Microbial interactions affect the tempo and mode of antibiotic resistance evolution

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Competing interests

The authors declare no conflict of interest.

Abstract

The global rise of antibiotic resistance impedes the treatment of bacterial infections. To limit the emergence and evolution of antibiotic resistance it is important to understand how bacterial interactions in multispecies communities affect the course of evolution. We investigated how ecological interactions between microbes derived from polymicrobial urinary tract infections affect the tempo and mode of antibiotic resistance evolution. We show that for representative strains of three uropathogens, *Escherichia coli, Klebsiella pneumoniae* and *Enterococcus faecium,* the rate and evolutionary trajectories towards antibiotic resistance depend on interactions with community members that mediate their growth and antibiotic tolerance. Replicate lineages of the same species evolved under similar ecological conditions show parallel evolutionary trajectories. Our findings demonstrate that bacterial interactions determine the evolutionary potential of antibiotic resistance evolution.

Introduction

Bacterial pathogens often live in multispecies communities. In such microbial communities bacteria interact, which affects their growth, survival and coexistence (1–5). Bacterial infections are often treated with antibiotics, whose efficacy may also depend on ecological interactions with community members (6). For instance, co-existing bacterial species in pathogenic consortia can mediate the growth and survival of community members in the presence of antibiotics (7–9) through antibiotic degradation (10,11), change the metabolic landscape affecting pathogen fitness (12–14), lower antimicrobial efficacy or alter the transport of antibiotics (7,15), or change the induction of signal molecules that induce resistance mechanisms (16).

Ecological interactions can therefore affect the evolvability of antibiotic resistance in several ways. Firstly, they may alter the mutation supply, by affecting the population size or mutation rate (17–20). Additionally, ecological interactions may alter the fitness effect of mutations, mediating the selective accessibility of adaptive mutations, as genetic and environmental factors are known to modulate the effect of mutations (21–23) and therefore potentially morph the shape of the adaptive landscape (24,25).

In clinical practice, urinary tract infections (UTIs) are treated with antibiotics. Recurrence of infection after the discontinuation of the antimicrobial therapy in treated patients can be high (up to 50% in the elderly population) (26). This is not surprising, as infections are often polymicrobial and many UTI isolates are resistant to commonly prescribed antibiotics (1,27,28). It is therefore important to understand how antibiotic resistance evolves in a community context. To investigate the role of ecological interactions on the evolution of antibiotic resistance, we evolved three focal species (*Escherichia coli, Klebsiella pneumoniae* and *Enterococcus faecium*) in the conditioned medium of several species (*K. pneumoniae*, *E. coli*, *E. faecium*, *Staphylococcus haemolyticus* and *Pseudomonas aeruginosa*). The bacteria were isolated from elderly patients diagnosed with

polymicrobial urinary tract infections (1). These pathogens are part of the so-called ESKAPEE bacteria, which are known for their ability to evade antibiotics due to their increasing multi-drug resistance (29). We hypothesize that ecological interactions between uropathogens determine the evolutionary potential of antibiotic resistance evolution.

Materials and Methods

Bacterial isolates and growth medium

The five uropathogen isolates used in this study were previously collected anonymously from elderly patients (>70 years old) suffering from urinary tract infection (1). These five isolates (*E. coli*, *K. pneumonia*, *E. faecium*, *S. haemolyticus*¸ and *P. aeruginosa*) were selected based on their consistent planktonic growth in Artificial Urine Medium (AUM) and for their observed interactions in a previous study (2,6). Isolates were cultured in Artificial Urine Medium (AUM) (30).

Preparation of conditioned medium

Spent medium of *E. coli*, *K. pneumonia*, *E. faecium*, *S. haemolyticus*¸ and *P. aeruginosa* isolates was prepared by culturing in AUM, followed by centrifugation and filtering. To prepare conditioned medium, the AUM spent medium fraction was mixed with AUM medium. The resulting conditioned medium contained metabolite concentrations 0.75x AUM if all of a metabolite was consumed during culturing and 1.25x AUM if nothing of a metabolite was consumed.

Serial dilution experimental evolution protocol

K. pneumoniae and *E. faecium* were evolved for 30 bi-daily transfers, *E. coli* was evolved for 35 bi-daily transfers. In 5 ml preheated conditioned medium, in 15 ml screw cap falcon tubes, with the caps slightly opened. Each transfer *K. pneumoniae* and *E. coli* were diluted 500 times (max

~9 generations per bi-daily growth cycle, 2^9=512), and *E. faecium* was diluted 100 times (max ~6/7 generations per bi-daily growth cycle, 2^7=128), due to its smaller population size in the artificial urine medium. The population sizes of *K. pneumoniae* and *E. coli* are ~10^8 CFU/ml, and *E. faecium* ~10^4 in AUM. *K. pneumoniae* and *E. coli* were evolved in the conditioned media of *K. pneumoniae*, *E. coli*, *E. faecium*, *Staphylococcus haemolyticus* and *E. faecium* also additionally evolved in *P. aeruginosa* conditioned medium. Each focal isolate-conditioned medium combination was replicated five times. The Gram-negative isolates *K. pneumoniae* and *E. coli* were evolved in trimethoprim-sulfamethoxazole (ratio 1:5, as in clinical use (31)) and *E. faecium* in vancomycin (32). The level of antibiotics at the start of the experiments was set at a 50% sub-MIC concentration. If the cultures exceeded a predetermined population size threshold, the antibiotic concentration was increased by 20%, otherwise the antibiotic concentration stayed at the same level as the previous transfer.

Growth Measurements

Three clones of three of the five evolved replicate lineages of each focal species, evolved in each conditioned medium, and the wild types of each focal species were tested in the phenotypic growth assays. Due to a -80°C freezer incident, only one lineage of *E. faecium* evolved in *P. aeruginosa* conditioned medium could be revived. Each isolate was incubated in three wells (triplicate cultures) of a preheated 96-well plate (transparent non-treated flat bottom, Brand) containing 200 µL of conditioned medium for 24 h. The plates were inoculated using a 96 solid pin multi-blot replicator tool, transferring ~1 µL of stock from a −80°C glycerol stock. The population size of wild type cultures was obtained using the average OD600 at stationary growth after 24h. Growth rates were obtained by incubation in a BMG Clariostar or Fluorostar microplate reader at 37°C under aerobic conditions and shaken continuously at 500 rpm. The optical density at 600 nm (OD600) was measured every 6–7 min in the microplate reader (three flashes, 10 ms settle time). Before each measurement, the plate was shaken with a double orbital pattern. The growth rate was

defined by the shortest obtained doubling time and quantified by the steepest linear fit of the exponential phase of log(OD600). Population density was inferred from OD600 absorbance measurements as well, taking the average for all the measurements of the stationary phase of the growth curve. Evaporation of media from the plates was not detected. 96-well plates also contained non-inoculated wells as a control for contamination of both the conditioned medium and the inoculation step. Contamination was found to be negligible.

Tolerance assays

Wild type isolates of *K. pneumoniae*, *E. faecium* and *E. coli* were inoculated with 2 ul of overnight cultures in triplicates in conditioned medium and AUM reference medium in a concentration gradient with two fold increases in antibiotic concentration in 96-well plates. Plates were incubated for 24h at 37°C. *K. pneumoniae* and *E. coli* were assayed in the presence of trimethoprim-sulfamethoxazole and *E. faecium* in the presence of vancomycin. The fold change tolerance was calculated by dividing the concentration that limited growth in a particular conditioned medium by the concentration of antibiotic that limited growth in AUM.

MIC assays

Starting Minimal Inhibitory Concentration (MIC) of the wild-types were measured in a serial dilution assay of the desired antibiotics. Bacterial cultures in AUM with a volume of 200 µL were created in a 96-well plate including a two-fold serial dilution of the desired antibiotic (vancomycin for *E. faecium* and a 1:5 mixture of trimethoprim and sulfamethoxazole respectively for *E. coli* and *K. pneumoniae*) ranging from 0.125 to 1 µg/mL for the vancomycin and 0.00625 to 0.2 µg/mL for trimethoprim:sulfamethoxazole in triplicate. Cultures without antibiotics were used as control. The serial dilution assay was grown over 24h at 37°C at 200 rpm. Whether or not bacteria had visibly grown was determined by measuring OD600 and subtracting AUM without bacteria as blank. OD600 values <0.05 were inspected visually to determine whether inhibition was complete or not.

MIC of final transfer mutants were determined on agar using a modified version of the TDtest described previously by Gefen et al. (33).

DNA extraction

Cultures of evolved clones were grown overnight in 5ml LB, cells were pelleted, and DNA was extracted using a Maxwell 16 Instrument in combination with the Maxwell® 16 Tissue LEV Total RNA Purification Kit Custom (XAS 1220) (Promega, Madison, WI, USA) following manufacturer's instructions. The DNA was eluted in 30 µL DNAse free water, and quantified using Nanodrop 2000c Spectrophotometer (Thermo Fisher Scientific, USA) and gel electrophoresis.

Sequencing

Three clones of three of the five evolved replicate lineages of each focal species, evolved in each conditioned medium, were prepared for sequencing. Sequencing was performed at Baseclear BV Leiden. Paired-end sequence reads were generated using the Illumina HiSeq2500 or MiSeq system.

Calculation *H*-index

To quantify how repeatable the evolution of the mutants was, the *H*-index of genome repeatability previously described by Schenk et al. (17) was used. The *H*-index was calculated on the functional (COG) and at the gene level. All mutants were compared pairwise, calculated using (17):

$$H_{A,B} = \sum_{j=1}^{m}\sum_{k=1}^{n}\left(\frac{|A_j \cap B_k|}{|A_j|}\right) / \sum_{j=1}^{m}\sum_{k=1}^{m}\left(\frac{|A_j \cap A_l|}{|A_j|}\right) \qquad (1)$$

Which compares genotype *A* and *B* with mutations *m* and *n* respectively. The *H*-index is calculated for both genotypes separately ($H_{A,B}$ and $H_{B,A}$) and compared using (17):

$$H = \frac{H_{A,B} + H_{B,A}}{2} \qquad (2)$$

The final *H*-index value would fall between 0 (no repeated evolution) and 1 (complete parallelism). When the *H*-index is 0.5, it indicates that the average of the two compared mutants have half of their mutations shared between them.

Statistical analysis

For each genotype, measurements (of phenotypes such as on growth rate, population size, resistance or tolerance levels) were performed in either triplicates or quadruplicates. Throughout all of the analyses, whenever the values between two conditions of mutants were compared, a two-tailed Welch's *t*-test was performed to test the null hypothesis that the two parameters do not have significantly different values (hypothesis of neutrality). We used a Poisson-test to assess whether the number of mutations in the mutators was significantly different in the hypermutators and the non-hypermutators. We used a Pearson correlation coefficient and corresponding *p*-values to determine the level of correlation. The null hypothesis was rejected if the *p*-value obtained from the test was lower than a significant level of 0.05.

Additional information on Materials and Methods can be found in Supplementary text 1.

Results

Conditioned media promote the adaptive radiation of antibiotic resistance evolution

To investigate the effect of ecological interactions on the evolution of antibiotic resistance, we evolved single isolates of focal species *K. pneumoniae*, *E. coli* and *E. faecium* by bi-daily serial transfers in conditioned artificial urine medium (AUM) supplemented with increasing concentrations of antibiotics. Conditioned medium was used as a proxy for microbial interactions, which was prepared by culturing isolates in AUM followed by filtering out bacteria and replenishment of the medium with fresh nutrients (Materials and Methods) (6). When the evolving

isolates had reached a predetermined population size threshold at the moment of transfer, the antibiotic concentration was increased by 20% (Materials and Methods). Each combination of focal isolate and conditioned medium was replicated five times. The two Gram-negative species, *K. pneumoniae* and *E. coli*, were evolved in the presence of trimethoprim-sulfamethoxazole (ratio 1:5, similar to clinical use (31)), which inhibits DNA synthesis (34). *E. faecium* was evolved in the presence of the Gram-positive specific antibiotic vancomycin, which inhibits cell wall synthesis (35); initial antibiotic concentrations were 50% of the minimal inhibitory concentration (MIC) in all cases. *E. coli* was evolved over 70 days (35 transfers) and *K. pneumoniae* and *E. faecium* for 60 days (30 transfers) (Materials and Methods).

We find that the ecological interactions alter the course of antibiotic resistance evolution. Specifically, these interactions affect the rate and final level of antibiotic tolerance increase (Figure 1). Notably, the evolutionary trajectories of the replicates per conditioned medium are highly parallel, and the parallel trajectories in each conditioned medium diverge from other conditioned media. This is especially the case for *K. pneumoniae* and *E. faecium* (Figure 1B,C). The evolutionary trajectories of *E. coli* per conditioned medium environment also display parallelism (Figure 1A), but are more variable compared to *K. pneumoniae* and *E. faecium* (Figure 1B,C).

Generally, we find that the rate and level of antibiotic resistance evolution is slowest in the conditioned medium of the focal isolate (e.g. *K. pneumoniae* in *K. pneumoniae* conditioned medium, and *E. faecium* in *E. faecium* conditioned medium, Figure 1B,C). Moreover, the rate of evolution is often fastest in the conditioned medium of the isolate that is phylogenetically least related, e.g., *E. faecium* evolves fastest in the Gram-negative conditioned media, and the *E. coli* evolves fastest in the Gram-positive conditioned media. This suggests that niche specific effects such as avoidance of competition promote the divergence of antibiotic resistance evolution, as also observed in other studies (36–38).

Response to selection correlates with ecological effects on population size

Bacterial interactions, due to for instance metabolic interactions, can induce niche specific effects that dictate the course of evolution. Such effects can be mediated by the specific chemical composition of the conditioned media affecting the population size and/or growth rate, and therefore the mutation supply rate (20). To investigate whether ecological interactions mediate the evolutionary potential by affecting the mutation supply rate of evolving populations, we correlate the maximum population sizes, as inferred by OD600 measurements, of the ancestors in conditioned medium, with the final antibiotic levels tolerated in the evolution experiments (Figure 2A-C). Indeed, the antibiotic concentration at the final transfer correlates significantly with the population size in conditioned medium for all three focal species. We find a strong positive correlation for *E. coli* and *E. faecium*, and a moderate correlation for *K. pneumoniae* (Pearson correlation coefficient 0.86, 0.93 and 0.45, for *E. coli*, *E. faecium* and *K. pneumoniae* resp., $p < 0.001$ for *E. coli* ($N = 20$) and *E. faecium* ($N = 25$), and $p = 0.017$ for *K. pneumoniae* ($N = 20$)) (Figure 2A-C).

We previously found that growth-mediating effects of bacterial interactions in the absence of antibiotics correlate with antibiotic tolerance levels (6). The fold change in antibiotic tolerance was calculated based on the change in growth inhibiting antibiotic concentration of conditioned media compared to unconditioned medium. Here, we find that the concentration of antibiotics at the last transfer of the experiment correlates positively and significantly with the antibiotic tolerance levels conferred by the conditioned media (Pearson correlation coefficients, 0.50, 0.68 and 0.64 for *E. coli* ($N = 20$), *K. pneumoniae* ($N = 20$), and *E. faecium* ($N = 25$) resp., for all $p < 0.001$, Figure 2D-F). This suggests that ecological interactions may accelerate antibiotic resistance evolution via increased antibiotic tolerance, which enhances the mutation supply by allowing a larger standing population size.

Ecological interactions do not affect the number of genetic changes

To investigate the consequences of ecological interactions on mutational trajectories, we determined the genetic changes in these evolved lineages through Illumina sequencing of three clones per evolved lineage (Materials and Methods). A fundamental determinant of evolutionary trajectories is the population size, as it affects both the supply of mutations and the efficacy of natural selection to select beneficial mutations (17,20,39). Therefore, effects on population size may lead to different mutations being selected (40).

To quantify the impact of natural selection in our populations, we measured the dN/dS ratio (41). Generally, we find dN/dS ratios >> 1, resp. *K. pneumoniae* 7, *E. faecium* 17, and *E. coli* 5, indicating strong positive selection in all populations.

We did not find a strong positive correlation between the number of SNPs in the evolved lineages correlates and population size in conditioned media in the absence of antibiotics. We find a weak positive correlation for *E. coli* and *E. faecium* (Pearson correlation coefficient 0.18, 0.47, $p = 0.17$, 0.20, resp., Figure 3A,C), and a negative correlation for *K. pneumoniae* (Pearson correlation coefficient, -0.70, $p < 0.001$, Figure 3B). SNPs outnumbered other mutations in the evolved isolates (Figure 3D-F). Yet other genetic differences were also observed in the evolved lineages, such as deletions, inversions and copy-number differences. Moreover, in several of these *K. pneumoniae* lineages one of the plasmids was entirely lost. Especially for the second plasmid (Supplementary Figure 1B), we observed that blocks of the plasmid were deleted during evolution. In general, mutational patterns did not seem related to the specific conditioned media.

Additionally, hyper-mutator lineages appeared. These lineages accumulated many more mutations compared to the other evolved lineages of these species: around 60 mutations in comparison to the average of ≈10, for *E. coli* and *K. pneumoniae* (Figure 3D, E, Poisson-test, $p < 0.001$). For this reason, these lineages were removed from the SNP count analysis. The

sequences of the *E. coli* hyper-mutator lineages showed large deletions (>3KB) in the region which includes *mutH*, a gene involved in mismatch repair (42), which points to a role for *mutH* in the generation of these hyper mutator lineages. The *K. pneumoniae* hyper-mutator lineage appeared in *E. faecium* conditioned medium, and was associated with a missense SNP mutation in *mutS*.

We do not find a strong relationship between the total number of genetic changes in evolved lineages, and the population size in conditioned media (Pearson correlation coefficient, 0.023 and 0.03, 0.15, p = 0.68, 0.59, 0.19 for *E. coli, K. pneumoniae* and *E. faecium* lineages respectively, Supplementary Figure 2A-C). This suggests an important role for clonal interference during adaptation, which affects the type and not the number of mutations in populations with different mutation supplies (17).

Ecological interactions do affect the types of genetic changes

To test whether different conditioned media caused different adaptive trajectories in the three focal species, we compared the genetic similarity of isolates evolved in the same with those evolved in different conditioned media. To this aim we calculated the *H*-index (17), i.e. a measure for the fraction of mutated genes that two evolved isolates have in common (Materials and Methods). If we calculate this index at the level of Clusters of Orthologous Groups (COGs) (43), i.e. genes grouped by function, we find more parallelism among replicate populations evolved within the same conditioned media compared to those same lineages in different conditioned media (p = 0.043, 0.043, 0.032 (*Welch's t-test*) for *E. coli*, *K. pneumoniae* and *E. faecium,* respectively, Figure 4). This indicates that the parallel evolution observed (Figure 1), compliments the parallelism observed at the functional level in the same conditioned media.

For *E. coli*, particularly the lineages that evolved in the Gram-positive conditioned media of *S. haemolyticus* and *E. faecium* display a high degree of parallelism at the COG-level (H > 0.6, Figure 4A). This pattern is conserved at the gene-level for lineages evolved in *S. haemolyticus*

conditioned medium (Supplementary Figure 3A). The high genetic parallelism corresponds to a similar phenotypic growth response of *E. coli* replicates to these conditioned media (Figure 2D), and to their similar evolutionary trajectories (Figure 1A). Interestingly, the population size of *E. coli* in the conditioned medium of itself is also similar to the population size in the conditioned medium of *S. haemolyticus* (Figure 2A). Yet, the *H*-index values that correspond to the *E. coli* lineages evolved in these Gram-positive (*S. haemolyticus)* and Gram-negative (*E. coli)* conditioned media are relatively low ($H < 0.6$), indicating lower parallelism within these conditions. This shows that population size alone cannot explain the level of parallelism, which suggests that the particular metabolic constituents of the conditioned medium, also play a role in the genetic divergence of these evolutionary trajectories.

For *E. faecium*, we find a particularly strong parallelism at the COG-level for evolved lineages in *P. aeruginosa* conditioned medium, but less so for other conditioned media (Figure 4C). At the gene-level, this strong parallelism for evolved lineages in *P. aeruginosa* conditioned medium is not present (Supplementary Figure 3C). The high level of parallelism among *E. faecium* lineages evolved in *P. aeruginosa* conditioned medium coincides with their highest rate of evolution (Figure 1C). The level of parallelism of *E. faecium* evolved in the conditioned media of *K. pneumoniae* and *S. haemolyticus* is lower, despite similar resistance trajectories and population sizes (*H*-index means of 0.75 for *P. aeruginosa,* and 0.35 ($p = 0.022$) and 0.48 ($p = 0.013$) for *K. pneumoniae* and *S. haemolyticus* resp., *Welch's t*-tests *H*-index comparison of *E. faecium* in the respective conditioned media) (Figure 1B, 4B). This again suggests that particular metabolic constituents of the conditioned medium play a role in the genetic divergence.

For *K. pneumoniae*, we find consistently high *H*-indices ($H > 0.6$) at the COG-level across conditions, except for the comparison of the isolates evolved in *E. faecium* and *E. coli* conditioned media (Figure 4B). In addition, at the gene-level we also find relatively high parallelism (although slightly lower than at the COG-level), especially for the isolates evolved within *S. haemolyticus*

and *K. pneumoniae* conditioned medium (Supplementary Figure 3B). Interestingly, the generally high parallelism at the COG-level for *K. pneumoniae* is associated with small population size differences due to the exposure to these conditioned media (approximately two-fold). These combined highlight the rather limited effect of conditioned media on the antibiotic resistance evolution of *K. pneumoniae* (Figure 2B).

As observed before (17,44), the *H*-indices at the COG-level show a higher parallelism than at the gene-level (Figure 4, Supplementary Figure 3). This indicates that there is genetic redundancy in the functionalities that are selected for under these particular ecological circumstances. Generally, the higher *H*-index of lineages evolved within the same conditioned medium (along the diagonal axis in Figure 4) shows that the antibiotic resistance evolution in the conditioned media leads to parallel genotypic (Figure 4) and phenotypic (Figure 1) adaptation under the influence of species-specific bacterial metabolites.

Conditioned medium causes specific genetic changes

Next, we investigated whether the species-specific ecological interactions also lead to the selection of different resistance mechanisms. We do find that in particular conditioned media specific mutations occur repeatedly, whereas others do not (Figure 5A-C). We hypothesized that in larger populations, larger effect-size mutations are selected (17). Indeed, if we look at the resistance conferring mutations of *E. faecium*, we find that the lineages with the smallest population size, which are those cultured in its own conditioned medium, repeatedly had substitutions in *ompR*, a two-component response regulator of *ompF* involved in the transport of antibiotics 41 42 (45,46) (Figure 5C). Efflux-enhancing mutations have previously been associated with lower selective pressures (47). *E. faecium* lineages evolved in other conditioned media reached higher levels of resistance and incorporated mutations in well-known vancomycin resistance targets, *VanR, VanS* (45,48,49), or both. *VanS* is a sensor histidine kinase that detects

vancomycin and activates *VanR* (45,48,50). *VanR* is a transcriptional activator, regulating the transcription of the vancomycin-resistance operon together with regulating transcription of the vanHAX operon, resulting in higher tolerance due to changes in peptidoglycan formation (45,49). Indeed, lineages that had mutations in these targets generally had higher resistance levels and larger population sizes (Figure 5C, 2C).

The two Gram-negative lineages (*K. pneumoniae* and *E. coli*) were selected for resistance to trimethoprim-sulfamethoxazole. All sequenced isolates had mutations in *folA*, dihydrofolate reductase (Figure 5A,B), which is involved in the folate pathway that is essential for DNA synthesis and is inhibited by trimethoprim-sulfamethoxazole (51). *FolA* is the direct target of trimethoprim. One lineage of *E. coli* evolved in *E. coli* conditioned medium even had a 20-fold amplification of the copy-number of this gene.

Both *K. pneumoniae* and *E. coli* incorporated *folM* mutations. *FolM* encodes an isozyme of dihydrofolate reductase and is also involved in DNA synthesis. Inhibition of this gene causes the upregulation of *folA,* but usually also requires resistance mutations in *folA* to be effective (52). *E. coli* incorporated *folM* mutations only in lineages that evolved slowly in the *K. pneumoniae* conditioned medium (Figure 5A). In *K. pneumoniae*, *folM* mutations appeared in lineages that were evolved in *K. pneumoniae* medium, but also in the *E. coli* and *S. haemolyticus* conditioned media, which allowed fast resistance evolution.

In *K. pneumoniae* in *E. faecium* conditioned medium, *folX* was targeted instead of *folM* (Figure 5B). *E. coli* lineages evolved in *E. coli* and *S. haemolyticus* conditioned medium incorporated mutations in yet another gene in the folate pathway, *folP*, (53) (Figure 5A). All these mutations in the folate pathway correspond to clinically relevant mutations (Supplementary text 2). The observation that different genes within the folate pathway are targeted in the different conditioned

media suggests that ecological interactions may cause different trajectories of antibiotic resistance evolution.

The observed mutations also confer resistance to antibiotics in the absence of conditioned media (Supplementary text 2, Supplementary figure 5). Specifically, for *E. coli* and *E. faecium*, the antibiotic level tolerated by the populations at the end of the serial transfer experiment correlated positively with the measured MICs, while for *K. pneumoniae* we found this relationship to be inverted. Moreover, the growth rate and population size of evolved lineages is mediated by exposure to specific conditioned media (Supplementary text 3, Supplementary Figure 6-8) and antibiotics interactions (Supplementary text 3, Supplementary Figure 6E). Concluding, specific antibiotic resistance genes were targeted repeatedly within, but not across conditioned media (Figure 5). This shows that the genetic basis of antibiotic resistance evolution is mediated by the genetic background of the focal species, as well as by the ecological interactions.

Discussion and conclusion

We find that the bacterial interactions mediated by conditioned medium altered the rate and mutational trajectories of antibiotic resistance evolution of uropathogenic isolates *E. coli*, *K. pneumoniae* and *E. faecium*, derived from polymicrobial urinary tract infections. The conditioned medium approach has some important caveats. In the conditioned media environment, cultured bacteria were not in physical contact with the bacteria used to create conditioned media. Such contact-mediated interactions may affect eco-evolutionary outcomes, since two populations of bacteria can respond to the presence and the produced metabolites of the other when in the same environment (54,55). Moreover, higher-order interactions between more than two bacterial species could potentially affect growth (56,57). Also, cross-feeding metabolites may mediate ecological interactions by altering gene expression in both interaction partners, in these experiment cross-feeding was only limited to metabolites produced by the donor in conditioned

media. Future studies are needed to investigate such additional factors that can affect the evolution of antibiotic resistance in the context of ecological interactions.

We find a positive relationship between the level of evolved antibiotic resistance and the ecological interactions in the absence of the antibiotics: the larger the population size of the unevolved strains in the conditioned medium and the higher the tolerance conferred by the conditioned medium, the higher the final concentration of antibiotic that can be sustained in the serial dilution experiment. Population size is known to affect the sensitivity to antibiotics known as the inoculum effect, and although this is usually observed for β-lactamases (11), there is evidence that it plays a role for antibiotics like trimethoprim as well, even though these are not degraded enzymatically (58,59). This is also in line with other work that showed that community composition can alter the selection for antibiotic resistance in a community context, for instance through modulating population densities via competitive or cooperative interactions (58,60–65).

Overall, we found parallelism in lineages evolved under the same ecological conditions or in the same conditioned medium (Figure 1), and that resistance mutations and other functional targets selected were dependent on these conditions (Figure 4, 5). Parallelism has been observed to depend on the environmental conditions before, such as resource availability (66), ecological interactions (67) and population size of evolving lineages (17), but the phenotypic parallelism does not always correspond to molecular parallelism (68). This work shows that ecological interactions, which may be caused by avoidance of resource competition, cross-feeding and potentially other antibiotic tolerance effects (6,38,61), can drive both phenotypic and genetic parallel antibiotic resistance evolution.

It is important to recognize that only the final transfer replicates were sequenced, meaning that it is unclear when specific mutations fixed in the population and how those affected the evolutionary trajectories and by extent the adaptive landscape. Following mutational trajectories during the

emergence of antibiotic resistance evolution could lead to more insight in the environmental impact on resistance by identifying genotypes that alter these trajectories (69–73).

For *E. coli* and *E. faecium*, the antibiotic level tolerated by the populations at the end of the serial transfer experiment correlated with their respective MICs, while for *K. pneumoniae* we found this relationship to be inverted. The *K. pneumoniae* lineages with the highest evolved MIC corresponded to those cultured in the lowest concentration of antibiotics. Potentially, larger population sizes corresponded to more 'greedy' adaptation, promoting faster initial adaptation, but lower final adaptation, as measured by evolved MIC levels (Supplementary Figure 4B) (74). Such balance between greedy and slower-and-steady evolution may have led to higher resistance levels for the initially smallest population sizes, such as *K. pneumoniae* in *K. pneumoniae* conditioned medium (Figure 1). Additionally, we showed that evolved resistance levels depend on the environment. Ecological interactions, mediating population sizes and other ecological factors, affect the antibiotic resistance levels as measured in isolation (e.g., in the clinic), which is in accordance with other studies (6,64,75).

Concluding, antimicrobial resistance is turning into a 'silent pandemic' (47), as infections caused by resistant microorganisms fail to respond to conventional treatment, resulting in prolonged illness, greater risk of death and higher healthcare costs (76). We find that ecological interactions between bacteria, by altering population size and conferred antibiotic tolerance, affect the mode, the genetic functions selected, and the tempo, the rate of antibiotic resistance evolution. The identification of the nature of such ecological interactions can aid in forecasting the evolutionary potential of antibiotic resistance evolution. Further knowledge of such predictive parameters can improve decision making, which will be crucial for developing interventions, and for curbing the emergence and further evolution of antibiotic resistance (77).

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Contributions

Conceived and designed the study: LEZ JAGMDV BJZ MGJDV. Performed the experiments: LEZ ADF MGJDV. Analyzed the data: LEZ JVDH MGJDV. Wrote the manuscript: LEZ JVDH ADF JAGMDV MGJDV.

Data availability statement

Bacterial sequence data have been deposited with links to BioProject accession number PRJNA1103400 in the NCBI BioProject database. The other datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Figure legends

Figure 1 | Evolutionary trajectories of uropathogenic bacteria in conditioned media. A, The phenotypic trajectories of *E. coli* evolution in the presence of sulfamethoxazole-trimethoprim, with ratio sulfamethoxazole:trimethoprim 5:1. *E. coli* was evolved in the presence of the conditioned media of *K. pneumoniae, E. coli, E. faecium* and *S. haemolyticus*. The five replicates evolved along similar trajectories, but are more divergent than those of *K. pneumoniae* and *E. faecium*. **B,** The phenotypic trajectories of *K. pneumoniae* evolution in the presence of sulfamethoxazole-trimethoprim. Ratio sulfamethoxazole:trimethoprim is 5:1. *K. pneumoniae* was evolved in the presence of the conditioned media of *K. pneumoniae, E. coli, E. faecium* and *S. haemolyticus*. Five replicates were evolved in each conditioned medium and all replicate trajectories overlapped. **C,** The phenotypic trajectories of *E. faecium* evolution in the presence of vancomycin. *E. faecium* was evolved in the presence of the same conditioned media as *K. pneumoniae* and *E. coli*, and also in the conditioned medium of *P. aeruginosa*. The trajectories of all five replicates per conditioned medium overlapped.

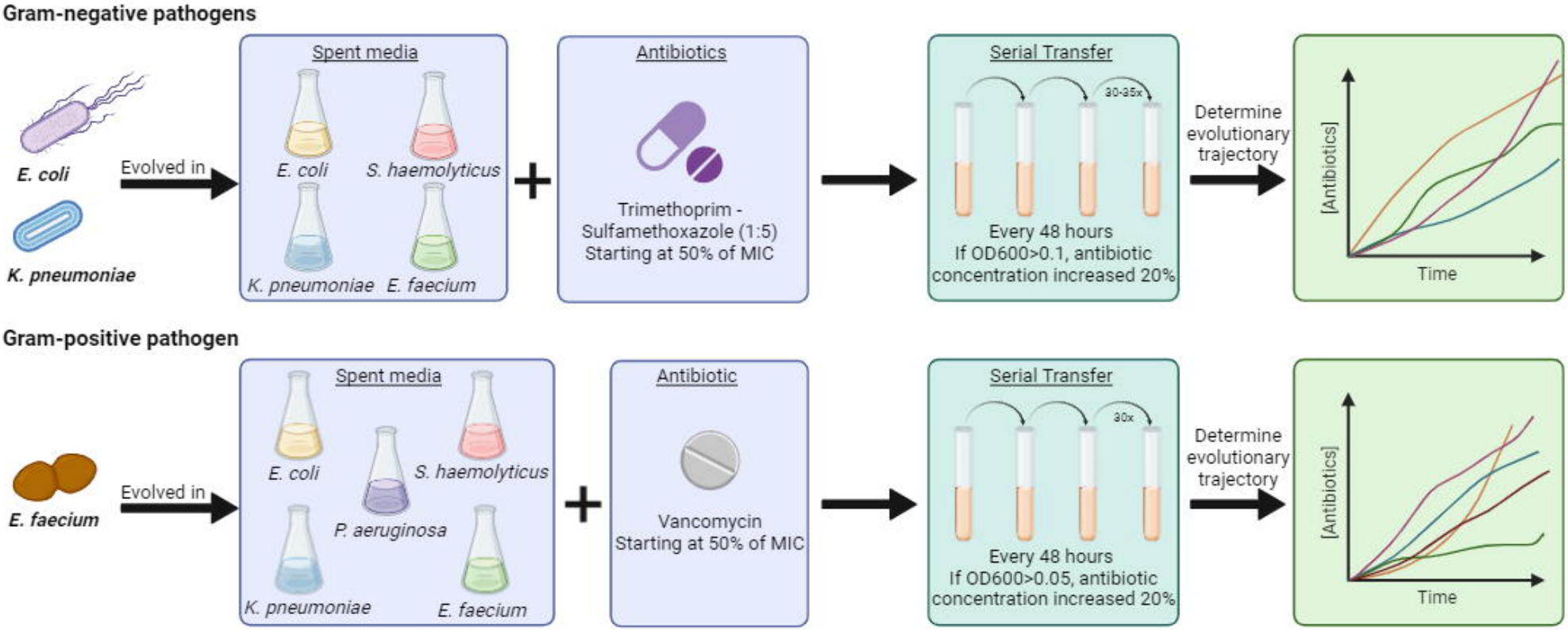
Figure 2 | Correlation of antibiotic concentration at the final transfer with ecological interactions; WT population size in conditioned medium, and the fold change of antibiotic tolerance in conditioned medium. Colors indicate the conditioned medium in which the focal species were cultured, with blue indicating *K. pneumoniae* conditioned medium, red *E. coli* conditioned medium, yellow *E. faecium* conditioned medium and green *S. haemolyticus* conditioned medium. The *E. faecium* mutants were also evolved in *P. aeruginosa* conditioned medium, represented by orange markers. **A**, Trimethoprim concentration (ratio sulfamethoxazole:trimethoprim is 5:1) at final transfer of *E. coli* lineages in the serial propagation experiment correlated with the population size interactions of the wild type in the absence of antibiotics (Pearson correlation coefficient of 0.8556, $N = 20$, $p < 0.001$, means ± s.e.m.). **B**, Trimethoprim concentration (ratio sulfamethoxazole:trimethoprim is 5:1) at final transfer of *K. pneumoniae* lineages in the serial propagation experiment correlated with the population size interactions of the wild type in the absence of antibiotics (Pearson correlation coefficient of 0.4509, $N = 20$, $p = 0.017$, means ± s.e.m.). **C**, Vancomycin concentration at final transfer of *E. faecium* lineages in the serial propagation experiment correlated with the population size of the wild type in the absence of antibiotics (Pearson correlation coefficient of 0.9259, $N = 25$, $p < 0.001$, means ± s.e.m.). **D**, Correlation between antibiotic concentration at final transfer of *E. coli* mutants of in the serial propagation experiment versus the fold change of the antibiotic tolerance of the wild-type *E. coli* in the conditioned media compared to artificial urine medium (Pearson correlation coefficient of 0.4986, $N = 20$, $p < 0.001$). **E**, Correlation between antibiotic concentration at final transfer of *K. pneumoniae* mutants of in the serial propagation experiment versus the fold change of the antibiotic tolerance of the wild-type *K. pneumoniae* in the conditioned media compared to artificial urine medium (Pearson correlation coefficient of 0.6757, $N = 20$, $p < 0.001$). **F**, Correlation

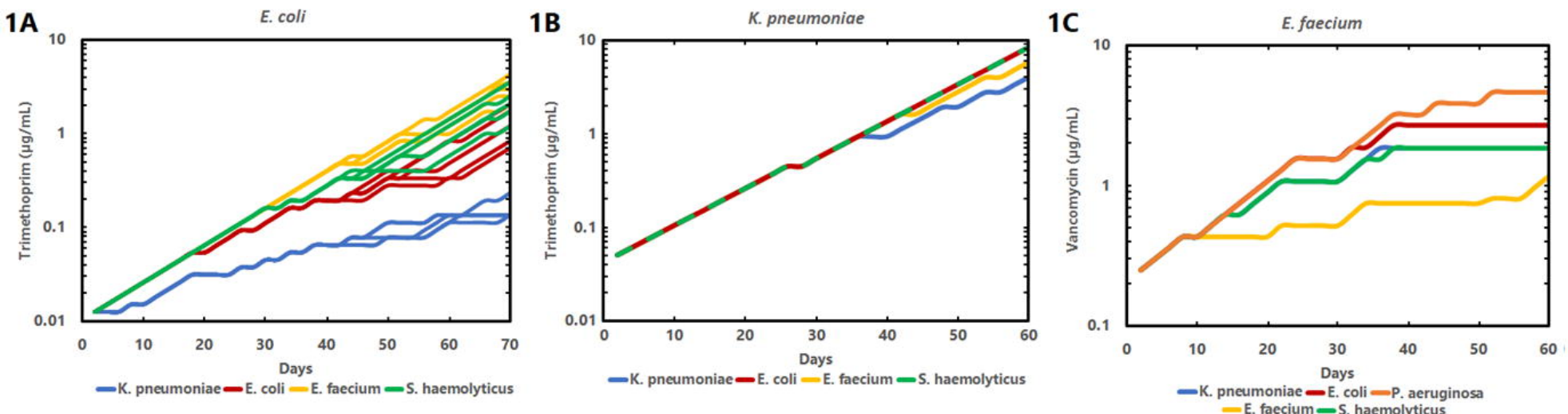
between antibiotic concentration at final transfer of *E. faecium* mutants of in the serial propagation experiment versus the fold change of the antibiotic tolerance of the wild-type *E. faecium* in the conditioned media compared to artificial urine medium (Pearson correlation coefficient of 0.6418, $N = 25$, $p < 0.001$).

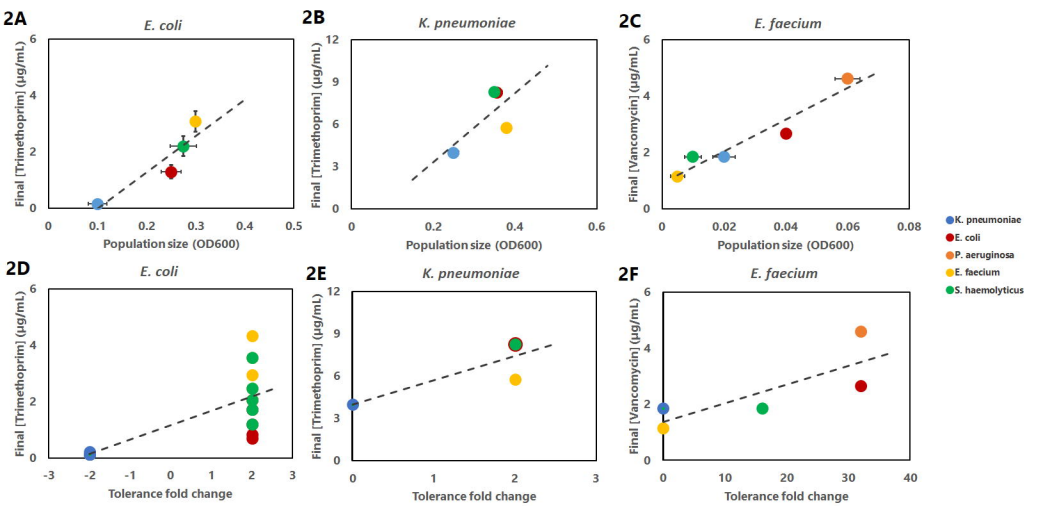
Figure 3 | Mutation supply, number of SNPs and type of mutations in final propagation step mutants. Colors indicate the conditioned medium in which the focal species were cultured, with blue showing mutants evolved in *K. pneumoniae* conditioned medium, red showing mutants evolved in *E. coli* conditioned medium, yellow showing mutants evolved in *E. faecium* conditioned medium, and green showing mutants evolved in *S. haemolyticus* conditioned medium. For *E. faecium* mutants were also evolved in *P. aeruginosa* conditioned medium, which is shown with the orange marker. The error bars show the standard error of the mean for the number of SNPs in the non-mutator trains. **A,** The average number of single nucleotide polymorphisms (SNPs) in the clones of evolved *E. coli* in the conditioned media correlated with the population size of the wild type in those conditioned media (Pearson correlation coefficient = 0.1792, $N = 12$, $p = 0.17$, means ± s.e.m.). **B,** The average number of SNPs in the clones of *K. pneumoniae* in the conditioned media correlated with the population size of the wild type in those conditioned media (Pearson correlation coefficient = 0.7045, $N = 12$, $p < 0.001$, means ± s.e.m.). **C,** The average number of SNPs in the clones of evolved *E. faecium* in the conditioned media correlated with the population size of the wild type in those conditioned media (Pearson correlation coefficient =0.7616, $N = 16$, $p = 0.1989$, means ± s.e.m.). **D,** All mutations in the *E. coli* mutants evolved in the conditioned media shown on the x-axis per replicate lineage. Two mutator strains were identified, shown here by the broken y-axis/bars. **E,** All mutations in the *K. pneumoniae* mutants evolved in the conditioned media shown on the x-axis per replicate lineage. One mutator strain was identified, shown here by the broken y-axis/bars. **F,** All mutations in the *E. faecium* mutants evolved in the conditioned media shown on the x-axis per replicate lineage.

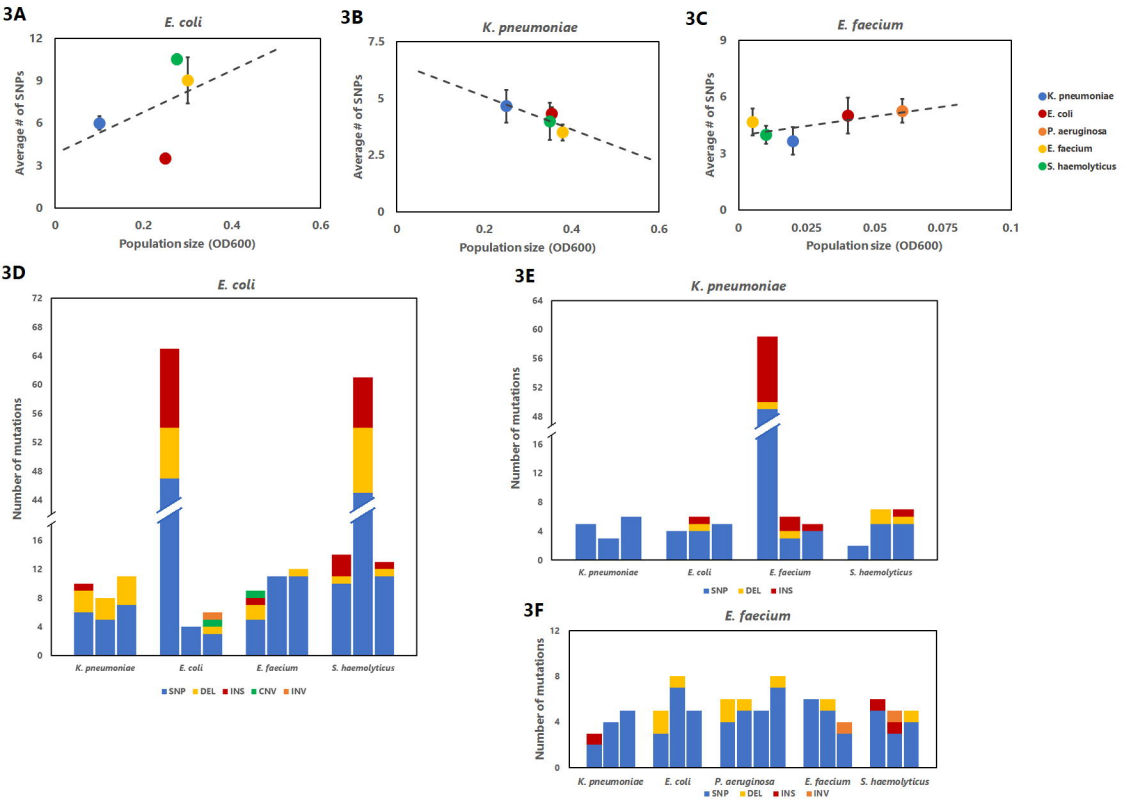
Figure 4 | Clustering and mutation repeatability of the final propagation step mutants clustered on COG-level. Higher values indicate a higher parallelism of the two genotypes compared. Three mutant lineages were sequenced per conditioned medium (four for *E. faecium* mutants evolved in *P. aeruginosa* conditioned medium). Comparisons in the blocks around the diagonal in each figure show the intra-conditioned medium comparisons, while the other blocks show the inter-conditioned media comparisons. **A,** *H*-index of mutation repeatability at COG-level of *E. coli* evolved in the four conditioned media. Mutator lineages are not taken into account (Figure 3D). **B,** *H*-index of mutation repeatability at COG-level of *K. pneumoniae* evolved in the four conditioned media. Mutator lineages are not taken into account (Figure 3F). **C,** *H*-index of mutation repeatability at COG-level of *E. faecium* evolved in the five conditioned media.

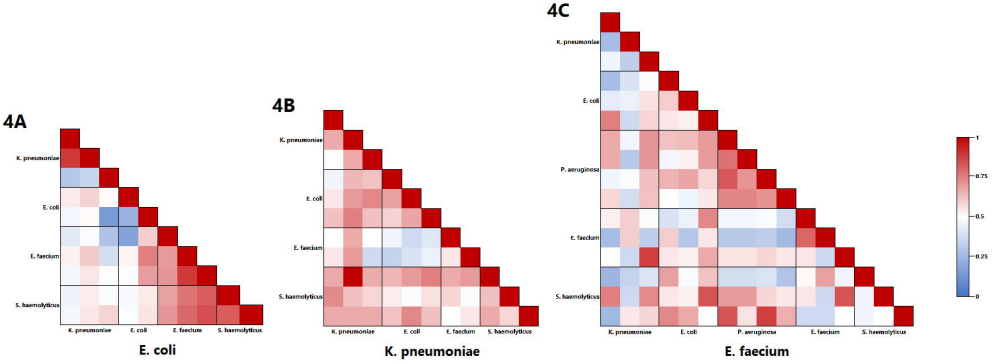
Figure 5 | Evolved genetic targets separated by type of mutation. Genetic targets shown were hit multiple times (at least twice) in either replicate populations or environment, or both. Size of the spheres indicate the number of unique mutations in the specific gene that were observed. Genes are separated into their respective COG functional group as shown on the right side of the figures, with each letter corresponding to a different COG-group. COG-groups: C – Energy production and conversion, E – Amino acid transport and metabolism, F – Nucleotide transport and metabolism, G – Carbohydrate transport and metabolism, H – Coenzyme transport and metabolism, K – Transcription, L – Replication, recombination and repair, M – Cell wall/membrane/envelope biogenesis, O – Posttranslational modification, protein turnover, chaperones, P – Inorganic ion transport and metabolism, Q – Secondary metabolites biosynthesis, transport and catabolism, R – General function prediction only, T Signal transduction mechanisms, and V – Defense mechanisms. **A,** Genetic targets in *E. coli* evolved in four different conditioned media. See Supplementary Table 2 for a list of the genes and proteins. **B,** Genetic targets in *K. pneumoniae* evolved in four different conditioned media. See Supplementary Table 3 for a list of the genes and proteins. **C,** Genetic targets in *E. faecium* evolved in five different conditioned media. See Supplementary Table 4 for a list of the genes and proteins.

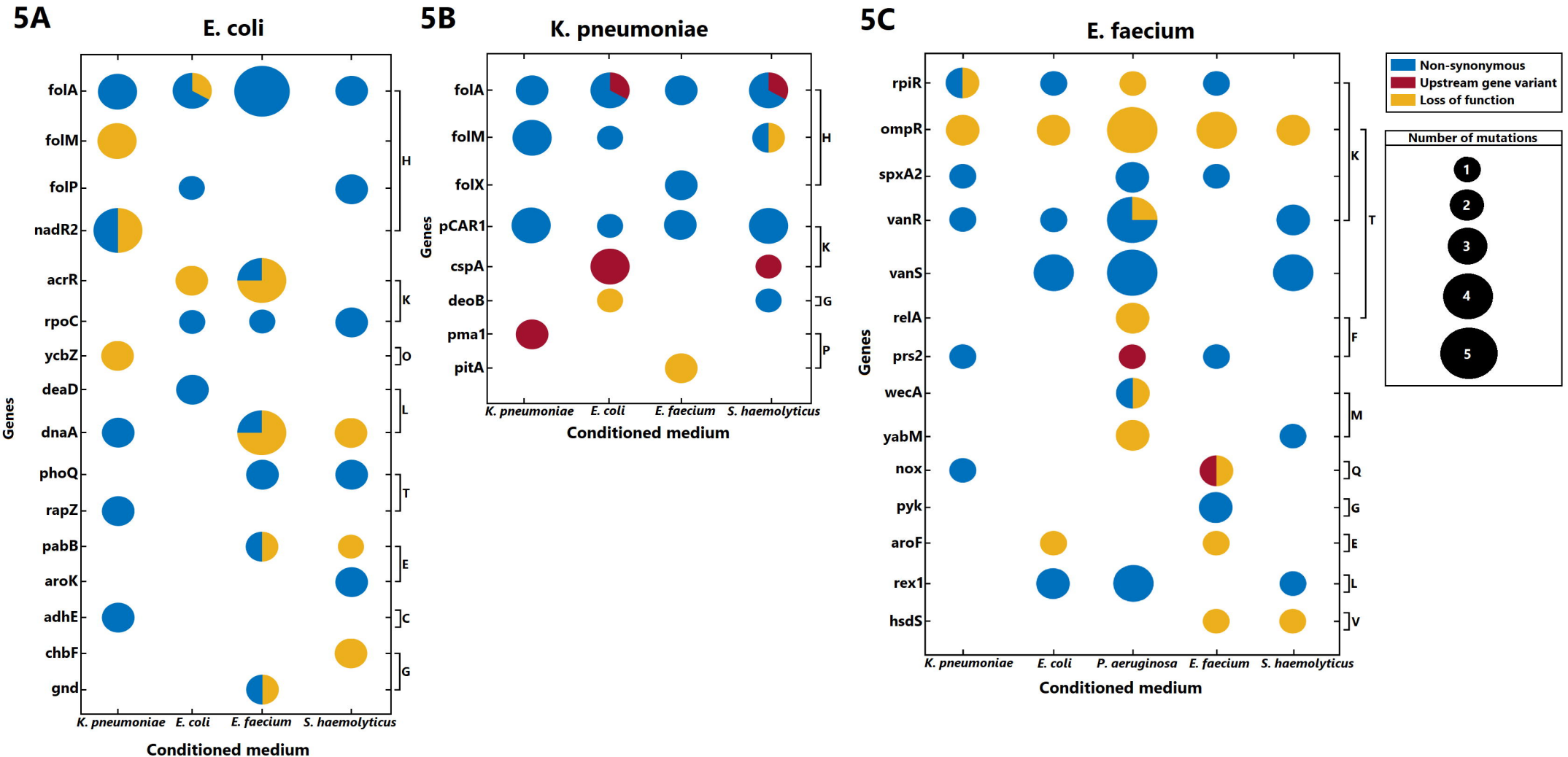

Gram-negative pathogens
E. coli
K. pneumoniae
Evolved in
Spent media
E. coli
S. haemolyticus
K. pneumoniae
E. faecium
Antibiotics
Trimethoprim - Sulfamethoxazole (1:5)
Starting at 50% of MIC
Serial Transfer
30-35x
Every 48 hours
If OD600>0.1, antibiotic concentration increased 20%
Determine evolutionary trajectory
[Antibiotics]
Time
Gram-positive pathogen
E. faecium
Evolved in
Spent media
E. coli
S. haemolyticus
P. aeruginosa
K. pneumoniae
E. faecium
Antibiotic
Vancomycin
Starting at 50% of MIC
Serial Transfer
30x
Every 48 hours
If OD600>0.05, antibiotic concentration increased 20%
Determine evolutionary trajectory
[Antibiotics]
Time


1A
E. coli
Trimethoprim (µg/mL)
10
1
0.1
0.01
0
10
20
30
40
50
60
70
Days
K. pneumoniae
E. coli
E. faecium
S. haemolyticus
1B
K. pneumoniae
Trimethoprim (µg/mL)
10
1
0.1
0.01
0
10
20
30
40
50
60
Days
K. pneumoniae
E. coli
E. faecium
S. haemolyticus
1C
E. faecium
Vancomycin (µg/mL)
10
1
0.1
0
10
20
30
40
50
60
Days
K. pneumoniae
E. coli
P. aeruginosa
E. faecium
S. haemolyticus


2A
E. coli
Final [Trimethoprim] (µg/mL)
Population size (OD600)
2B
K. pneumoniae
Final [Trimethoprim] (µg/mL)
Population size (OD600)
2C
E. faecium
Final [Vancomycin] (µg/mL)
Population size (OD600)
K. pneumoniae
E. coli
P. aeruginosa
E. faecium
S. haemolyticus
2D
E. coli
Final [Trimethoprim] (µg/mL)
Tolerance fold change
2E
K. pneumoniae
Final [Trimethoprim] (µg/mL)
Tolerance fold change
2F
E. faecium
Final [Vancomycin] (µg/mL)
Tolerance fold change


3A
E. coli
Average # of SNPs
Population size (OD600)
3B
K. pneumoniae
Average # of SNPs
Population size (OD600)
3C
E. faecium
Average # of SNPs
Population size (OD600)
K. pneumoniae
E. coli
P. aeruginosa
E. faecium
S. haemolyticus
3D
E. coli
Number of mutations
K. pneumoniae
E. coli
E. faecium
S. haemolyticus
SNP DEL INS CNV INV
3E
K. pneumoniae
Number of mutations
K. pneumoniae
E. coli
E. faecium
S. haemolyticus
SNP DEL INS
3F
E. faecium
Number of mutations
K. pneumoniae
E. coli
P. aeruginosa
E. faecium
S. haemolyticus
SNP DEL INS INV


4A
K. pneumoniae
E. coli
E. faecium
S. haemolyticus
K. pneumoniae
E. coli
E. faecium
S. haemolyticus
E. coli
4B
K. pneumoniae
E. coli
E. faecium
S. haemolyticus
K. pneumoniae
E. coli
E. faecium
S. haemolyticus
K. pneumoniae
4C
K. pneumoniae
E. coli
P. aeruginosa
E. faecium
S. haemolyticus
K. pneumoniae
E. coli
P. aeruginosa
E. faecium
S. haemolyticus
E. faecium
1
0.75
0.5
0.25
0


5A
E. coli
folA
folM
folP
nadR2
acrR
rpoC
ycbZ
deaD
dnaA
phoQ
rapZ
pabB
aroK
adhE
chbF
gnd
H
K
O
L
T
E
C
G
Genes
K. pneumoniae
E. coli
E. faecium
S. haemolyticus
Conditioned medium
5B
K. pneumoniae
folA
folM
folX
pCAR1
cspA
deoB
pma1
pitA
H
K
G
P
Genes
K. pneumoniae
E. coli
E. faecium
S. haemolyticus
Conditioned medium
5C
E. faecium
rpiR
ompR
spxA2
vanR
vanS
relA
prs2
wecA
yabM
nox
pyk
aroF
rex1
hsdS
K
T
F
M
Q
G
E
L
V
Genes
K. pneumoniae
E. coli
P. aeruginosa
E. faecium
S. haemolyticus
Conditioned medium
Non-synonymous
Upstream gene variant
Loss of function
Number of mutations
1
2
3
4
5