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Cross-Feeding Does Not Affect the Evolution of Antibiotic Tolerance

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Abstract: Antibiotic tolerance refers to the ability of bacteria to survive short periods of high antibiotic concentration. This is distinct from antibiotic resistance, the ability of bacteria to survive indefinitely under a limited antibiotic concentration. Emerging evidence suggests antibiotic tolerance may be supported by increases in lag time, a delay before growth that can be quantified by the time taken for colonies to become visible. While tolerance is studied less than resistance, ecological influences from between-species interactions upon it are even less frequently studied. Cross-feeding is one such interaction, and a common occurrence in nature. To investigate the impact of cross-feeding on tolerance, the evolution of antibiotic tolerance was induced in both *Escherichia coli* monoculture and a mutualistic coculture of *E. coli* and *Salmonella enterica.* Cyclic exposure to ampicillin followed by growth in antibiotic-free media yielded evolved isolates of *E. coli* with delayed colony appearance time but no change in growth rate, minimum inhibitory concentration (MIC), or competitive advantage against the ancestral strain. However, the rate of tolerance evolution was unaffected by culture type. Additionally, a computational population model was able to replicate culture-independent tolerance evolution when lag time was the only mutable trait. Antibiotic tolerance appears to be uniquely unaffected by species interactions, demonstrating the need for ecological considerations in the study of tolerance and resistance.

Introduction

Among the many challenges in medical biology, antimicrobial resistance (AMR) is a pressing threat. The discovery of antibiotics in the 20th century marked a leap in the progress of human medicine, as well as the beginning of a microscopic war. Evolutionary pressures have led pathogenic microbes to develop defense mechanisms against drugs, reducing the impact of treatments and elevating the risk of infection. Analysis of global literature, hospital systems, and other sources revealed an estimated 5 million AMR-associated deaths in 2019[2]. The

economic consequences of AMR could be revealed in coming years in the form of increased poverty due to heightened treatment costs and longer hospital stays[3]. The World Health Organization released the first list of urgently threatening antibiotic-resistance species in 2017[4], calling for action as AMR evolution observably accelerates, with some species developing resistance within a year of antibiotic exposure[5].

There are many mechanisms that microbes use to overcome the effects of antibiotics. From preventing initial uptake through the lack of certain binding proteins to generalized membrane channel-blocking by efflux pumps[6], the quantity and diversity of microbes have allowed them ingenious ways of antibiotic neutralization. Under AMR there are sub-categories of mechanisms to combat antibiotics, known as antibiotic resistance and antibiotic tolerance[7]. While resistance generally refers to the ability of bacteria to survive indefinitely under a certain concentration of antibiotic, tolerance refers to their ability to survive short-term exposure to high, lethal levels of antibiotic concentrations. Resistance is the more studied of the two, but tolerance is comparably important for various reasons. First, laboratory[7] and clinical[8] trials reveal that not only does tolerance usually develop quicker than resistance, but it facilitates evolution of the latter. Secondly, the methodology of typical prescription antibiotic treatment involves cyclical exposure to high antibiotic concentrations and thus mimics conditions that facilitate tolerance[9].

Among antibiotic resistance and tolerance studies, a major caveat is that most are conducted on single species in monoculture conditions, overlooking the reality that bacteria live in microbial communities rife with ecological interactions. Cross-feeding is an interaction common in nature, in which species participate in a mutualistic exchange of nutrients due to natural auxotrophy[10]. The ability of a microbe to acquire nutrients it cannot metabolize and the alleviation of its burden to produce them are factors that result in enhanced growth while cross-feeding[11]. However, interdependency may force microbes to essentially wait for each other when evolving antibiotic resistance, a process known as a "weakest-link hypothesis"[1]. In accordance with this hypothesis, resistance was observed to evolve slower in a mutualistic *E. coli* and *S. enterica* coculture compared to monoculture with *E. coli* only[1]. The effects of cross-feeding on antibiotic tolerance are unknown.

In 2014, Fridman et al. elucidated a possible mechanism for antibiotic tolerance known as tolerance by lag[12]. The "lag" phase of microbial growth is relatively understudied, but assumed to be a period of adjustment to a new environment which precedes the exponential growth of "log" phase[13]. Growth of bacterial colonies is not visible until the cells have exited the lag phase. Fridman et al. discovered that increasing the length of time of high antibiotic exposure led *E. coli* to evolve longer lag times to match, which improved their survival[12]. This suggests lag time is an underlying mechanism unique to tolerance, which may be impacted differently than resistance mechanisms in the presence of cross-feeding. In this paper, tolerance evolution both *in* and *ex silico* is

compared across *E. coli* monoculture and *E. coli* and *S. enterica* coculture (Fig. 1).

Results

To determine how cross-feeding affects antibiotic tolerance, methionine-auxotrophic *E. coli* either in monoculture or in cross-feeding mutualism with *S. enterica* was exposed to high concentrations of ampicillin (100 μg/mL) in shaken liquid conditions (Fig. 2A). Samples were taken over time and the fraction of *E. coli* remaining from the original amount was calculated. Overall, tolerance measured after 5-hour treatment with ampicillin yielded no significant difference between monoculture and coculture (One-way ANOVA, p=0.332), suggesting that cross-feeding does not influence the level of antibiotic tolerance.

To study how cross-feeding affects the evolution of antibiotic tolerance over time, an evolution experiment was conducted. Following a previously established protocol[12,23] (Fig. 2A), *E. coli* in monoculture and coculture were grown to the stationary phase. The bacteria were then diluted and cyclically exposed to ampicillin (100 μg/mL) for 5 hours, washed twice in saline solution and allowed to grow for 42-hours in antibiotic-free liquid media. At the end of each cycle, half of the culture was frozen for characterization, and the remaining half carried into subsequent cycles of antibiotic exposure and antibiotic-free growth. Survival fraction at 5 hours, calculated as the ratio of final cell density after 5 hours of antibiotic treatment to the cell density of untreated colonies, was used to quantify the level of antibiotic tolerance. Over 10 cycles, tolerance increased greater than 100-fold for both monoculture and coculture (Fig. 2C). There was no significant difference in rate of tolerance evolution between the cultures (One-way ANOVA, p=0.952) (Fig. 2B).

To test for convolution via concurrent evolution of antibiotic resistance, cycle-10 evolved *E. coli* were exposed to ampicillin to determine MIC. Definitively, *E. coli* was not found to be resistant to ampicillin in either monoculture or coculture (Student's t-test, p=0.421 for both) (Fig. 2B). These results confirm that tolerance was the primary mechanism behind the increased survival of *E. coli* to ampicillin in this experiment.

To determine the mechanism of tolerance, evolved *E. coli* isolates drawn from the end populations of the evolutionary experiment, as well as a wildtype isolate, were subjected to quantifications of tolerance, resistance, growth rate, lag time, and fitness. A significant increase in tolerance, measured in survival fraction after 5 hours of treatment, was observed in the evolved isolates compared to the wildtype ancestor (One-way ANOVA, p=0.00253) (Fig. 2C). In contrast, no significant increase in resistance, measured as MIC, was observed (One-way ANOVA, p=0.952).

Results from this experiment demonstrate that antibiotic tolerance is not caused by slowed growth. Only one coculture isolate showed a significant decrease in growth rate compared to the ancestor strain (Pairwise one-way ANOVA, p=0.0163), and all other strains did not (Pairwise one-way ANOVA, p>0.0758) (Fig. 2C). One the other hand, lag time appeared to play an important role in the evolution of tolerance in *E. coli*. All evolved isolates exhibited a shift toward later appearance

times (Fig. 2C) compared to the ancestor strain. A competition assay revealed that evolved *E. coli* did not exhibit higher fitness than the ancestor strain when plated with kanamycin (Fig. 2C), supporting the idea that lag time is the primary mechanism behind antibiotic tolerance.

Computational Model

The processes of the computational model of the tolerance evolution experiment are based closely on the liquid experiment procedure, though some parameters differ, such as initial resource ratios and relative initial cell abundances. Most importantly, bacteria *in silico* are only allowed to mutate by increasing or decreasing their lag time (though not below 1 hour, a baseline set to prevent mathematical complications). The results of the model replicate that of the liquid experiment; there was no significant difference in the rate of tolerance evolution measured as either MDK99 or 5-hour survival fraction (One-way ANOVA, MDK99: $p=0.609$, 5-hour survival fraction: $p=0.224$) (Fig. 3). This suggests that the mechanism of lag time, isolated from other factors, can be modeled in a way that accurately reproduces the evolution of bacterial tolerance.

Discussion

Despite the attention antimicrobial resistance has garnered, the fast-developing mechanism of antibiotic tolerance receives much less attention than antibiotic resistance. This study sought to examine tolerance through the impact of cross-species interactions, a factor often overlooked in laboratory studies. The results of this study revealed that a cross-feeding mutualism between *E. coli* and *S. enterica* does not impact antibiotic tolerance compared to *E. coli* in monoculture. These results are contrary to the codependence that has been observed regarding antibiotic resistance (MIC), in which cocultures developed resistance at a slower rate than monocultures[1].

In addition, the role of lag time in tolerance was confirmed by shifts in appearance time for tolerance-evolved isolates without changes in growth rate, resistance, or fitness on kanamycin. A simple computational model replicated the results of culture-independent tolerance evolution by simulating mutational variation in lag time. This provides additional evidence pointing to lag time as the underlying mechanism for antibiotic tolerance. Resistance in coculture appears to follow a theorized "weakest-link hypothesis"[1], but the mechanism of extended lag time may bring tolerance out of that dependency by temporarily reducing nutrient reliance. During antibiotic exposure, rather than depending on their struggling mutualism partners for nutrient provision, lagging cells pause their nutrient needs along with their growth. The results of this study provide an ecological perspective on AMR, and confirm that antibiotic tolerance is a mechanism of microbial pushback that must be studied separately from antibiotic resistance.

Methods

Hypho Minimal Media

E. coli and *S. enterica* were grown in Hypho minimal media[14] containing phosphate, nitrogen, and sulfate salt solutions, and varying carbon sources based whether they were in monoculture or coculture[15]. Phosphate salt solution (145.2 mM K_2HPO_4 and 187.5 mM NaH_2PO_4) and sulfate salt solutions (37.8 mM

$(NH_4)_2SO_4$ and 8.1 mM $MgSO_4$) were autoclaved in preparation. Monoculture medium contained lactose (2.78 mM) and methionine (0.08M) carbon sources. Coculture medium contained lactose (2.78 mM) as the only carbon supplementation. Henceforth, when media is mentioned in this paper, it is implied that it is Hypho minimal media containing the carbon sources respective to the type of culture grown in it.

Bacterial Strains

The strain of *Escherichia coli* used in the liquid experiments was engineered by Harcombe et al. from the methionine-auxotrophic (ΔmetB knockout) K-12 strain (BW25113) of the Keio collection[16]. It is an Hfr strain incorporating a lac operon through bacterial conjugation, which grants it the ability to metabolize lactose. As a result, this strain can grow in Hypho minimal media as a monoculture with methionine supplementation, or in mutualism (coculture) with a methionine-secreting *S. enterica*. The cooperating *Salmonella enterica* LT224 strain was a previously characterized synthetic mutant evolved to secrete methionine for cross-feeding with *E. coli*[17] (Fig. 1). Both strains were labeled with fluorescent proteins through genetic transduction; the *E. coli* was labeled with cyan fluorescent protein and the *S. enterica* with yellow fluorescent protein.

Antibiotic Tolerance Evolution Experiment in Liquid

To understand the effect of cross-feeding on antibiotic tolerance evolution, an experiment was conducted in liquid media using methods developed in Fridman et al.[12]. *E. coli* in monoculture and in coculture were grown to the stationary phase overnight in antibiotic-free liquid media. 500µl of the culture was transferred to 10mL of fresh media, which was exposed to 100 µg/mL ampicillin for 5 hours at 37℃ in shaking incubator. Cultures were then washed two times with saline via high-speed centrifugation (4700 RPM for 20 min) and resuspension, then transferred into a 1 mL of ampicillin-free media for ~42 hours to grow. Of the culture, 500 µl was frozen at -80 ℃, and the other 500 µl transferred to 10 mL of ampicillin-containing media to repeat 5-hour treatment. Ten of these cycles of ampicillin treatment, washing, and freezing/transfer were performed. Similarly, a negative control experiment was conducted wherein each cycle, only 10 µL of bacterial culture was transferred into fresh media and the rest frozen for future characterization[12] (Fig. 2).

Survival Curve in Liquid

To construct a survival curve for each culture type, wild-type *E. coli* in monoculture and coculture were first grown to stationary phase overnight in antibiotic-free media. Subsets of each culture were transferred to 10 mL of fresh antibiotic-free media in a shaking incubator for 1 hour at 37 ℃, before the addition of 100 µg/mL ampicillin. At various time points after ampicillin addition (0 min, 40 min, 80 min, 2 h, 5 h, 24 h), serial dilutions of each culture were plated on agar with lactose and agar with galactose to promote growth of *E. coli* and *S. enterica* colonies, respectively. Colonies for each serial dilution were counted after a ~48-hour incubation period at 37 ℃ and used to calculate the survival fraction at all six

time points. A logistic kill curve was fitted to the data.

Antibiotic Tolerance

To quantify antibiotic tolerance, survival fractions were measured for evolved samples. Frozen isolate end samples and each cycle's populations were incubated in media overnight at 37 ℃. After cultures reached the stationary phase, they were treated with 100 ug/mL ampicillin in fresh media for 24 hours. *Survival Curve in Liquid* methodology was used on all samples to construct survival curves over a 24-hour period and quantify survival fraction.

Antibiotic Resistance

Minimum inhibitory concentration (MIC) was assessed per cycle for monoculture and coculture populations. Samples of each culture were diluted to a density of OD600=0.001 per well in a 96-well plate with a 2-fold ampicillin concentration gradient (from 100ug/mL to 0.098ug/mL) in Hypho media which allowed for *E. coli* growth only. Samples were incubated at 37 ℃ for 24 hours, and MIC for each sample was defined as the lowest ampicillin concentration at which turbidity (growth) was absent[18]. This test was performed in two repetitions. The rate of evolution of antibiotic resistance was measured as the slope of log-transformed MIC change in respect to cycle.

Population-level Growth Rate

Population growth curves were determined from fluorescence signaling. The constitutively expressed cyan fluorescence protein in E. coli and the yellow fluorescence protein in S. enterica were measured as an indicator of growth in each species. Samples were grown overnight to log phase at 37 ℃ and transferred to fresh liquid media. Using a continuous shaking microreader plate (TECAN Tradings, Switzerland), fluorescence signaling of cyan and yellow fluorescence proteins were measured every 20 minutes for 250 cycles at an excitation wavelength of 430 and 500 nm and emission wavelength of 490 and 530 nm respectively.

Appearance Time and Growth Rate

Time-lapse imaging was used to measure bacterial appearance time. *E. coli* isolates from end populations of evolved liquid monoculture (XY004-006) and coculture (XY001-003) were grown in monoculture and coculture conditions. At 37 ℃, 100-200 colonies of *E. coli* from either culture were spread on agar. An Epson Perfection V600 Photo office scanner (Epson America Inc, CA) was programmed to take hourly images of the agar plate. Using programs similar to that of Levin-Reisman et al.[7] and Chacón et al.[19], the computer was able to distinguish *E. coli* and *S. enterica* morphologically. Colony area size was measured over time using pixelated units. Appearance time was measured as the earliest incubation duration at which the colony area became non-zero. Growth rate was measured by fitting a log-linear line to a growth curve obtained in liquid culture following previous work[20].

Competitive Advantage

To determine why antibiotic-surviving strains have high frequency, a competition assay was run by competing isolates against their ancestor strain. With change in frequency in the population as a measure of fitness, the

mutualistic *E. coli* ancestor strain (WRH224) and 6 end population isolates (XY001-006), all fluorescent and kanamycin-resistant, were each grown with a non-fluorescent, kanamycin-sensitive *E. coli* strain (WRH221) at OD=0.001. These samples were plated on LB plates both with and without kanamycin. Using the non-fluorescent strain as a control, CFU/mL was measured to determine the change in frequency within the population in the presence/absence of kanamycin as a ratio to the non-fluorescent strain.

Computational Model for the Antibiotic Tolerance Evolution Experiment

The antibiotic tolerance experiment in liquid was modeled computationally in Python, using ordinary differential equations for each strain of each species (*E. coli, S. enterica*) and the three resources (methionine, lactose, acetate):

$$\frac{dE_{gi}}{dt} = (1-\alpha) r_E \left(\frac{M}{M+K_M}\right)\left(\frac{L}{L+K_L}\right) E_{gi} - \kappa_E E_{gi} + \frac{E_{li}}{\tau_{Ei}}$$

$$\frac{dE_{li}}{dt} = \frac{-E_{li}}{\tau_{Ei}}$$

$$\frac{dS_{gi}}{dt} = (1-\alpha) r_S \left(\frac{A}{A+K_A}\right) S_{gi} - \kappa_S S_{gi} + \frac{S_{li}}{\tau_{Si}}$$

$$\frac{dS_{li}}{dt} = \frac{-S_{li}}{\tau_{Si}}$$

$$\frac{dM}{dt} = p_M r_S \left(\frac{A}{A+K_A}\right) \sum S_{gi} - c_M \left(\frac{M}{M+K_M}\right)\left(\frac{L}{L+K_L}\right) \sum E_{gi} - \kappa_M M$$

$$\frac{dL}{dt} = -c_L \left(\frac{M}{M+K_M}\right)\left(\frac{L}{L+K_L}\right) \sum E_{gi} - \kappa_L L$$

$$\frac{dA}{dt} = p_A r_E \left(\frac{M}{M+K_M}\right)\left(\frac{L}{L+K_L}\right) \sum E_{gi} - c_A \left(\frac{A}{A+K_A}\right) \sum S_{gi} - \kappa_A A$$

E and S are the abundances (cell units) of *E. coli* and *S. enterica* while M, L and A are the abundances (cell-equivalents/mL, or density of cells a unit of resource can produce) of methionine, lactose, and acetate, respectively. For each of *i* strains of bacteria emerging from mutation, there exists a growing and lagging population, notated by subscript *g* or *l*. The death constant α determines the rate of cell death relative to growth rate, which is determined by maximum growth rate *r* times resources limited by half-saturation constants *K* (e.g. K_M) times the current growing population *Eg/Sg*. In each cycle of the simulation, bacterial populations underwent a Phase 1 of rapid death (α = 3; death occurs twice as fast as growth does in antibiotic-less conditions) to simulate antibiotic exposure, followed by a Phase 2 of growth with new resources and no antibiotic exposure, and finishing with random mutation to produce new strains with differing lag times. Half of the cells and nutrients from each cycle are transferred to the next cycle, to replicate the process of transferring half of a growth solution into a new well of liquid. Default parameter values and parameter sources are listed in Supplementary Table 1.

Two measures of tolerance were observed: Minimum Duration of Killing of 99% of the *E. coli* population (MDK99) and the fraction of *E. coli* remaining of its original population after 5-hour exposure to antibiotics (5-hour survival fraction). To calculate MDK99, separate code constructed a 1000-cell population for each cycle and repetition of the original simulation, made up of all the strains in their relative frequencies at the end of that cycle. This model population was subjected to a Phase 1 (death under antibiotic growth) until 1% of the original *E. coli* population remained. Hours taken until this point were recorded.

To calculate the 5-hour survival fraction, the same 1000-cell population was constructed, but subjected to only 5 hours of Phase 1, and the

fraction of *E. coli* remaining of its original population was recorded.

Acknowledgement

This work is jointly supervised by Xianyi Xiong and Dr. William R. Harcombe at the University of Minnesota BioTechnology Institute. This work was supported by the National Institutes of Health R01-GM121498 to Will R. Harcombe.

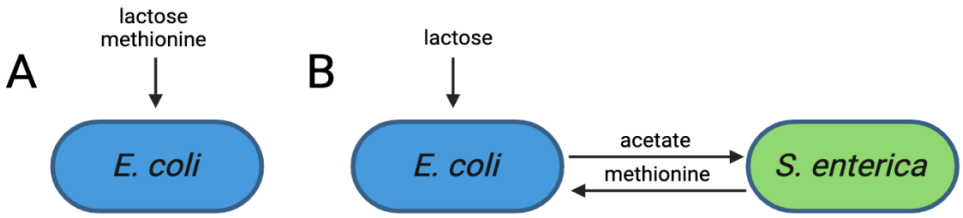


Figure 1. **Cross-feeding experimental set-up. A** *E. coli* monoculture experimental set-up with lactose and methionine supplementation. **B** *E. coli* and *S. enterica* experimental coculture cross-feeding set-up with only lactose supplementation.

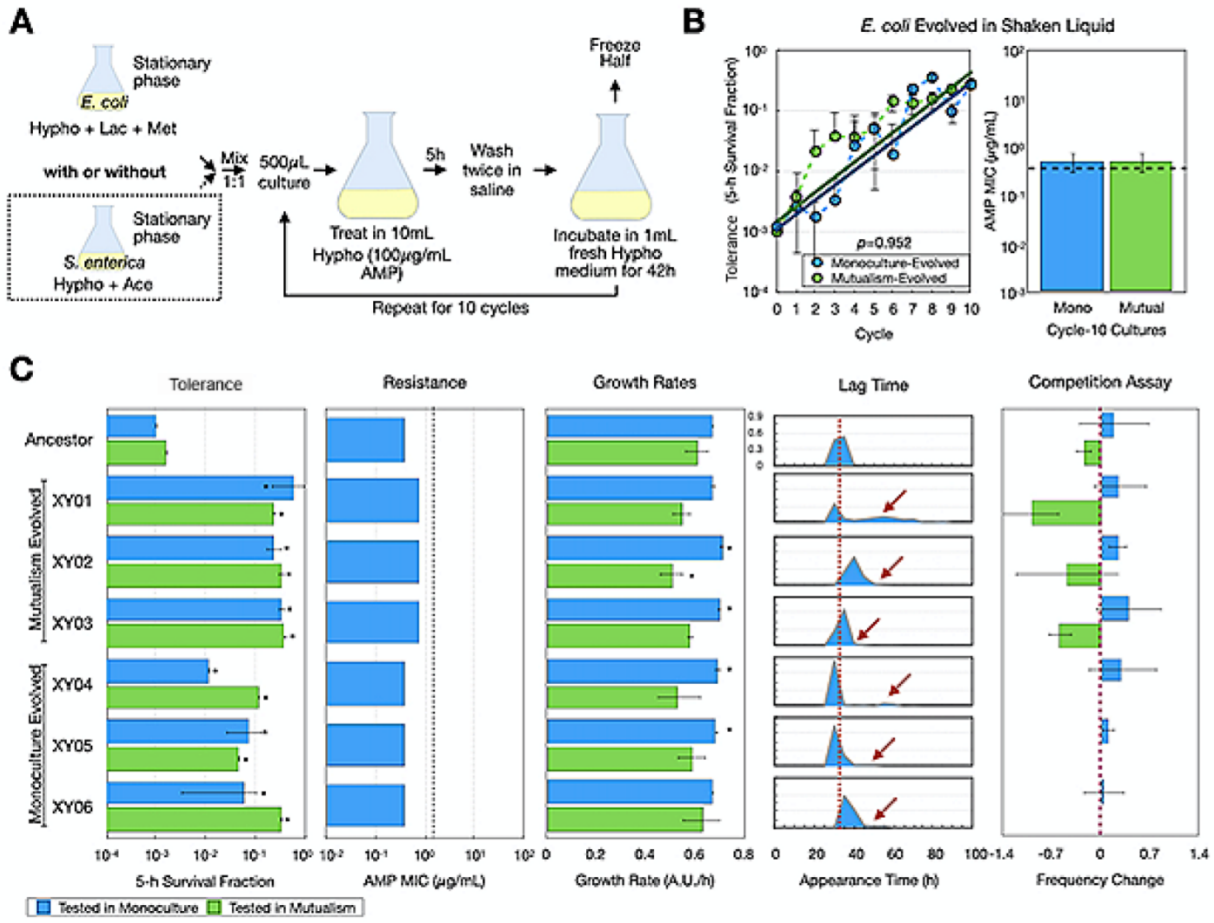


Figure 2. **Longer Lag Time is an Underlying Mechanism for Tolerance Evolution in Liquid Monoculture and Coculture. A** Schematic of the Tolerance Evolution in Liquid experiment. *E. coli* and/or *S. enterica* was grown to stationary phase, treated with ampicillin for 5 hours, washed to remove antibiotics, then transferred to fresh media for 42 hours. Each cycle, half of this grown culture was exposed to the next cycle of ampicillin treatment and the other half was frozen. **B** Greater than 100-fold increased relative population tolerance (5-hour survival fraction) over 10 consecutive cycles of exposure to antibiotics. No significant difference in the rate of tolerance evolution between monoculture and

coculture/mutualism populations (One-way ANOVA, p=0.952, n=3 biologically independent evolution experiments). Tolerance-evolved *E. coli* did not evolve to be resistant to ampicillin in either monoculture or mutualistic coculture conditions (T-test, p=0.421 for monoculture and coculture). Error bars indicate one standard deviation from the mean. Ancestral MIC is denoted by black dashed line. **C** Isolate tolerance, resistance, growth rate, lag time and competitiveness were tested from end populations of mutualistic coculture (XY01-03) and monoculture (XY04-06). All isolates showed a significant increase in tolerance and no significant increase in resistance. Black dashed lines denote 4x the ancestral MIC. Isolates were generally not found to have decreased growth rates. Isolates showed right-ward skewed lag time, with red dashed lines denoting mean lag time, and red arrows showing a fraction of cells with longer lag times. No increased fitness when competing against a kanamycin-sensitive ancestral strain. Error bars show standard deviations from 3 biological replicates. The "*" symbols indicate p<0.05 in pairwise one-way ANOVA tests with comparable data for the ancestral *E. coli* strain.

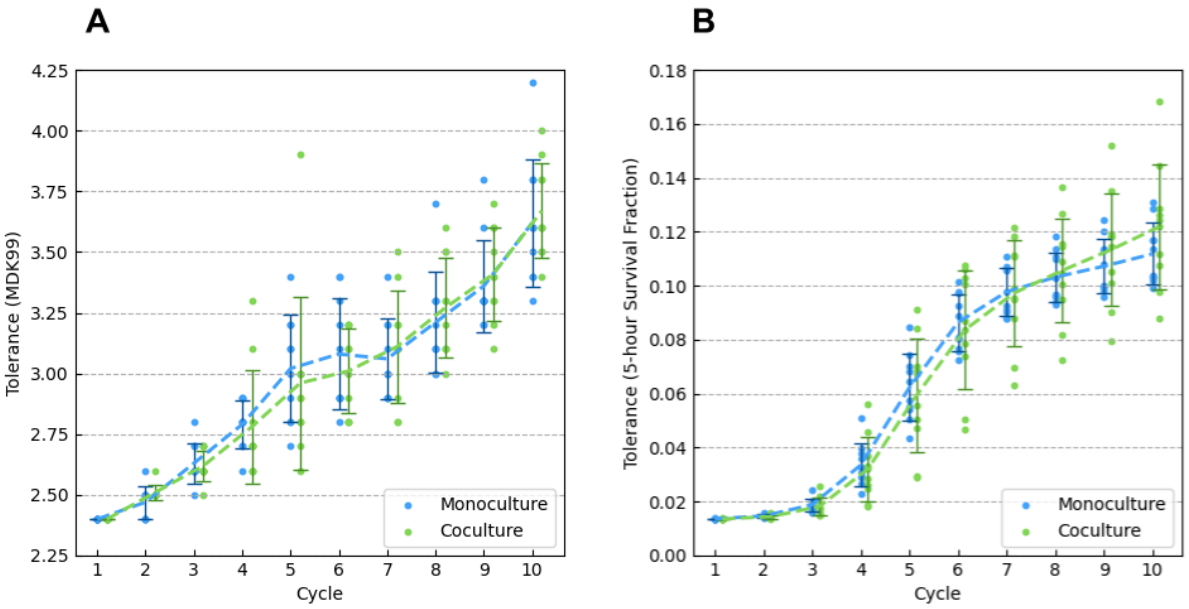


Figure 3. **Computational Model, Impact of Culture Type on Tolerance by Cycle** Default values for resources were: methionine: 1000 (monoculture) or 1 (coculture), lactose: 1000, acetate: 0. Error bars represent one standard deviation from the mean, and the means over all repetitions are represented by dashed lines. **A** No significant difference between cultures with *E. coli* MDK99 as the tolerance metric (one-way ANOVA, p=0.431). **B** No significant difference between cultures with *E. coli* 5-hour survival fraction as the tolerance metric (one-way ANOVA, p=0.235).

Supplemental Information

Model Robustness

Parameters for the computational model of the tolerance evolution experiment were varied to evaluate the effect on the model-produced rate of the antibiotic tolerance, measured as the slope of the log-transformed 5-hour *E. coli* survival fraction over 10 experimental cycles.

Initial Resources

Initial resources refer to the amounts of methionine, lactose, and acetate provided in "fresh media" at the beginning of each Phase 1 (simulated antibiotic exposure for 5 h) and Phase 2 (simulated antibiotic-free growth for 42 h), though half of Phase 2 resources are carried into the next cycle's Phase 1. In the following robustness tests, initial acetate was always maintained at 0 and coculture methionine at 1 to imitate the evolution experiment in liquid (while in the liquid experiment, coculture contains no initial methionine at all, *in silico* a value of 1 is used to emulate *S. enterica* "kicking off" the mutualism as happens in nature). Varying initial methionine yields a clear division of impact: while up to a value of about 20 units, methionine was directly and positively correlated with the rate of tolerance evolution, above 20 units, evolution rate stagnated (Supplementary Fig. 1A). For initial lactose, this plateau occurs at about 250 units (Supplementary Fig. 1B). The values of methionine: 1000 and lactose: 1000 used in the computation model fall within the plateau range.

Post-Phase 2 Mutation

In the computational model, mutation is a simplified process occurring all at once at the end of Phase 2 in a cycle, based on a set mutation rate and the current cell population. In mutation, singular cell units are selected to mutate before half the entire population is transferred to the next cycle, where it immediately enters Phase 1. Newly mutated cells, despite being present in an abundance of 1, do not go extinct in Phase 1 even if their lag time is shorter than the duration of the phase - that is, they immediately die by antibiotic exposure upon emerging from the lag phase. This is due to the differential equations that make up the model, in which death rate is proportional to current population size.

To show that the simplified mechanism of mutation does not impact evolution of tolerance, a "Phase 0" was introduced, in which cells were allowed to grow in the absence of antibiotic between each mutation phase and Phase 1 of the next cycle. Varying the length of this pre-antibiotic growth phase did not appear to impact the rate of tolerance evolution (Supplementary Fig. 2). This suggests that the immediate repression of new mutant strains does not affect their overall relative frequencies nor the eventual dominance of strains with longer lag times.

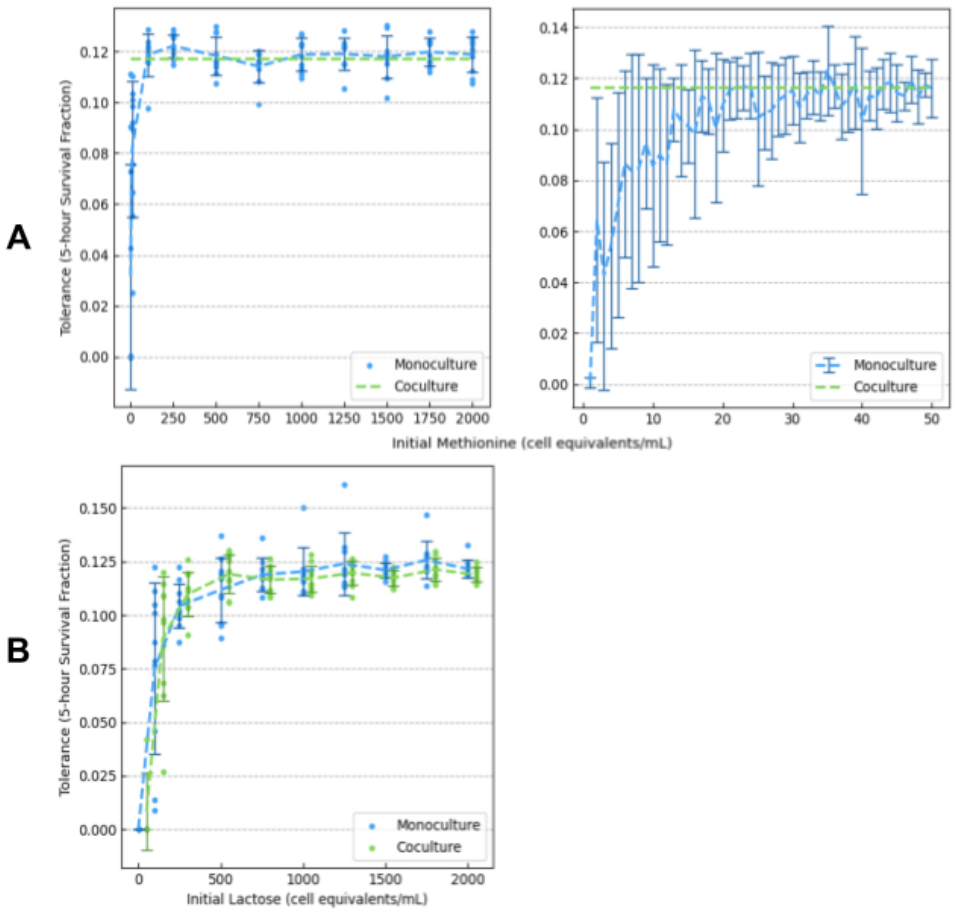
Parameter	Unit	Value	Biological Interpretation	Source

α	Unitless	phase 1: 3 phase 2: 0	Non-decay death constant	
r_E	h^{-1}	1	*E. coli* maximum growth rate	Hammarlund et al. (2021)
K_M, K_L, K_A	Cell unit/mL	1	Half-saturation methionine, lactose, acetate concentration for bacterial growth	Hammarlund et al. (2021); Xiong et al. (2023)
κ_E, κ_S, κ_M, κ_L, κ_A	h^{-1}	5E-09	Natural decay rate of *E. coli*, *S. enterica*, methionine, lactose, acetate	Xiong et al. (2023)
τ_E, τ_S	h	-	Partially randomly generated lag time of *E. coli*, *S. enterica*	
r_S	h^{-1}	0.5	*S. enterica* maximum growth rate	Hammarlund et al. (2021)
p_M	Unitless	1.56	Production rate of methionine by *S. enterica*	Hammarlund et al. (2021); Xiong et al. (2023)
p_A	Unitless	1.01	Production rate of acetate by *E. coli*	Hammarlund et al. (2021); Xiong et al. (2023)
c_M	h^{-1}	0.1	*E. coli* consumption of methionine	Hammarlund et al. (2021); Xiong et al. (2023)
c_L	h^{-1}	1.0	*E. coli* consumption of lactose	Hammarlund et al. (2021); Xiong et al. (2023)
c_A	h^{-1}	1.0	*S. enterica* consumption of acetate	Hammarlund et al. (2021); Xiong et al., (2023)

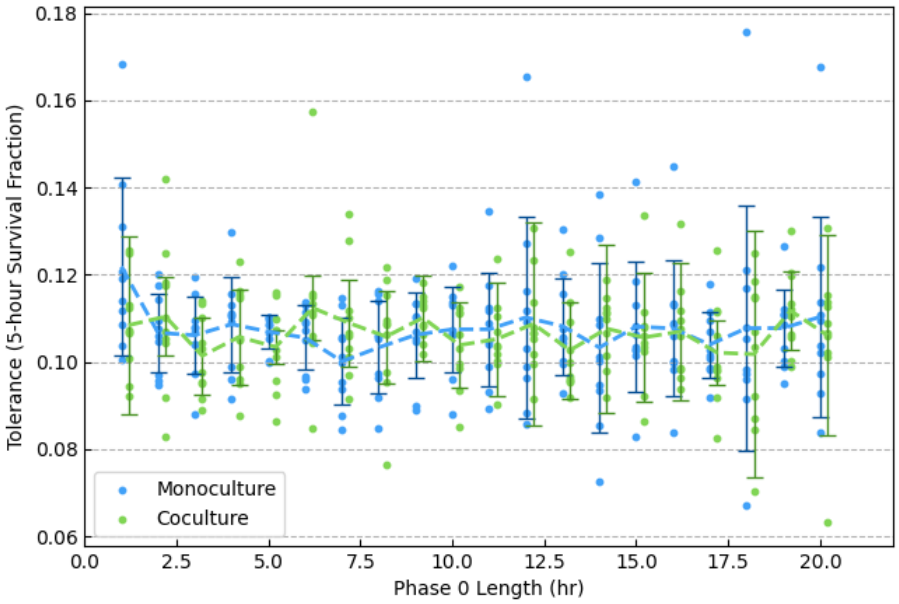
***Supplementary Table 1A*. Equation Constants.**

Parameter	Unit	Value	Biological Interpretation
init_n	Arbitrary unit of cell abundance as (E, S)	co: (5, 5) mono: (10, 0)	Initial population at beginning of simulation's current repetition
init_lag	h	1.0	Initial lag time for ancestor strain
n_cycles	Cycle	10	Number of cycles in the current repetition
init_R	Arbitrary unit of nutrient abundance as (M, L, A)	co: (1, 1000, 0) mono: (1000, 1000, 0)	Nutrient abundances in growth medium
μ	Cell unit/mL	0.003	Mutation rate
max_lag_change	h	1.1	Multiplied by a random proportion to produce the difference in lag time of a mutant from its ancestor

Supplementary Table 1 B. **Experiment Parameters.**



***Supplementary Figure 1*. Computational Model, Impact of Initial Methionine or Lactose on 5-hour Survival Fraction** The measure of tolerance is the slope of the regression line of the log-transformed 5-hour survival fractions for each cycle. Default values for initial resources, when not varied, were: methionine: 1000 (monoculture) or 1 (coculture), lactose: 1000, acetate: 0. Error bars represent one standard deviation from the mean, and the means over all repetitions are represented by dashed lines. **A** Coculture methionine was not varied to remain true to the liquid experiments. The single value of coculture tolerance at initial methionine: 1 is represented as a line for comparison purposes. Left: methionine values from 1 to 2000. Right: methionine values from 1 to 50 in increments of 1. Positive, logarithmic correlation was observed with methionine in monoculture until a plateau at about 20 units. **B** Positive, logarithmic correlation was observed with lactose in both cultures until a plateau at about 250 units.



Supplementary Figure 2. **Computational Model, Impact of Phase 0 on 5-hour Survival Fraction** Phase 0 length refers to the amount of time cells spend in an antibiotic-free pre-Phase 1 (antibiotic exposure) period of growth, with re-initialized nutrients, following the last cycle. Longer Phase 0 times allow newly mutated cells a greater opportunity to proliferate before antibiotic exposure. Error bars represent one standard deviation from the mean, and the means over all repetitions are represented by dashed lines. Phase 0 length does not impact 5-hour survival fraction.

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