with increasing concentrations or media only (MA). Each plate also included negative controls (NC). Four replicate populations were created for each condition. The highest concentration where growth was present was used to inoculate the next concentration challenge. *E. faecium*, *S. aureus*, and *K. pneumonia* were evolved for 18 days while *A. baumannii*, *P. aeruginosa*, and *E. cloacae* were evolved for 12 days. (B) The top table lists Day 1 drug concentrations. OD values of the evolved lineages minus the negative control OD values are shown in the second table. OD values of the evolved mutants normalized by the OD value of the media only evolved lineage are show in the third table. The fourth table shows the calculated percent inhibition for each well. Wells with the best growth and less than 60% inhibition were
selected as inoculum for the next resistance evolution challenge. These wells are highlighted in green. The bottom plate lists concentrations used in the next challenge.

Figure 2. Change in drug tolerance over the course of the resistance evolution period. Each graph shows the resistance improvement, the concentration tolerated by the isolate relative to the corresponding WT IC90 value, over the course of the resistance evolution period. All normalized values were log transformed. Drugs used in the experiments are color coded and run down while species investigated run across the figure. Each graph is comprised of three to four replicates. Each point reflects the mean resistance improvement of the isolates on a specific day. Error bars represent a single standard deviation. *E. faecium, S. aureus, and K. pneumoniae* were evolved for 18 days while *A. baumannii, P. aeruginosa, and E. cloacae* were evolved for 12 days.

Figure 3. Resistance improvement of drug evolved isolates relative to their corresponding populations. Four biological isolates from each of the evolved populations were evaluated to determine the extent of their individual resistances. These values were converted to resistance improvement by normalizing to their corresponding WT IC90 value and then compared to resistance improvement of the population. The final resistance evolution concentration of the corresponding population was used to generate a resistance improvement value. Isolates from all media evolved lineages had their IC90 values tested to determine how the media chosen may have affected resistance evolution of each species. All normalized values were log transformed and error bars represent a single standard deviation. The bars at the top of each graph represent the resistance improvement of the isolates. The middle bars show the improvement of the evolved populations. The bars at the bottom of each graph show the resistance improvement of the media evolved lineages. If no media bar is visible it is because the IC90 value is the same as the WT. The resistance improvement of the isolates closely mirrored that of their corresponding populations. The * denotes a statistically significant difference between the resistance improvement of the population and isolates.
Figure 4. Collateral interactions resulting from evolution to a single antibiotic.

Isolates from the most evolved lineages were tested against the other antibiotics used in this study. (a) Heatmap results are grouped according to resistance group. Rows identify the species tested while the challenge drug are listed by column. Each individual square represents the results of a single lineage. A collateral response was decided for each species of a resistance group when \( \geq 50\% \) the species' lineages exhibited resistance or sensitivity. Blue indicates CS while red represents CR. (b) A high degree of cross resistance agreement was observed among the different species with a total of 14 relationships being found. The robustness of the relationship is conveyed by the line style. Solid red lines reflect all species of a resistance group sharing the same response. Short red dashed lines represent responses where five out of six species shared the same response. Dotted red lines show responses where four of the six species shared the same response. Two global CS relationships were found and are shown as blue lines. CS relationships were less robust with only half the species of the resistance group exhibiting the response. (c,d) The number of collateral interactions as a function of drug evolved to (c) and drug tested against (d). (e) The average number of collateral interactions per species.

Figure 5. Beta-lactams and tetracycline provoke divergent genomic responses.

Single isolates from each of the most evolved populations were sequenced and compared to the ancestral WT and media evolved lineages to identify mutations that arose from resistance evolution. (a) The average number of non-silent (NS) mutations per drug. (b-d) NS mutations observed in lineages evolved to (b) cefepime, (c) meropenem, and (d) tetracycline. The mutations shown were found in genes associated with resistance determinants as defined by The Comprehensive Antibiotic Resistance Database (CARD)(71). Columns detail the species while rows list genes where mutations were observed. Genes are grouped by resistance mechanism. Color intensity reflects the number of the lineages carrying a mutation in the listed gene per species. A black background indicates an absence of gene mutation. *E. faecium* lineages evolved to cefepime did not include mutations in genes associated with resistance determinants defined by CARD therefore this species is not included in Figure 5b.
Figure 6. Resistance evolution to gentamycin is characterized by localized mutations in fusA gene.

Single isolates from each of the most evolved populations were sequenced and compared to the ancestral WT and media evolved lineages to identify mutations that arose from resistance evolution. NS mutations observed in lineages evolved to (a) ciprofloxacin and (b) gentamycin. The mutations shown were found in genes associated with resistance determinants as defined by The Comprehensive Antibiotic Resistance Database (CARD)(71). Columns detail the species while rows list genes where mutations were observed. Genes are grouped by resistance mechanism. Color intensity reflects the number of the lineages carrying a mutation in the listed gene per species. A black background indicates an absence of gene mutation. (c) Schematic of the fusA gene highlighting the mutations that arose during resistance evolution. The table below the gene schematic lists the species in which the mutations are observed, provide a color code for each species, supply the number of amino acids (AAs) present in the gene, identify the AAs changed, and supply information about the gene region in which the AA change is located. The majority of mutations in fusA were found between amino acid (AA) 516 and 608 (numbered according to PAO1) in Domain IV of the gene. Domain IV is home to two highly conserved loops where aminoglycosides are known to bind. (d) A mutation in the fusA gene was associated with at least 100-fold improvement in IC90 relative to the WT for all species expect E. faecium. A. baumannii isolates with fusA mutations had 1000-fold improvement.

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### Species X Day 1 concentrations

| Replicate | FEP  | CIP  | GEN  | MEM  | TET  | Media |
|-----------|------|------|------|------|------|-------|
| A         | 16   | 32   | 16   | 8    | 4    | 2     |
|           | 1    | 0.5  | 0.50 | 0.25 | 0.13 | 0.50  |
|           | 0.5  | 0.25 | 0.13 | 0.0625 |      |       |

### Day 1, OD minus negative control

| Antibiotic | FEP  | CIP  | GEN  | MEM  | TET  | Media |
|------------|------|------|------|------|------|-------|
|            | 0.03 | 0.02 | 0.12 | 0.01 | 0.01 | 0.00  |
|            | 0.13 | 0.14 | 0.25 | 0.01 | 0.02 | 0.01  |
|            | 0.20 | 0.78 | 0.33 | 0.40 | 1.36 | 0.41  |

### Day 1, OD mutant/avg OD positive control

| Antibiotic | FEP  | CIP  | GEN  | MEM  | TET  | Media |
|------------|------|------|------|------|------|-------|
|            | 0.09 | 0.07 | 0.44 | 0.01 | 0.03 | 0.28  |
|            | 0.46 | 0.46 | 0.68 | 1.36 | 1.43 | 0.30  |
|            | 2.67 | 0.87 | 0.77 | 1.43 | 1.38 | 0.30  |

### Day 1, inhibition

| Antibiotic | FEP  | CIP  | GEN  | MEM  | TET  | Media |
|------------|------|------|------|------|------|-------|
|            | 91.4%| 92.8%| 55.8%| 54.1%| 32.0%| -167% |
|            | 92.1%| 66.0%| 52.4%| 49.0%| 29.2%| -11.6%|
|            | 89.0%| 59.2%| 12.5%| 11.1%| 13.9%| -12.3%|

### Media well selected as innoculum in next challenge

### Day 2 concentrations

| Replicate | FEP  | CIP  | GEN  | MEM  | TET  | Media |
|-----------|------|------|------|------|------|-------|
| A         | 32   | 64   | 64   | 4    | 2    | 1     |
|           | 16   | 32   | 32   | 1    | 0.5  | 0.5   |
|           | 8    | 16   | 16   | 0.5  | 0.25 | 0.25  |
|           | 4    | 8    | 8    | 0.25 | 0.125| 0.125 |
|           | 2    | 4    | 4    | 0.125| 0.0625| 0.0625|
|           | 0.5  | 1    | 1    | 0.0625| 0.0313| 0.0313|
|           | 0.25 | 0.5  | 0.5  | 0.0313| 0.016| 0.016 |

| Antibiotic | FEP  | CIP  | GEN  | MEM  | TET  | Media |
|------------|------|------|------|------|------|-------|
|            | 0.28 | 0.30 | 0.30 | 0.30 | -42.9%| -42.9%|
|            | 0.30 | 0.30 | 0.30 | 0.30 | -36.1%| -36.1%|
|            | 0.30 | 0.30 | 0.30 | 0.30 | -37.8%| -37.8%|

### Antibiotic concentration

| Antibiotic | Media  |
|------------|--------|
|            |        |
|            |        |
|            |        |
|            |        |
|            |        |
|            |        |
|            |        |

### Overnight Isogenic Wild Type Culture of Species X

| Stock | Volume | Temperature |
|-------|--------|-------------|
| WT    | 50 mL  | 37℃         |
|       |        |             |
|       |        |             |
|       |        |             |
|       |        |             |

### Day 2, OD mutant/avg OD positive control

| Antibiotic | FEP  | CIP  | GEN  | MEM  | TET  | Media |
|------------|------|------|------|------|------|-------|
|            | 0.09 | 0.07 | 0.44 | 0.01 | 0.03 | 0.28  |
|            | 0.46 | 0.46 | 0.68 | 1.36 | 1.43 | 0.30  |
|            | 2.67 | 0.87 | 0.77 | 1.43 | 1.38 | 0.30  |

### Day 2, inhibition

| Antibiotic | FEP  | CIP  | GEN  | MEM  | TET  | Media |
|------------|------|------|------|------|------|-------|
|            | 91.4%| 92.8%| 55.8%| 54.1%| 32.0%| -167% |
|            | 92.1%| 66.0%| 52.4%| 49.0%| 29.2%| -11.6%|
|            | 89.0%| 59.2%| 12.5%| 11.1%| 13.9%| -12.3%|

### Media well selected as innoculum in next challenge

### Day 2, OD mutant/avg OD positive control

| Antibiotic | FEP  | CIP  | GEN  | MEM  | TET  | Media |
|------------|------|------|------|------|------|-------|
|            | 0.09 | 0.07 | 0.44 | 0.01 | 0.03 | 0.28  |
|            | 0.46 | 0.46 | 0.68 | 1.36 | 1.43 | 0.30  |
|            | 2.67 | 0.87 | 0.77 | 1.43 | 1.38 | 0.30  |

### Day 2, inhibition

| Antibiotic | FEP  | CIP  | GEN  | MEM  | TET  | Media |
|------------|------|------|------|------|------|-------|
|            | 91.4%| 92.8%| 55.8%| 54.1%| 32.0%| -167% |
|            | 92.1%| 66.0%| 52.4%| 49.0%| 29.2%| -11.6%|
|            | 89.0%| 59.2%| 12.5%| 11.1%| 13.9%| -12.3%|
Figure 2

Log resistance improvement

E. faecium
S. aureus
K. pneumoniae
A. baumannii
P. aeruginosa
E. cloacae
TET
MEM
GEN
CIP
FEP
| Target alteration | Efflux pumps | Reduced permeability | Drug resistance |
|-------------------|--------------|----------------------|-----------------|
| E. faecium        |              |                      |                 |
| S. aureus         |              |                      |                 |
| K. pneumoniae     |              |                      |                 |
| A. baumannii      |              |                      |                 |
| P. aeruginosa     |              |                      |                 |
| E. cloacae        |              |                      |                 |

Figure 5

A

No. NS mutations

1000

10

1

B

CIP FEP GEN MEM TET

1

10

100

1000

No. NS mutations

1

2

3

4

D

30S ribosomal protein S10

ermB

rpoC

dltA

AcrR family

transcriptional regulator

mexR

mexT

parS

E. faecium
S. aureus
K. pneumoniae
A. baumannii
P. aeruginosa
E. cloacae

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http://aac.asm.org/Downloaded from
**fusA Gene - Elongation factor G (EFG)**

**EF Tu**

**domain 2**

**EF Tu GTP binding domain**

**EFG II**

**EFG IV**

**C terminus**

### Table: Species and AA Changes

| Species          | Color | No. AA | AA Change | Region          |
|------------------|-------|--------|-----------|-----------------|
| E. faecium       | I     | 695    | 596, 164, 595 | IV, TU/GTP, IV |
| S. aureus        | I     | 693    | 598, 460, 578, 415 | IV, II, IV, II |
| K. pneumoniae    | I     | 704    | 638, 461, 606 | IV, II, IV     |
| A. baumannii     | I     | 712    | 620, 600, 606 | IV, IV, IV     |
| P. aeruginosa    | I     | 705    | 603, 603, 516, 679 | IV, IV, C-terminus |
| E. cloacae       | I     | 704    | 652, 506, 516, 577 | C-terminus, IV, IV, IV |

### Figure 6

- **A**: Target alteration
- **B**: Efflux pumps
- **C**: Drug resistance
- **D**: Log Improvement

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**CIP**

**GEN**

**Figure 6**

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Table 1. List of antibiotics used in this study.

| Antibiotic     | Abbreviation | Class (sub-class)             | Target                                      |
|----------------|--------------|-------------------------------|---------------------------------------------|
| Cefepime       | FEP          | B-lactam (cephalosporin)      | cell wall synthesis                         |
| Ciprofloxacin  | CIP          | Fluoroquinolone               | DNA gyrase                                  |
| Gentamicin     | GEN          | Aminoglycoside                | protein synthesis, 30S                      |
| Meropenem      | MEM          | B-lactam (carbapenem)         | cell wall synthesis                         |
| Tetracycline   | TET          | Tetracycline                  | protein synthesis, 30S                      |
Table 2. Starting concentrations for adaptation experiments.

| Antibiotic | E. faecium | S. aureus | K. pneumoniae | A. baumannii | P. aeruginosa | E. cloacae |
|------------|------------|-----------|---------------|--------------|---------------|------------|
| FEP        | 0.25       | 0.125     | 0.002         | 0.125        | 0.125         | 0.0156     |
| CIP        | 0.25       | 0.0039    | 0.001         | 0.0039       | 0.0039        | 0.002      |
| GEN        | 0.0313     | 0.0156    | 0.0039        | 0.0313       | 0.0625        | 0.0625     |
| MEM        | 0.25       | 0.0039    | 0.002         | 0.0039       | 0.0625        | 0.0156     |
| TET        | 0.0039     | 0.0039    | 0.0078        | 0.0156       | 0.5           | 0.125      |