# The effects of antibiotic combination treatments on *Pseudomonas aeruginosa* tolerance evolution and coexistence with *Stenotrophomonas maltophilia*

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## Abstract

*Pseudomonas aeruginosa* bacterium is a common pathogen of Cystic Fibrosis (CF) patients due to its ability to evolve resistance to antibiotics during treatments. While *P. aeruginosa* resistance evolution is well characterised in monocultures, it is less well understood in polymicrobial CF infections. Here, we investigated how exposure to ciprofloxacin, colistin, or tobramycin antibiotics, administered at sub-MIC doses alone and in combination, shaped the tolerance evolution of *P. aeruginosa* (PAO1 lab and clinical CF LESB58 strains) in the absence and presence of a commonly co-occurring species, *Stenotrophomonas maltophilia*. Increases in antibiotic tolerances were primarily driven by the presence of that antibiotic in the treatment. We observed a reciprocal cross-tolerance between ciprofloxacin and tobramycin, and when combined these antibiotics selected increased MICs for all antibiotics. Though the presence of *S. maltophilia* did not affect the tolerance or the MIC evolution, it drove *P. aeruginosa* into extinction more frequently in the presence of tobramycin due to its relatively greater innate tobramycin tolerance. In contrast, *P. aeruginosa* dominated and drove *S. maltophilia* extinct in most other treatments. Together, our findings suggest that besides driving high-level antibiotic tolerance evolution, sub-MIC antibiotic exposure can alter competitive bacterial interactions, leading to target pathogen extinctions in multi-species communities.

## Importance

Cystic Fibrosis (CF) is a genetic condition resulting in thick mucus secretions in the lungs that are susceptible to chronic bacterial infections. The bacterial pathogen *Pseudomonas aeruginosa* is often associated with morbidity in CF and difficult to treat due to high resistance to antibiotics. *Pseudomonas aeruginosa* resistance evolution is poorly understood in polymicrobial infections typical of CF. To study this, we exposed *P. aeruginosa* to sublethal concentrations of ciprofloxacin, colistin, or tobramycin antibiotics in the absence and presence of a commonly co-occurring CF species, S*tenotrophomonas maltophilia*. We found that low-level antibiotic concentrations selected for high-level antibiotic resistance. While *P. aeruginosa* dominated in most antibiotic treatments, *S. maltophilia* drove it into extinction in the presence of tobramycin due to an innately higher tobramycin resistance. Our findings suggest that, besides driving high-level antibiotic tolerance evolution, sub-lethal antibiotic exposure can magnify competition in bacterial communities, leading to target pathogen extinctions in multi-species communities.

## Keywords

Experimental evolution, interspecies interactions, Cystic Fibrosis

## Introduction

Cystic Fibrosis (CF) is a genetic condition that is characterised by impaired chloride ion channel function, resulting in thick mucus secretions in the lungs that are susceptible to chronic bacterial infection (1)⁠. Of the bacterial species that infect adult CF patients, *Pseudomonas aeruginosa* is the most prevalent pathogen associated with morbidity (2, 3)⁠ and is difficult to treat due to its intrinsic resistance to many antibiotics and its ability to readily evolve resistance to new antibiotics (4, 5)⁠. Though usually dominated by *P. aeruginosa*, CF infections are often polymicrobial and many different bacterial species co-occur with *P. aeruginosa* in the CF lungs (6–10)⁠.

Over the course of their lives patients with CF will be treated with a number of different antibiotics; for example, during treatment to eradicate *P. aeruginosa* or to help resolve pulmonary exacerbations (11, 12)⁠. Antibiotics are administered at high concentration multiple times per day to maintain a therapeutic dose at a concentration greater than the minimum inhibitory concentration (MIC) required to inhibit bacterial growth (12–14)⁠. Antibiotic combinations are used to target multiple species simultaneously or to increase efficacy against a single species (15, 16)⁠. However, the thick mucus secretions and complex branching structure of the lungs themselves will likely result in bacterial populations experiencing a gradient of antibiotic concentrations (17, 18)⁠. Thus, despite the best efforts of the treatment regimens, pockets of bacteria within the lungs are likely to experience antibiotic concentrations below that required to inhibit those bacteria, and such subinhibitory concentrations have been shown to promote antibiotic resistance evolution (19, 20)⁠.

Selection for antibiotic resistance differs between antibiotics administered at-or-above MIC, and below MIC (14)⁠. At concentrations greater than MIC the driver of selection is whether the bacteria can survive the antibiotic challenge, and so any mutations in the bacterial population that increase MIC, regardless of the impact on other competitive growth traits, would be selected (19, 20)⁠. Conversely, below MIC the selective pressure differs, such that any mutation that confers an increase in growth in the presence of the low antibiotic concentration, and thus a competitive advantage, relative to other members of the population would be selected regardless of whether this mutation would increase the MIC (19, 20). This relatively weaker selection pressure increases the number of viable mutations, which in turn increases the likelihood that one such mutation could increase MIC through mechanisms not traditionally considered to be involved in resistance (20). The lack of antibiotic-mediated killing also results in a longer selective window during which more mutations can accumulate and either ameliorate the costs of higher-level resistance (21), or together confer high-level resistance via epistatic interactions (22)⁠. While effects of lethal concentrations (>MIC) of antibiotic combinations on individual bacterial species have been explored previously (23, 24), they are less well understood at sub-MIC concentrations in multi-species communities.

Competition with other bacterial species could change the trajectory of antibiotic resistance evolution in a focal pathogen species in various ways (25). Firstly, the presence of competitors more tolerant of an antibiotic treatment than the susceptible pathogen species could increase the strength of competition between the two and lead to a decrease in relative pathogen abundance, potentially triggering extinctions (26)⁠. Competitor-mediated reduction in the population density of the focal pathogen could further slow resistance evolution by reducing the mutation supply rate and the emergence of *de novo* resistance (27). When antibiotic resistance evolves it is often associated with metabolic costs, such as activation of efflux pumps or modification of the antibiotic target. These costs could reduce pathogen competitiveness in the presence of non-resistant mutants, or species unaffected by a given antibiotic, when antibiotic concentrations are low (28–30). While it has been suggested that bacterial interactions are predominantly competitive (31), it is also possible that other interacting bacteria could facilitate antibiotic resistance of the focal species via horizontal gene transfer of resistance genes (32). Alternatively, other species could provide protection from antibiotics via secretions that break down antibiotics (21, 33, 34)⁠ or create protective microenvironments via production of biofilms (30, 35). Despite being a ubiquitous selective force in nature, there are relatively few studies directly testing the effect of bacterial inter-species interactions on the evolution of antibiotic resistance.

Here we focused on studying how the evolution of antibiotic tolerance of *P. aeruginosa* is affected by the presence of *Stenotrophomonas maltophilia*, another CF-associated species that is increasing in prevalence among CF patients (36–41)⁠ and commonly co-occurs with *P. aeruginosa* (42). In order to investigate this, we performed a short-term *in vitro* serial transfer experiment in which we grew both of the lung-naïve laboratory *P. aeruginosa* strain PAO1 and the lung-adapted Liverpool Epidemic Strain B58 (LESB58; 42)⁠ either alone in monoculture or in the presence of *S. maltophilia* (resulting in four different cultures: PAO1, LESB58, PAO1 & *S. maltophilia*, and LESB58 & *S. maltophilia*; Figure 1). Two strains of *P. aeruginosa* were chosen to compare the potential effect of previous exposure to antibiotic treatments and other infecting bacteria on the evolution of antibiotic tolerance. Each of these cultures were treated with one of the eight combinations (see Methods) of the anti-Pseudomonal antibiotics ciprofloxacin, colistin, and tobramycin. These antibiotics were selected because of their use in either *P. aeruginosa* eradication therapy or for treatment of pulmonary exacerbations (11, 12, 44, 45), and for their differing modes of action (46–48)⁠. Each of the antibiotics were applied at a sub-MIC concentration that had small but contrasting effects on the growth of all three bacterial strains (Figure S1).

During the serial transfer experiment, which took place over 21 days, we tracked the presence of *P. aeruginosa* and *S. maltophilia* for any extinctions and monitored changes in total population densities across the 192 selection lines. Following the experiment, we measured the ability of the evolved focal *P. aeruginosa* isolates to grow in the treatment concentrations of the individual antibiotics relative to ancestral stock strains. Moreover, the MICs of each antibiotic were determined for all evolved *P. aeruginosa* isolates. We hypothesised that: i) antibiotic tolerance evolution could be constrained in the presence of a competitor but promoted in the presence of multiple antibiotics if cross-tolerance evolution is common; and ii) antibiotic exposure could change community composition due to differences between the species’ innate susceptibility to the antibiotics or due to evolution of tolerance-growth trade-offs.

We found increases in antibiotic tolerance or MIC were not generally enhanced by antibiotic combinations; rather increases in tolerance or MIC to a given antibiotic were driven by the presence of that antibiotic in the treatment combination, which occasionally led to cross-tolerance. Similarly, the presence of *S. maltophilia* did not affect antibiotic tolerance evolution or MIC with either *P. aeruginosa* strain, likely due to frequent extinctions during the early phase of the selection experiment. However, while both *P. aeruginosa* strains were able to dominate the “No Antibiotic” control and many antibiotic-containing coculture treatments, tobramycin-containing antibiotic treatments triggered *P. aeruginosa* extinctions in 15% of coculture populations, which were more common when *S. maltophilia* was cultured with PAO1 than LESB58. Together, these results suggest that the effects of sub-MIC antibiotic concentrations could be magnified in polymicrobial communities due to competition, asymmetry in innate antibiotic tolerances, and differential evolution of antibiotic tolerance and its associated costs.

## Materials and Methods

### Bacterial strains and culture conditions

Two strains of *Pseudomonas aeruginosa* were used as the focal pathogen species: PAO1, a lab adapted reference strain (ATCC 15692), and LESB58, a transmissible CF lung isolated strain (42). *Stenotrophomonas maltophilia* type strain ATCC 13637—isolated from the oropharyngeal tract of a cancer patient (49)—was used as the coculture competitor. The base media used throughout was a 50:50 mix of nutrient broth without NaCl (Sigma; 5 g/l peptic digest of animal tissue, 3 g/l beef extract, pH 6.9) and PBS (8 g/l NaCl, 2 g/l KCl, 1.42 g/l Na2HPO4, 2.4 g/l KH2PO4), hereafter ‘NB’, that allowed stable coexistence of both the focal pathogen (*P. aeruginosa*) and competitor (*S. maltophilia*) species over a single 72 h growth period. All cultures, unless otherwise stated, were grown at 37ºC with shaking at 180 rpm.

### Selection experiment

During the selection experiment, a focal bacterium (either *P. aeruginosa* strain PAO1 or LESB58) was grown in a culture either alone (monoculture) or with *S. maltophilia* (coculture) and treated with subinhibitory concentrations of ciprofloxacin (CIP), colistin (CST), and tobramycin (TOB) antibiotics in all one-, two- and three-way combinations (“No Antibiotic”, CIP, CST, TOB, CIP+CST, CIP+TOB, CST+TOB, CIP+CST+TOB). Each treatment was replicated 6 times for both focal pathogens in the absence and presence of *S. maltophilia* resulting in a total of 192 selection lines. During the initial setup overnight cultures, from frozen stocks, of PAO1, LESB58, and *S. maltophilia*, were diluted down to the same optical density at 600 nm (OD600; approximately 0.17 at 600 nm), corresponding to cell densities of 7.4x106, 2.2x107, and 4.6x106 CFU/ml respectively. Monocultures consisted of 20 µl of the *P. aeruginosa* strain, while cocultures mixed 10 µl of the *P. aeruginosa* strainwith 10 µl of *S. maltophilia*, each in 200 µl of NB supplemented with one of the eight antibiotic treatments for a total volume of 220 µl. The concentrations of the antibiotics (0.03215 μg/ml ciprofloxacin [Sigma Aldrich], 2 μg/ml colistin [Acros Organics], and 0.5 μg/ml tobramycin [Acros Organics]) were chosen to be below the minimum inhibitory concentration of all three species (as determined below; Table 1, Figure S1), and were kept constant across each combination. Only one concentration of each antibiotic was used to model antibiotic therapy in the clinic, where antibiotics are applied without necessarily knowing the variation in existing levels of resistance within the infecting populations. Selection lines were grown in 96-well plates. Following setup plates were incubated for 72 hours, after which each replicate was homogenised by mixing and OD600 was measured (Tecan Infinite 200), as a proxy measure of bacterial population densities. After measurement, each replicate was again mixed and 20 μl of each culture was transferred to 200 μl of fresh media with the same antibiotic treatment. These fresh plates were incubated for 72 hours until the next serial transfer. Presence or absence of each species in each culture was determined following each transfer by growing subsamples of the 72-hour cultures on different selective agar; *Pseudomonas* selective agar (Oxoid; *Pseudomonas* agar base: 16 g/l gelatin peptone, 10 g/l casein hydrolysate, 10 g/l potassium sulphate, 1.4 g/l magnesium chloride, 11 g/l agar, 1% vol/vol glycerol; *Pseudomonas* CN selective supplement: 200 µg/ml centrimide, 15 µg/ml sodium nalidixate), and *S. maltophilia* selective agar: LB agar (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, 15 g/l agar) supplemented with 64 μg/ml tobramycin incubated at 30ºC rather than 37ºC—*S. maltophilia* is innately resistant towards tobramycin at 30ºC (50)⁠. Some monoculture replicates were contaminated with *S. maltophilia* (14 PAO1 selection lines, and 9 LESB58 selection lines), and these were excluded from the analyses. Whole population bacterial samples were picked from the agar plates for each replicate, grown overnight in NB, and cryopreserved in 20% glycerol to be frozen at -80ºC. The selection experiment was carried out for 21 days, equalling 6 serial transfers. See Figure 1 for a schematic of the treatment and culture combinations and experimental procedures.

### Determination of MIC and antibiotic tolerance

Both prior to and following the selection experiment, the minimum inhibitory concentration (MIC) of each of the three antibiotics—ciprofloxacin, colistin, and tobramycin—was determined by broth microdilution for the three bacteria strains. Briefly, overnight cultures, from frozen samples, were diluted 1 in 10 in PBS and further diluted 1 in 10 into NB with antibiotic concentrations ranging from 32 µg/ml to 0.015625 µg/ml (25 to 2-6) and grown in static conditions, in triplicate. OD600 was measured after 24 hours (Tecan Sunrise). MIC was defined as the lowest concentration of antibiotic at which there was no growth. We found no considerable difference between shaken and static conditions when determining MIC in our preliminary experiments. For the evolved strains, the MIC50 of a bacterial population was defined as the MIC required to inhibit half of the replicates of that population. We also grew all bacteria (evolved and ancestral) without antibiotic in NB for 24 hours, using the same protocol as above.

To assess the difference in tolerance of the antibiotics for each individual evolved replicate and ancestral strain, at the treatment concentrations used during the selection experiments, we define a growth proxy, , as

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where is the endpoint OD600 from growth after 24 hours of the replicate in a given antibiotic, and analogously for growth of the same replicate without antibiotic, i.e., in NB. Such a measure is a proxy for growth of the bacteria that accounts for differences in media adaptation when comparing antibiotic tolerance. We use this measure accordingly in Figure 2 and its associated statistics. Tolerance of evolved bacteria from a selection regimen as a whole was calculated as

where we calculate the errors in our measure by computing the standard error of the mean (SEM). The sum is over all relevant strains evolved in the selection regimen of interest, e.g., monoculture PAO1 treated with CIP, and N ≤ 6 replicates dependent on extinctions.

We use a similar growth proxy, , to assess the difference in media adaptation for each individual replicate compared to the respective ancestor, defined as

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where is the endpoint OD600 from growth after 24 hours of the evolved strain without antibiotic, and analogously for the ancestral strain. We use this measure in Figure 3 and its associated statistics. We calculate the mean and SEM of for a given selection regimen as with above.

### Statistical analyses

All data were analysed in R version 4.1.0 (51)⁠. Data manipulation and graphing were performed using the *tidyverse* suite of packages (52)⁠, along with *egg* for figure assembly (53), and *ggbeeswarm* for point plotting (54). Regarding the tolerance dataset, separate linear regression models for each *P. aeruginosa* species were used when analysing each response variable (i.e., for growth in each antibiotic, and for growth without antibiotic). Here, the response variable was difference in growth in antibiotic relative to growth without antibiotic () for antibiotic tolerance (Figure 2), and difference in growth relative to the ancestor () for media adaptation (Figure 3). Two-way type II ANOVA was performed using the *car* package (55). Post-hoc pairwise comparisons were computed from estimated marginal means and *p* values were adjusted using Šidák correction, through the emmeans and contrast functions of the *emmeans* package (56). Pairwise comparisons were computed between treatments alone, after observing no effect of competitor nor interaction.

Regarding the MIC dataset, individual Pearson Chi-squared tests of independence were performed for each *P. aeruginosa* strain in each antibiotic. MIC values were represented as ordered nominal variables, and frequency of observed MIC for each replicate in each treatment was tabulated. Chi-squared tests were computed using the chisq\_test function from the *coin* package (57)⁠. Pairwise tests of independence with Benjamini-Hochberg false discovery rate corrections were performed between each treatment using the pairwiseOrdinalIndependence function from the *rcompanion* package (58)⁠.

With the population density dataset, two linear regression models were fit, one to each *P. aeruginosa* strain. The response variable was natural logarithm transformed OD600 values, and antibiotic treatment and competitor as the predictor variables. Two-way type II ANOVA and post-hoc pairwise comparisons were performed as with the tolerance data.

### **Data availability**

Population density and species presence absence data from the transfer experiment, ancestral and evolved strain growth with and without antibiotic, and MIC data have been deposited in Dryad (DOI: 10.5061/dryad.83bk3j9tn).

## Results

### Effects of antibiotic treatments on *P. aeruginosa* antibiotic tolerance and relative cost of tolerance

To test potential tolerance evolution, we compared the evolved *P. aeruginosa* populations’ ability to grow (as optical density at 600 nm; OD600) in the presence of the treatment concentration of each of the antibiotics and compared this to growth without the antibiotic (see Methods). Some *P. aeruginosa* monoculture replicates were removed from the analyses due to contamination with *S. maltophilia* (see Methods). Moreover, as *P. aeruginosa* went extinct in some of the tobramycin-containing treatments (24/143 selection lines), the evolution of tolerance was compared using only surviving treatment replicates.

Firstly, the antibiotic tolerances of both strains were not affected by previous exposure to *S. maltophilia* (Figure 2, *p* > 0.05, Tables S1 & S2). With regard to the control treatments, in the case of the clinical isolate LESB58 the “No Antibiotic” control treatment resulted in increased susceptibility to antibiotics relative to the ancestor, while the antibiotic treatments maintained the ancestral-level tolerances of ciprofloxacin and tobramycin. In contrast, the “No Antibiotic” control treatment of the lab strain PAO1 maintained ancestral-level tolerance, while the antibiotic treatments further increased the tolerance of the evolved isolates (Figure 2).

For both strains there was a significant increase in ciprofloxacin tolerance when treated with the ciprofloxacin (CIP) mono-, CIP+colistin (CST) and CIP+tobramycin (TOB) treatments compared to the “No Antibiotic” control treatment, as well as with the CIP+CST+TOB treatment in LESB58 (Post-hoc pairwise comparisons, PAO1: *t*(48) = 11.78 (CIP); 13.76 (CIP+CST); 11.42 (CIP+TOB), *p* < 0.001; LESB58: *t*(66) = 4.73 (CIP); 4.82 (CIP+CST), *p* < 0.001; *t*(66) = 4.42, *p* = 0.001 (CIP+TOB); *t*(66) = 3.46, *p* = 0.026 (CIP+CST+TOB); Figure 2A, B). Similarly, the TOB and CIP+TOB treatments significantly increased tobramycin tolerance in PAO1 (Post-hoc pairwise comparisons, *t*(48) = 7.71 (TOB); 6.20 (CIP + TOB), *p* < 0.001; Figure 2E), while all tobramycin containing treatments significantly increased tolerance in LESB58 (Post-hoc pairwise comparisons, *t*(66) = 6.39 (TOB); 6.53 (CIP+TOB); 6.92 (CST+TOB); 6.51 (CIP+CST+TOB), *p* < 0.001; Figure 2F). In contrast, no colistin-containing treatment resulted in increased colistin tolerance in PAO1, while only the CST+TOB and CIP+CST+TOB treatments significantly increased colistin tolerance in LESB58 compared to the “No Antibiotic” control treatment (Pairwise post-hoc comparisons, *t*(66) = 3.30, *p* = 0.043 (CST+TOB); *t*(66) = 3.39, *p* = 0.032 (CIP+CST+TOB); Figure 2C, D).

We also observed cross-tolerance between ciprofloxacin and tobramycin, i.e., the CIP mono-treatment provided tobramycin tolerance, and vice versa (Figure 2). In PAO1, TOB mono-treatment gave a significant increase in ciprofloxacin tolerance, and to a similar degree as the CIP mono-treatment, when compared to the “No Antibiotic” control treatment (Post-hoc pairwise comparisons, *t*(48) = 9.68, *p* < 0.001; Figure 2A). And though not significant, the CIP mono-treatment increased tobramycin tolerance (Pairwise post-hoc comparisons, *t*(48) = 3.07, *p* = 0.093; Figure 2E). A similar pattern emerged in LESB58, where the CIP mono-treatment resulted in a significantly higher tobramycin tolerance than the “No Antibiotic” control (Post-hoc pairwise comparisons, *t*(66) = 3.73, *p* = 0.011; Figure 2F). And though the TOB mono-treatment did not significantly increase ciprofloxacin tolerance the CST+TOB treatment increased tolerance compared to the “No Antibiotic” control (Pairwise post-hoc comparison, *t*(66) = 3.94, *p* = 0.006; Figure 2B). Additionally, the CIP+TOB combination treatment resulted in cross-tolerance to colistin in both strains (Post-hoc pairwise comparisons, PAO1: *t*(48) = 5.04, *p* < 0.001; LESB58: *t*(66) = 4.21, *p* = 0.002; Figure 2B, F). The CST mono-treatment did not provide any cross-tolerance towards the other antibiotics. These results suggest that while colistin tolerance evolution was rare, both pathogen strains readily evolved tolerance to ciprofloxacin and tobramycin, which was driven by prior exposure to these antibiotics and reciprocal cross-tolerance.

To test whether selection in different antibiotic treatments led to a cost of tolerance, we grew each of the surviving evolved replicates in media without antibiotic and compared their growth relative to the respective ancestors (Figure 3A, B). The majority of both *P.* *aeruginosa* genotype replicates across all treatments evolved to grow better in the growth media relative to their ancestors (Figure 3A, B), and the increase in growth, relative to the ancestor, was greater in the lung-adapted LESB58 than the lab-adapted PAO1. However, this increase clearly varied between the antibiotic treatments. In the case of both genotypes, the TOB mono-treatment constrained adaptation, resulting in a significantly reduced growth compared to the “No Antibiotic” control treatment (Post-hoc pairwise comparisons, PAO1: *t*(44) = 4.47, *p* = 0.001; LESB58: *t*(66) = 3.40, *p* = 0.03). Moreover, the growth of evolved LESB58 populations treated with any tobramycin-containing antibiotic treatment were significantly below the “No Antibiotic” control treatment (Post-hoc pairwise comparisons, *t*(66) = 3.58, *p* = 0.01 (CIP+TOB); 4.81, *p* < 0.001 (CST+TOB); 3.89, *p* = 0.005 (CIP+CST+TOB); Figure 3H). These results suggest that adapting to tolerate tobramycin reduced the growth and potential competitive ability of *P. aeruginosa* strains.

### Changes in the MIC of antibiotics with evolved *P. aeruginosa* populations

We measured changes in the Minimum Inhibitory Concentration (MIC) of each antibiotic for evolved *P. aeruginosa* replicate populations, and MIC50—the MIC capable of inhibiting 50% of replicates—for each treatment to quantify whether exposure to low antibiotic concentrations led to increased MIC. For both *P. aeruginosa* strains across all three antibiotics, there was no effect of previous exposure to *S. maltophilia* on the MICs. However, the MICs of the evolved populations changed considerably with both *P. aeruginosa* strains in response to all antibiotics (Table S3).

#### Changes in ciprofloxacin MIC

Both evolved *P. aeruginosa* strains showed large increases in MIC to ciprofloxacin (Figure 4A, B). The MIC of ciprofloxacin for PAO1 replicates from the “No Antibiotic” control treatment remained mostly unchanged relative to the ancestor, at 0.125 µg/ml, though a pair of individual replicates increased their MIC by three-fold (Figure 4A). In comparison, in LESB58 the baseline effect of the “No Antibiotic” control was a three-fold decrease in MIC compared to the ancestor, from 1 µg/ml to 0.125 µg/ml—to the same MIC as the laboratory PAO1 strain (Figure 4B). Pairwise Chi-squared tests showed that the CIP, CIP+CST, and CIP+TOB treatments all resulted in significantly greater MIC values, compared to the control treatment, amongst isolates of both strains (Pairwise independence, PAO1—CIP: *X*2(1, N = 22) = 15.61, *p* = 0.002; CIP+CST: *X*2(1, N = 22) = 10.77, *p* = 0.010; CIP+TOB: *X*2(1, N = 15) = 8.34, *p* = 0.018; Figure 4A; LESB58—CIP: *X*2(1, N = 24) = 19.03, *p* > 0.001; CIP+CST: *X*2(1, N = 24) = 13.71, *p* = 0.0019; CIP+TOB: *X*2(1, N = 22) = 13.31, *p* = 0.0019; Figure 4B). Indeed, in LESB58 the triple antibiotic treatment also significantly increased MIC values (Pairwise independence: *X*2(1, N = 21) = 9.95, *p* = 0.0064; Figure 4B), such that all ciprofloxacin-containing treatments increased ciprofloxacin MIC. Moreover, many of the TOB mono-treated isolates from both strains had high MIC values, and in LESB58 the MICs for both these and the CST+TOB treated isolates were significantly different to the “No Antibiotic” control treatment (Pairwise independence, TOB: *X*2(1, N = 21) = 7.34, *p* = 0.019; CST+TOB: *X*2(1, N = 19) = 10.03, *p* = 0.0064), further suggesting that there is some cross-tolerance provided by tobramycin as also seen in the growth measurements (Figure 4). The MIC values for evolved LESB58 isolates reached higher levels than in PAO1, with 18 LESB58 isolates reaching 4 or 8 µg/ml compared with one PAO1 isolate, and there was also greater variation in MIC values among the LESB58 isolates of a given treatment than PAO1. Overall, both *P. aeruginosa* strains evolved an increase in ciprofloxacin MIC, which was driven mostly by the previous exposure to ciprofloxacin.

#### Changes in colistin MIC

As opposed to ciprofloxacin, the MICs of colistin did not increase as a result of prior colistin exposure during the selection experiment (Figure 4C, D). In the case of PAO1, the majority of treatments resulted in no change to the ancestral MIC of 4 µg/ml (Figure 4C). Slightly more variation was observed amongst the LESB58 isolates that had been exposed to CIP, CST, or TOB mono-treatments, though no changes greater than one-fold for more than a single replicate were found. However, the MICs of isolates treated with the combinations CIP+TOB and CST+TOB were both significantly higher compared with the “No Antibiotic” control treatment (Pairwise independence, CIP+TOB: X2(1, N = 22) = 6.13, p = 0.037; CST+TOB: X2(1, N = 19) = 6.21, p = 0.037; Figure 4C). Overall, only small changes in colistin MIC were observed, which were indirectly driven by other antibiotics.

#### Changes in tobramycin MIC

The MIC changes for tobramycin were similar between both *P. aeruginosa* strains (Figure 4E, F). The “No Antibiotic” control treated isolates of PAO1 maintained the ancestral MIC of 1 µg/ml (Figure 4E), whereas the “No Antibiotic” control treated LESB58 isolates decreased in MIC relative to their ancestor (from 2 µg/ml down to 0.5 and 1 µg/ml; Figure 4F). For both strains, the TOB and CIP+TOB treatments resulted in significant increase in MIC compared with the “No Antibiotic” control treatment (Pairwise independence, PAO1—TOB: *X*2(1, N = 20) = 8.54, *p* = 0.0035; CIP+TOB: *X*2(1, N = 15) = 12.00, *p* < 0.001; Figure 4E; LESB58—TOB: *X*2(1, N = 21) = 12.67, *p* = 0.0035; CIP+TOB: *X*2(1, N = 22) = 11.35, *p* = 0.0053; Figure 4F), and for LESB58 this was the also the case with the CST+TOB and triple antibiotic treatments (Pairwise independence, CST+TOB: *X*2(1, N = 19) = 13.77, *p* = 0.0035; CIP+CST+TOB: *X*2(1, N = 21) = 13.22, *p* = 0.0035). Both strains also had a significant increase in MIC as a result of the CIP mono-treatment compared to the “No Antibiotic” control treatment (Pairwise independence, PAO1: *X*2(1, N = 22) = 7.98, *p* = 0.019); LESB58: *X*2(1, N = 24) = 10.58, *p* = 0.0064). However, this increase did not reach the same values as the tobramycin containing treatments did; the MIC of the CIP mono-treated LESB58 isolates was significantly lower compared to TOB, CST+TOB, and CIP+CST+TOB treatments (Pairwise independence, TOB: *X*2(1, N = 21) = 7, *p* = 0.021; CST+TOB: *X*2(1, N = 19) = 8.05, *p* = 0.014; CIP+CST+TOB: *X*2(1, N = 21) = 6.64, *p* = 0.023), suggesting that the cross-tolerance provided by ciprofloxacin was weaker than that of tobramycin. Overall, both *P. aeruginosa* strains evolved increases in tobramycin MIC, which was primarily driven by the previous exposure to tobramycin during the selection experiment.

### The effect of antibiotic treatments on bacterial densities and coculture composition

We measured a proxy of total population density (OD600)of each bacterial population at the final timepoint of the selection experiment to determine the extent to which antibiotics inhibited bacterial growth (measurements were taken at the population level and did not differentiate species frequencies). For both *Pseudomonas* strains, there was a significant effect of antibiotic treatment on total population density (Table S4, *p* < 0.001), and with the exception of the CST mono-treatment, antibiotics generally decreased the total population density relative to the “No Antibiotic” control treatment (Figure 5A–D). However, post-hoc pairwise comparisons showed that this effect was driven by the cocultures as none of the monocultures differed significantly between any of the treatments with either strain (Figure 5A, B). The combination of CST+TOB was particularly effective in the cocultures of both strains, reducing the population density significantly compared with the “No Antibiotic” control (Post-hoc pairwise comparison, PAO1: *t*(65) = 4.21, *p* = 0.005; LESB58: *t*(71) = 3.98, *p* = 0.010; Figure 5C, D). There was no effect of growing in monoculture vs. coculture for PAO1 (Table S4, *p* > 0.05), and though there was a significant effect in LESB58 (Table S4, *p* = 0.022) this was likely driven by the large difference in population density between the two CST-mono treated cultures (Post-hoc pairwise comparison, *t*(71) = 3.62, *p* = 0.034; Figure 5B, D). Together, these results show that the antibiotics reduced bacterial population densities compared with the “No Antibiotic” control treatment regardless of the presence of *S. maltophilia*, and that the combination of CST+TOB was highly effective at reducing total bacterial population densities.

We also compared the composition of *P. aeruginosa* and *S. maltophilia* cocultures at the end of the experiment to examine the effects of antibiotics on the species coexistence. We found that *P. aeruginosa* survived in all monocultures across the different treatments (except for a single PAO1 replicate that went extinct under CST+TOB treatment). This suggests that the low concentrations of the antibiotic were not sufficient to kill *P. aeruginosa*, even when applied in combination. However, extinctions of *P. aeruginosa* were more common in the presence of *S. maltophilia* (Figure 5E, F). In the absence of antibiotics both *P. aeruginosa* genotypes were able to dominate the cocultures, driving *S. maltophilia* extinct in all replicates. The same was true in the CIP and CST mono-treatments. Moreover, in these treatments *S. maltophilia* became undetectable at early timepoints of the selection experiment (Figure S2). In contrast, the TOB mono-treatment allowed prolonged coexistence between the two bacteria and *S. maltophilia* was able to survive with both *P. aeruginosa* genotypes in two of the six TOB mono-treatment replicates, and fully outcompeted *P. aeruginosa* in a further three replicates (Figure 5E, F). The two *P. aeruginosa* genotypes differed in their capacity to coexist with *S. maltophilia* across the antibiotic combination treatments. The laboratory strain PAO1 was driven extinct more often than the clinical strain LESB58; PAO1 was only able to survive in two CIP+TOB treated replicates and was otherwise driven extinct in the remaining 22 replicates across the other tobramycin-containing combination treatments (Figure 5E). In contrast, the LESB58 strain dominated *S. maltophilia* in all combination treatments except for the CST+TOB treatment, in which *P. aeruginosa* was driven extinct in two replicates (Figure 5F). Together, these results suggest that while *P. aeruginosa* was able to outcompete *S. maltophilia* in most of the environments, this relationship was reversed in the presence of tobramycin, leading to either *P. aeruginosa* extinction or coexistence with *S. maltophilia*.

## Discussion

While antibiotics are routinely used to treat *P. aeruginosa* infections within the polymicrobial communities in CF patient lungs, it is unclear how low antibiotic concentrations affect antibiotic resistance evolution in the presence of naturally occurring CF lung microbiota. Here we studied this by exposing *P. aeruginosa* to sub-MIC concentrations of ciprofloxacin, colistin, tobramycin and their combinations in the presence and absence of a commonly co-occurring CF species, *S. maltophilia*. We observed *P. aeruginosa* tolerance evolution to all antibiotics and a clear cross-tolerance between tobramycin and ciprofloxacin. Moreover, antibiotic exposure often led to an increase in MIC, suggesting that sub-MIC selection can lead to high levels of antibiotic tolerance. While the presence of a competitor had no effect on antibiotic tolerance evolution, antibiotic exposure had a strong effect on species community composition: even though *P. aeruginosa* dominated most of the treatments, it coexisted with-, or was driven into extinction by, *S. maltophilia* in the presence of tobramycin, likely due to drastic innate differences in tobramycin resistance. Thus, even low doses of antibiotics could significantly change antibiotic tolerance evolution and composition of multi-species communities.

We predicted that antibiotic tolerance evolution could be constrained by the presence of *S. maltophilia* as a competitor, for example due to negative effects on *P. aeruginosa* population densities and mutation supply rate (27)⁠. However, we found that the presence of *S. maltophilia* did not alter the trajectory of antibiotic tolerance evolution. Instead, *P. aeruginosa* evolved increased tolerance to all antibiotics regardless of the competitor presence, which was driven primarily by previous exposure to the same antibiotics during the selection experiment. The minimal effect of the competitor can in some cases be explained by *S. maltophilia* extinction early during the selection experiment, as was the case in the “No Antibiotic” control, CIP and CST mono-treatments (Figure S2). However, when *S. maltophilia* survived for longer, such as in the TOB mono-treatment or the CST+TOB treatment for LESB58 (Figure S2), there remained no significant effect of competition on tolerance or MIC evolution compared with monoculture growth. This suggests that the presence of *S. maltophilia* indeed had no effect on *P. aeruginosa* tolerance evolution. But due to the nature of our experimental setup, wherein evolutionary dynamics are inherently linked with ecological dynamics, it is difficult to isolate the effect that *S. maltophilia* may have had on the evolution of tolerance in *P. aeruginosa*. An alternative experimental setup, such as introducing fresh populations of *S. maltophilia* at each timepoint, could isolate the evolutionary effect of a competitor. Alternatively, it is possible that the effect of *S. maltophilia* occurred at an earlier timepoint. Quinn *et al.* (59)⁠found that the presence of *S. maltophilia* increased the rate at which imipenem resistance evolved in *P. aeruginosa* strain PAO1 when compared to *P. aeruginosa* monoculture. As such, though the final timepoint tolerances and MICs were similar between our monoculture and coculture *P. aeruginosa*, it is possible that they developed at differing rates.

We predicted that presence of multiple antibiotics could select for increased levels of antibiotic tolerance potentially due to cross-tolerance or via selection for generalised resistance mechanisms such as efflux pumps (4, 5). In support of this we found that increasing the numbers of antibiotics in the treatments resulted in higher level MICs compared to mono-antibiotic treatments. We also found a reciprocal cross-tolerance between ciprofloxacin and tobramycin in both *P. aeruginosa* strains. We recognise that our measurements have some limitations, as our tolerance measurements were obtained using optical density which can be biased by changes in cellular morphology unrelated to tolerance evolution, or by the presence of unviable cells. This bias is mitigated somewhat by calculating our proxy measurement that compared growth with antibiotic to growth without (see Methods), as it compares the growth of the same replicate in different conditions thus accounting for any morphological changes that have arisen by adaptation to the growth media. To fully compensate for this in the future we could perform direct counts of viable cells, such as counting colony forming units.

While ciprofloxacin and tobramycin resistance can be mediated by the same mechanism in *P. aeruginosa*—upregulation of the MexXY-OprM efflux pump (60, 61)⁠—previous studies have suggested that *in vitro* selection for such mutations are rare (62, 63)⁠. As a result, other antibiotic-specific resistance mechanisms could have evolved, such as mutations in *fusA1* for tobramycin (63–65)⁠ and *gyrAB* for ciprofloxacin (29, 66)⁠, even though these mutations are not known to provide cross-tolerance to the other antibiotic. Interestingly, in LESB58 the CST+TOB combination resulted in high levels of MICs for all three antibiotics, providing a cross tolerance to ciprofloxacin. Colistin and tobramycin resistance can be mediated by outer membrane modifications via activation of the PmrAB (67–69)⁠ and ParRS (70, 71)⁠ two component systems. Gain-of-function mutations in either *pmrB* or *parS* can result in increased tolerance to both antibiotics and have been observed in *P. aeruginosa* treated with aminoglycosides *in vitro* (63, 72)⁠ and in the clinic (22, 69)⁠. However, whether the decrease in membrane permeability that these systems provide is sufficient to prevent entry of ciprofloxacin is unclear. We also found that sub-MIC antibiotic selection often led to a clear increase in MIC, providing tolerance to much higher concentrations of antibiotics than the bacteria experienced during the selection experiment. With PAO1, this was especially clear with the ciprofloxacin and tobramycin MICs, while LESB58 showed increase in MIC to all antibiotics but mainly when exposed to antibiotic combinations. It has been shown previously that low levels of antibiotic selection can lead to high levels of resistance due to epistasis (20)⁠, or that antibiotic resistance can evolve *de novo* even in the absence of antibiotic selection due to adaptation to the growth media (73)⁠. Further genetic analyses would be of interest to ascertain the genetic mechanisms of antibiotic resistance in sub-MIC concentrations and to better understand the molecular basis of cross-tolerance.

We also predicted that antibiotic exposure could alter the community composition via potential differences in innate antibiotic sensitivity or tolerance-growth trade-offs. The baseline interaction between *P. aeruginosa* and *S. maltophilia* in the “No Antibiotic” control cocultures was antagonistic, whereby *P. aeruginosa* competitively excluded *S. maltophilia*. Indeed, it has previously been shown that *P. aeruginosa* can kill *S. maltophilia* via a contact dependent mechanism during planktonic growth, and that *P. aeruginosa* outcompetes *S. maltophilia* when grown in dual-species biofilms (74, 75)⁠⁠. However, while competitive exclusion of *S. maltophilia* was observed in the absence and presence of most antibiotic treatments, this pattern was reversed in the presence of tobramycin. In these treatments we observed coexistence of *P. aeruginosa* with the innately tobramycin tolerant *S. maltophilia* (50)⁠, or extinction of *P. aeruginosa*. Though no follow-up data exist for the extinct *P. aeruginosa* replicates in the tobramycin-containing cocultures, it is possible that they were unable to evolve tolerance to tobramycin, compared to surviving replicates, and were thus competitively excluded from cocultures. Moreover, evolution of tolerance by *P. aeruginosa* did not restore competitive dominance, as surviving coculture replicates that evolved tobramycin tolerance most frequently coexisted with *S. maltophilia*. The most probable explanation for this was the cost of tobramycin tolerance, which resulted in reduced growth of evolved *P. aeruginosa* isolates and thus likely less intense competition between the two species, though other mechanisms cannot be ruled out. We found that antibiotic treatments containing tobramycin were more effective at driving competitive exclusion in the naïve PAO1 strain, suggesting that combination antibiotic treatments may be more effective at clearing *P. aeruginosa* than individual treatments. Of the antibiotic combinations, colistin and tobramycin together resulted in extinction of both strains of *P. aeruginosa*, adding support for their efficacy in clinic (15)⁠. This would be particularly important during early infection, as failure to eradicate *P. aeruginosa* can select for mutations that aid establishment of long-term chronic infections (76)⁠⁠. Together, our findings suggest that the efficacy of antibiotic combinations can be magnified in polymicrobial infections, leading to higher clearance of the target pathogen.

Competitive exclusion of *P. aeruginosa* differed between the two strains: the laboratory strain PAO1 was driven extinct in each tobramycin combination except for two CIP+TOB replicates, whereas the clinical strain LESB58 was only affected in the CST+TOB treatment. A number of different factors may have contributed to this outcome. Firstly, LESB58 had a relatively greater initial tolerance of ciprofloxacin and tobramycin (Figure S1), which could have reduced the negative effect of antibiotics in the CIP+TOB and triple antibiotic treatments compared to PAO1. The initial differences in antibiotic susceptibility could reflect contrasting evolutionary histories between these two strains. PAO1 is highly lab-adapted due to repeated culturing in lab media while LESB58, a transmissible strain isolated in 1988 from CF patients (43)⁠, is adapted to the CF lungs by producing more biofilm than PAO1 (77)⁠ and lacks motility (42)⁠. A further contributing factor is that LESB58 has a greater interbacterial competitive ability—producing greater amounts of competitive factors, such as pyocyanin and proteases, and secreting these earlier in its growth phases (78, 79)⁠—than PAO1, which is likely beneficial during polymicrobial CF infections when in competition with other bacteria (80, 81)⁠. LESB58 was thus likely pre-adapted to compete with other species such as *S. maltophilia*, which could have contributed to the lower frequency of extinctions. Finally, in our experimental setup the initial inoculant of LESB58 was three-fold greater than PAO1, which may have increased the likelihood early in the selection experiment that LESB58 survived in coculture treatments where PAO1 did not. Further work would be required to disentangle each of these possibilities.

In summary, while the presence of competitor did not affect the trajectory of antibiotic tolerance evolution in *P. aeruginosa,* we found that exposure to sublethal antibiotic concentrations led to more frequent extinctions of the target pathogen in the presence of an antibiotic resistant competitor. Specifically, tobramycin played a key role in this eco-evolutionary process where the negative effect of this antibiotic persisted despite *P. aeruginosa* tolerance evolution, likely due to the associated growth costs. Somewhat worryingly, *P. aeruginosa* populations often evolved increases in MIC to all antibiotics, leading to resistance against much higher concentrations of antibiotics than experienced during the selection experiment. In conclusion, our results suggest that differences in antibiotic susceptibility can magnify competition in bacterial communities, leading to changes in community composition. The efficiency of the antibiotic treatment is then determined by both the surrounding community as well as efficacy of delivery, choice of antibiotic, and antibiotic concentration, further complicating treatment design.

## Author Contributions

All authors conceived and designed the study, JPL collected and analysed the data, and all authors wrote the manuscript.

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## Conflicts of Interest

The authors declare that there are no conflicts of interest.

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# Tables

Table 1: Minimum inhibitory concentrations for the three ancestral bacterial strains, along with the experimental concentrations used of each antibiotic.

|  |  |  |
| --- | --- | --- |
| **Antibiotic** | **Treatment concentration (µg/ml)** | **Minimum inhibitory concentration (µg/ml)[treatment concentration as proportion of MIC]** |
| PAO1 | LESB58 | *S. maltophilia* |
| Ciprofloxacin | 0.03125 | 0.0625[1/2] | 1[1/32] | 0.125[1/4] |
| Colistin | 2 | 4[1/2] | 4[1/2] | 8[1/4] |
| Tobramycin | 0.5 | 1[1/2] | 2[1/4] | 8[1/16] |

# Figures

Figure 1: Methods schematic showing (A) the **combinations of antibiotic treatment and bacterial culture that were used during the selection experiment; and (B) the procedure followed during the selection experiment.**

Figure 2: Growth of each evolved *P. aeruginosa* replicate population in the treatment concentrations of antibiotic relative to their **growth without antibiotic**, measured in separate growth assays at the end of the selection experiment.

Panel columns show the two *P. aeruginosa* strains, while panel rows show growth in the presence of different antibiotics. Each point represents the mean growth in antibiotic for three technical replicates of one replicate population, minus the growth without antibiotic of the same replicate population (, as defined in Methods). Boxes show mean of all replicates (centre line; , as defined in Methods), and upper and lower limits represent ±SEM. The horizontal dashed line represents the of the ancestor. The solid dark grey line represents growth equal to that without antibiotic (i.e., relative change in OD600 = 0). Shapes show mono- (circle; ‘Mono’) and cocultures (triangle; ‘Co’); colours show antibiotic treatment, with lighter and darker shades representing the absence and presence of the *S. maltophilia* competitor. \* represents significant difference (*p* < 0.05) of antibiotic treatment to the “No Antibiotic” control treatment by post-hoc pairwise comparison. Cross tolerance between ciprofloxacin and tobramycin can be seen in panels A, B and E, F, comparing “No Antibiotic” treatment in columns 1, 2, to CIP and TOB treatments in columns 3, 4 and 7, 8. Cost of tobramycin tolerance can be seen in panels G, H, comparing “No Antibiotic” in columns 1, 2, to TOB treatments in columns 7, 8.

**Figure 3: Growth of each evolved *P. aeruginosa* replicate population without antibiotic relative to the respective ancestor, measured in separate growth assays at the end of the selection experiment.**

Panel columns show the two *P. aeruginosa* strains, while panel rows show growth in the presence of different antibiotics. Each point represents the mean growth without antibiotic for three technical replicates of one replicate population, minus growth of the ancestor in the same conditions (, as defined in Methods). Boxes show mean of all replicates (centre line; , as defined in Methods), and upper and lower limits represent ±SEM. The horizontal dashed line represents growth equal to that of the ancestor (i.e., relative change in OD600 = 0). Shapes show mono- (circle; ‘Mono’) and cocultures (triangle; ‘Co’); colours show antibiotic treatment, with lighter and darker shades representing the absence and presence of the *S. maltophilia* competitor. \* represents significant difference (*p* < 0.05) of antibiotic treatment to the “No Antibiotic” control treatment by post-hoc pairwise comparison.

**Figure 4: The MIC of the three individual antibiotics for each evolved replicate population of *P. aeruginosa* strains.**

Panel columns show *P. aeruginosa* strain and rows the MIC of each antibiotic. The dashed line represents the MIC of the respective ancestors; the grey line shows the MIC50 of each treatment across replicate populations, as defined in the methods; the dashed blue line shows the treatment concentration. The size of each point represents the number of replicates with the specified MIC. Colour of the points represents treatment; white centre dot represents monoculture and black represents coculture. Number of extinctions in each treatment and coculture shown beneath the X-axis. MIC was measured in triplicate for each replicate. \* represents significant difference (*p* < 0.05) of antibiotic treatment to the “No Antibiotic” control treatment by post-hoc pairwise independence test. Cross tolerance between ciprofloxacin and tobramycin can be seen in panels A, B and E, F, comparing “No Antibiotic” treatment in columns 1, 2, to CIP and TOB treatments in columns 3, 4 and 7, 8.

Figure 5: **Optical density of** bacterial populations and composition of cocultures at the final timepoint of the selection experiment.

**(A–D)** Boxplots of optical density of bacterial populations (OD600) of each replicate population from eight treatments (see legend for boxplot fill colours). Panel columns show the *P. aeruginosa* strain, rows monocultures or cocultures. Points represent individual replicates; N = 6. Shapes show species present at the final timepoint: *P. aeruginosa* as circles, *S. maltophilia* as diamonds, and both as red squares. **(E–F)** The presence of surviving species in each coculture replicate (N = 6). Colours represent the surviving species as follows: *P. aeruginosa* in orange, *S. maltophilia* in blue, and both in red.