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(Article begins on next page)
Selection for Antibiotic Production

A dissertation presented
by
Ylaine Gerardin
to
The Committee on Higher Degrees in Systems Biology

in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy
in the subject of
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Selection for Antibiotic Production

Abstract

Antibiotic-producing microorganisms can gain a selective advantage by inhibiting nearby competing species. However, despite their genetic potential, natural isolates often make only small amounts of antibiotics and laboratory evolution can lead to loss rather than enhancement of antibiotic production. We sought to understand selection for antibiotic production in natural and engineered ecosystems. We show that, due to competition with antibiotic resistant cheater cells, increased levels of antibiotic production can actually decrease the selective advantage to producers. Competing fluorescently-labeled *Escherichia coli* colicin producers with non-producing resistant and sensitive strains on solid media, we found that while producer colonies can greatly benefit from the inhibition of nearby sensitive colonies, this benefit is shared with resistant colonies growing in their vicinity. Experimentally varying the amount of production shows a peak in selection for producers, reflecting a trade-off between benefit gained by inhibiting sensitive competitors and loss due to an increased contribution to resistant cheater colonies. A simple model, which accounts for such local competitive and inhibitory interactions, recapitulates the finding that the advantage of producers varies non-monotonically with the amount of production. These results help explain the low level of antibiotic production observed for natural species, and can help direct laboratory evolution experiments selecting for increased or novel production of antibiotics.
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To my friends and family.
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Microbial species naturally produce a variety of antibiotics. These diverse compounds range from sub-kilodalton small molecules to large proteins, but are functionally classified together due to their ability to kill or inhibit the growth of other microbial species or strains. Most of the antibiotic drugs used clinically are natural products (such as kanamycin and vancomycin) or semi-synthetic derivatives (such as ampicillin and doxycycline). Natural product antibiotics have been discovered through screening microbial isolates, and many more such compounds likely remain to be discovered. However, as the most commonly produced antibiotics have already been found, higher and higher-throughput screens are required to discover novel compounds. One way to bypass the need for screening individual isolates would be to harness the power of laboratory evolution to automatically select for increased antibiotic production. However, the conditions under which increased antibiotic production is a selective benefit are not well-understood.

Despite the ubiquity of antibiotic production, it remains unclear when toxicity towards other microbial species is an evolutionarily advantageous strategy. It is often assumed that antibiotics are used in ‘microbial warfare’ against competing strains, as the ability to inhibit nearby species could give a producer a competitive advantage. However, natural producers do not always maximize their production potential. Whole-genome sequencing of bacterial species known to produce antibiotics has revealed the presence of dozens of ‘cryptic’ gene clusters with sequence and structure resembling known biosynthetic gene clusters, but with no observed metabolic product. Efforts to
induce expression of these genes via external chemical or biological factors $^{82,70}$ and in heterologous hosts $^{35}$ have shown that some of these genes are indeed capable of producing novel compounds. In addition, metabolic engineering of natural species via modification of gene expression can increase production of antibiotics several-fold without significant reduction in growth $^{12,63}$. Given this potential, why are microbial species not producing greater amounts of antibiotics?

It is may be true that higher production levels are costly in nature, or that antibiotics are produced at small amounts not for inhibition of competitors but because they instead function as signaling molecules at sub-inhibitory concentrations $^{51,3}$. For example, sub-inhibitory levels of membrane pore-forming antibiotics have been shown to induce biofilm formation in *Bacillus subtilis* via activation of a potassium-sensing regulatory circuit $^{42}$. These antibiotics included surfactin, which is produced by *B. subtilis* itself, as well as natural products of other soil-dwelling species: the non-inhibitory properties of antibiotics may be intra- or inter-species. Another example of antibiotics that may have benefits unrelated to toxicity are the phenazines made by *Pseudomonas aeruginosa*. These molecules have a variety of proposed functions, including acting as electron shuttles $^{74}$, regulating quorum-sensing signals $^{29}$, or altering the cellular redox state to facilitate iron acquisition $^{75}$. Inter-species signaling via subinhibitory antibiotic concentrations has been studied by applying low levels of erythromycin and rifampicin, both of which are natural products of soil bacteria, to the animal pathogen *Salmonella enterica* $^{34}$. Both antibiotics were shown to modulate the expression of a wide variety of *Salmonella* genes, but the benefit to the producers from this cross-species gene regulation remains unknown.

Although it is difficult to determine the exact selective benefit of producing potentially toxic compounds that has been the most important through evolutionary history, for most antibiotics it is possible that their low production level does indeed stem directly from their inhibitory role. The environments in which microbial antibiotic production evolved, such as soil $^{69,80}$ or the mammalian gut $^{49}$, contain a variety of biotic and abiotic factors that fluctuate over time and space. In these complex ecosystems, high toxicity against competitors, even at no cost, may not always be the most advantageous strategy. It remains unclear whether there are indeed inherent limits on the optimal level of antibiotic production when focusing only on their toxic activity against competing species.

The species that compete with antibiotic producers in natural microbial ecosystems include both antibiotic-sensitive and resistant strains $^{27,32}$; producers do not necessarily prevent sensitive strains from growing in the same environment, and nor has antibiotic resistance obviated the use of antibiotics by their producers. While a study of *Vibrio* species in a marine microbial ecosystem has observed that antibiotic producers tend to share microhabitats with closely-related resistant strains $^{23}$, the fine-scale population structure of microbial ecosystems is complex. Compounds in the envi-
ronment, including the degradation products of antibiotics themselves, can selectively inhibit antibiotic-resistant strains, countering the selective effect of antibiotics. Studies of soil bacteria, primarily the prolific antibiotic-producing *Streptomyces*, have shown that different species may produce antibiotics that reciprocally inhibit one another, or secrete substances that attenuate the ability of antibiotics to inhibit other sensitive strains (e.g., via enzymatic degradation of the antibiotic). Antibiotic producers have even been observed to self-attenuate the toxicity of their own antibiotics, creating spatial or temporal heterogeneity in the inhibitory effectiveness of antibiotics. In addition, the observations that antibiotic resistance occurs in environments isolated from human impact and that antibiotic-resistance genes pre-date the widespread use of antibiotics by humans suggest that interactions between antibiotic producers and non-producers has long been of ecological importance.

Social evolution theory has been useful in understanding microbial interactions. In a well-mixed environment, the ability of antibiotic producers to inhibit competing non-producers (behavior termed ‘allelopathy’ or ‘interference competition’) can act as a public good: the benefit of decreasing competition from antibiotic-sensitive non-producers is shared among all the competitors in the environment. Other examples of the production of public goods, secreted molecules beneficial to other cells in the community, have been studied in microbial systems. These include iron-binding siderophores in *P. aeruginosa*, invertase (a sucrose-metabolizing enzyme) in *Saccharomyces cerevisiae*, and quorum-sensing autoinducers across many species of Gram-positive and Gram-negative bacteria. Antibiotic resistance can even act as a public good: when the resistance mechanism involves degradation of the antibiotic, other species in the environment can also benefit from the lowered global antibiotic concentration. Because antibiotic production can harm other species and incur a fitness cost to the producer, allelopathy has been called ‘spiteful’, but this term only applies when the relative benefit of production (i.e., from decreased competition) does not outweigh the direct costs to the producer.

As in other public-goods systems, spatial structure is key in determining the outcome of interactions between antibiotic producers and non-producers. Well-mixed environments present two obstacles to the evolution of antibiotic producers. First, microbially-produced antibiotics can be too dilute to inhibit competitor species and thus producers may only gain in fraction of the population when they are already above a critical abundance. Under these circumstances, a single mutant having increased production cannot invade a population. Second, regardless of their abundance, when producers simultaneously compete with both antibiotic-sensitive and resistant strains, the resistant strain can out-compete the producers. These resistant non-producers receive the same benefit of decreased competition without incurring any costs of antibiotic production and can thus
be thought of as ‘cheaters’. In a study of *Escherichia coli* bacteriocin producers, propagating the producers in a well-mixed system saw complete loss of toxicity after 253 generations. Relative to the ancestor, the evolved strain had an increased growth rate and decreased toxicity, suggesting that antibiotic production is costly and that producer populations in mixed cultures are vulnerable to lower-producing ‘cheater’ mutants. Invasion of a producer population by non-producing cheaters has also been studied in other public goods systems.

The inhibitory effect of antibiotic production can only be selected for in spatially structured environments, where diffusion limits how far antibiotic and nutrient molecules can travel. Spatially structured environments, in contrast to well-mixed environments, circumvent the problem of dilution by concentrating antibiotics around producer colonies, allowing producers to kill sensitive competitors in their immediate vicinity. As long as the benefit from decreased competition and the extent of inhibition of the competitors outweighs the costs of production, a single antibiotic-producing cell can thus have a relative advantage over its sensitive competitors. A study of *E. coli* bacteriocin production showed that low-abundance bacteriocin producers could invade a population of bacteriocin-sensitive non-producers when competing in the structured environment of agar plates, but not in well-mixed shaken flask culture. In addition, spatial structure also limits the success of resistant cheaters by restricting the selective advantage of inhibiting competitors to the locality of producers. Structured environments can thus theoretically allow coexistence of producers and non-producers.

Natural microbial environments are often highly structured. Soil is composed of interconnected networks of micron-scale pores between organic and inorganic particles, forming a high surface-area heterogenous environment that supports extremely diverse microbial ecosystems. Where microbial species grow in biofilms, as in the mammalian gut or microbial mats, differential oxygen penetration and nutrient availability create layered microenvironments dominated by characteristic species. Even in marine ecosystems, which may seem to be well-mixed, different types and sizes of organic particles form unique microhabitats. Spatial structure is therefore key in understanding the evolution of microbial social behaviors, such as antibiotic production.

There have been several attempts to evolve increased antibiotic production by competing antibiotic producers with sensitive strains in structured environments. In one study, *Streptomyces clavuligerus* colonies were grown on agar plates, then surrounded by an inoculation of methicillin-resistant *Staphylococcus aureus* (MRSA) on the remaining agar surface. On each cycle of the experimental evolution protocol, a sample of each *S. clavuligerus* colony was streaked for isolation (to prevent co-evolution of resistant MRSA) and a single colony picked to inoculate a new co-culture. After four months of 10-14 day cycles, 14 of 28 evolved lines showed zones of inhibition against
the co-cultured MRSA, attributed to increased holomycin production. It is unclear whether the 
*S. clavuligerus* colonies benefited from this inhibition, as the colonies were grown for three days 
prior to exposure to MRSA, the two species may not have been competing for the same resources 
(they occupy different habitats in nature), and the fitness of the evolved strain relative to its ancestor 
was not measured. Given these caveats, it is surprising that increased antibiotic production arose af-
fter only 8-12 rounds of selection, which suggests a very high strength of selection. One explanation 
could be that increased secondary metabolite production may have been a byproduct of a selection 
scheme unintentionally favoring early sporulation, since it is easier to collect spores from the surface 
of a *Streptomyces* colony than it is to break off a section of mycelium from the colony body. Alter-
natively, the manual picking of individual colonies for re-inoculation of the co-culture may have 
introduced an element of artificial selection.

Another experimental evolution study used only *E. coli* strains, competing producers of the bacterioci
n colicin E8 with sensitive non-producers both in mixed liquid media and on agar plate cul-
tures. On each cycle, the co-cultures were transferred to fresh media containing colicin-sensitive 
strains; transferring the agar plate cultures was done by replica plating to preserve the spatial rela-
tionships of the colonies. Contrary to expectation, antibiotic production, measured by the extent of 
inhibition of the evolved producers, declined in both the well-mixed and structured environments. 
The authors note that clumps of colicin producers are able to kill competitors several millimeters 
away, and that lower-producing, fitter mutants within that range may have been able to take advan-
tage of higher-producing cells. Thus, even in structured systems, evolution can lead to loss rather 
than enhancement of antibiotic production.

Here, we attempt to understand selection for antibiotic production in an engineered ecosys-
tem and in a natural soil environment through a combination of experimental and theoretical ap-
proaches. We created a synthetic ecosystem consisting of three strains of *E. coli* – producers and 
antibiotic-sensitive and resistant non-producers – competing on the surface of agar plates, a spatially 
structured environment. Using this system, we empirically mapped a parameter space to determine 
the conditions under which selection for antibiotic production is maximized. In addition, we de-
veloped static and dynamic mathematical models of antibiotic production and growth to test how 
physical parameters affect selection for antibiotic production. Finally, we present an experimental 
design for selecting specific antibiotic producers in their natural soil environment. Our results have 
implications in understanding the behavior of antibiotic-producing microorganisms in nature, as 
well as informing the design of laboratory evolution protocols for evolving enhanced antibiotic pro-
duction.
Microbial antibiotic production is common, but the selective benefit of such toxic behavior is unclear. We developed an experimental system to map the conditions allowing selection for antibiotic production. Because antibiotics can function as public goods, we hypothesized that cell densities would be important parameters, and found that selection for antibiotic production is only possible under a restricted set of initial density conditions. We also asked how the amount of antibiotic produced affects the selective advantage it confers, and found that competitive trade-offs lead to an optimal amount of antibiotic production that maximizes selection for production.

We used a colicin-based three-strain system in which producer and cheater strains compete in the presence of a sensitive strain. Colicins are plasmid-encoded, toxic proteins produced by *E. coli* that specifically target other *E. coli* and are thus classified with other narrow-spectrum peptide antibiotics as bacteriocins. Colicin-producing and non-producing *E. coli* have long served as model systems for studying the social evolution of antibiotic production. The three strains we used share the same genetic background and consist of colicin producers, colicin-resistant ‘cheaters’, and colicin-sensitive competitors. Producers carry the ColE2 plasmid, which encodes a gene for the colicin E2 toxin, a DNA endonuclease that is co-transcribed with immunity and lysis factors. The colicin operon is repressed by LexA, allowing its expression to be tuned by inducing DNA damage.
Figure 1.1: Three-strain colicin system. a. Architecture of the colicin E2 operon, containing toxin, immunity, and lysis genes under an SOS promoter induced by DNA damage. The immunity peptide gene has its own constitutive promoter and transcription terminator. b. Producer strain releases colicin (red hexagons) which kills a sensitive strain, but is ineffective against a resistant strain. Strains are differentially labeled with fluorescent reporters.

1. We use mitomycin C. When released, colicin can enter and kill the sensitive strain, but not the resistant strain (Fig. 1.1b).

We differentially labeled each of the three strains with plasmid-encoded fluorescent proteins – CFP, YFP, or mCherry. Plating the strains on agar and imaging in the three fluorescence channels allowed the abundance of each strain to be quantified (total fluorescence of colonies in the appropriate channel, see Methods). We defined selection for antibiotic production $\eta$ as the final abundance ratio of producers to cheaters, normalized to their seeding ratio (set to 1 to facilitate analysis). This definition of selection was chosen based on the phenotypic similarity between producers and cheaters: they are both resistant to the antibiotic and their only functional difference is antibiotic production. It also reflects the likely evolutionary history of antibiotic production first evolving in an already resistant background. However, the mechanism of resistance in our cheater strain is different from that of the producer. While the producer carries an immunity peptide within the colicin operon that binds to and inactivates the colicin toxin, the resistant strain was derived by selection on colicin extract, which most commonly results in loss-of-function mutations in the target receptor (for colicin E2, the vitamin B12 transporter BtuB) or in the translocation machinery (for colicin E2, the tol-dependent translocation system).

Competing the three strains on solid media confirmed that producers benefit from the inhibition of nearby sensitive colonies while also allowing cheater colonies in the vicinity to gain from this inhibition. Starting with pair-wise competition, we demonstrated that producer colonies make zones of inhibition and grow larger when competing against sensitive versus resistant competitors (Fig. 1.2a). When all three strains were grown together, we observed cheater colonies growing both outside and inside these inhibition zones with the colonies inside growing larger than the colonies outside (Fig. 1.2b).
Figure 1.2: Colicin producers inhibit nearby sensitive, but not resistant, competitors. a. Two-strain co-culture on solid media (containing 16 ng/mL mitomycin C) shows producer colonies inhibiting growth of nearby sensitive, but not resistant, colonies (scale bars = 1 mm). Sensitive colonies do not grow within the inhibition radius \( r \). b. Co-culture of all three strains together, showing the resistant strain can act as a production cheater. Left, growth across entire surface of plate; right, zoomed image of 1 cm\(^2\) box region (scale bar = 1 mm). Resistant colonies are small when competing with the sensitive strain (dash arrow) but they gain in size when growing in the vicinity of producer colonies (solid arrow).

1.2b). Proximity to a producer colony thus conferred a gain to these cheater colonies.

**Selection for production is density-dependent**

We next asked under what conditions producers win over cheaters. Because inhibition and cheating in a structured environment are limited by diffusion and thus governed by proximity between colonies, we surmised that colony densities could be important parameters determining the benefit of toxicity. Varying the seeding densities of the producers and sensitive strains over three orders of magnitude, we found that selection for production requires both high density of sensitive competitors and low density of producers (Fig. 1.3a). The requirement for a high density of sensitive competitors is consistent with the benefit of production stemming from the inhibition of sensitive species.

Indeed, in separate two-strain competition experiments, we found that the difference in growth of producers when competing against sensitive versus resistant strains appears only above a critical density of these competitors (Fig. 1.3b), where there is a significant chance of competitors falling within an inhibition zone (Fig. 1.2c). This difference widens at high competitor density. The growth
Figure 1.3: Producers have an advantage over resistant cheaters only at high densities of sensitive competitors and low densities of producers. a, Selection for production relative to the non-producing resistant cheater in three-way competitions on agar for varying seeding densities of sensitive and producer cells (producer density [P] = cheater density [C], mean of 4 replicates). See Fig. B.1 for data plotted as lines. b, Average producer growth per colony in pairwise co-culture with sensitive competitors (blue) versus resistant (green, line showing reciprocal fit, slope = -1). Gain from killing, measured as the difference between the two lines (arrows), appears and further increases as the density of competitors increases beyond a critical density equal to $1/\pi r^2$ (dotted line). c, The growth of individual cheater colonies (green dots) decreases with their distance to producer colonies ($d$ is distance to nearest producer colony; sensitive density [S] = 20,000 CFU/cm², [P] = [C] = 3 CFU/cm², combined data from 4 plates); line shows smoothed average by local linear regression. Inset: example of two cheater colonies indicated by symbols (scale bar = 1 mm). d, Cheater growth increases linearly with producers at high sensitive density, but is not helped by producers at low sensitive density. Each series is normalized to the mean growth at the lowest producer density.
of producers declined inversely with the density of resistant competitors, indicating a purely competitive interaction between these strains. Against sensitive competitors, a similar relationship was observed at low densities, but producer growth plateaued at high sensitive competitor density instead of continuing the inversely proportional relationship with competitor density. The transition between these two regimes corresponds to competitor densities equivalent to the inverse of the inhibition zone area. The zone of inhibition therefore ensures that a fixed amount of resources is available to producer colonies despite increased sensitive competitor density.

While selection for production increased with the density of sensitive colonies, it decreased with the density of producers due to elevated cheating. The relative growth of producers over resistant non-producers (cheaters) in the three-way competition declined with increasing producer density (Fig. 1.3a). At a high density of sensitive competitors, cheater colonies grow much better when they are near producer (Fig. 1.3c). As the chance of a cheater to be seeded close to a producer increases linearly with the producer density (up to a certain limit of producer density), the average growth across all cheater colonies is proportional to the producer density (Fig. 1.3d, darkest line). This relationship stems from the killing of sensitive cells; when the density of sensitive competitors is low, cheater colony growth is not helped by producer density (Fig. 1.3d, lightest line). Intermediate densities of sensitive competitors led to behaviors in between the linear and constant regimes. In sum, in a three-way competition, increasing producer density reduces selection for producers by increasing the chance that cheater colonies benefit from local inhibition of sensitive competitors by producers.

**Intermediate production maximizes selection**

To test how the amount of antibiotic produced affects selection for production, we performed competition experiments at varying levels of colicin expression. We varied the concentration of mitomycin C (MMC) in the growth media to tune colicin expression, restricting the range of concentrations to levels too low to affect growth of the non-producing strains (Fig. B.2). At each level of colicin induction, we measured the relative growth of producers to cheaters in the presence of sensitive competitors. Because producers still release colicin even in the absence of mitomycin C, we used resistant instead of sensitive competitors as a proxy for zero killing. Selection for production peaked at intermediate levels of colicin induction (Fig. 1.4a). The location and height of this fitness peak depended on producer density: higher density led to a lower selection peak and smaller optimal level of colicin induction.

The decline in selection for production at high colicin induction is not due to the cost of production, but rather the increased benefit gained by cheaters. Because inducing the colicin operon
Figure 1.4: The advantage of producers over cheaters is maximized at an intermediate level of antibiotic production. 

a. Selection for production in three-way competitions as a function of varying levels of colicin induction via mitomycin C ([S]=2,000 CFU/cm²; high density: [P]=[C]=20 CFU/cm², low density: [P]=[C]=2 CFU/cm²; mean of 4 replicate plates for each point). Leftmost points represent controls of no-killing (NK), where the sensitive competitor is replaced with resistant (competition between red producers and green resistant cheaters co-cultured with blue resistant competitors). 

b. The average growth of producer colonies increases monotonically with production level. Insets: representative colonies from highlighted data points. 

c. Growth of individual cheater colonies close to producer colonies increased with colicin induction (each series is combined data from 4 replicates in the high [P] condition; solid lines are smoothened averages calculated by local linear regression). See Fig. B.3 for low [P] data. 

d. Average growth of resistant colonies at low and high producer density for varying production levels.
causes cell lysis, production can incur a fitness cost (Fig. B.2). This cost increases with greater induction of the operon. However, the growth per producer colony increases monotonically over the range of mitomycin C concentrations where the peak is observed (Fig. 1.4b), demonstrating that the benefit of production was always higher than its direct cost. On the other hand, increasing colicin production also increased the growth of cheater colonies taking advantage of nearby producers (Fig. 1.4c), consistent with larger inhibition zones both increasing the probability of a resistant colony landing inside a zone, and freeing up more nutrients to these cheater colonies. Cheater colonies far away from producers were unaffected by changes in the mitomycin C concentration. The benefit gained by individual cheater colonies thereby increased the overall cheater growth (Fig. 1.4d). Thus, increased antibiotic production simultaneously decreases competition from sensitive cells and increases competition from resistant cheaters, leading to a competitive trade-off that sets the optimal level of antibiotic production.
Modeling Competition with Antibiotic Producers

The ability of microorganisms to inhibit competing strains can confer a selective benefit. Previously, we showed how cell densities and the amount of production affect selection for antibiotic production in an engineered ecosystem of competing *E. coli* strains. To understand how generalizable these findings are, we created mathematical models of antibiotic production and competition. Using a simple static model, we show how the experimental results can be qualitatively recapitulated with very few parameters. In addition, we used a dynamic model to investigate how physical parameters and spatial heterogeneity affect selection for antibiotic production.

### 2.1 Static Model of Competing Colonies

We developed a simple model to quantitatively understand how selection for producers varies with seeding density and the amount of antibiotic produced. Our model calculates the final growth of producers, cheaters, and sensitive colonies randomly seeded on a continuous two-dimensional space, interacting through inhibition and competition (Fig. 2.1a). Each producer colony is surrounded by a circular inhibition zone of radius $r_i$ where sensitive colonies are killed \(^{45,76}\). The remaining colonies compete for a single limiting resource evenly distributed across space: due to diffusion each colony
can access this resource only within a given “grazing zone” radius $r_g$ and the amount of resource that lies inside overlapping grazing zones is evenly shared. Each colony’s final growth is proportional to its total captured resources. As in our experiments, we define selection for antibiotic production $\eta$ as the ratio between the total growth of all producer colonies relative to that of cheater colonies, normalized by their initial ratio (set to 1).

We ran simulations of the model, testing how the selective advantage of production depends on the seeded densities of sensitive and producer colonies, as well as on the level of production. The model showed a monotonic increase in selection for production with the density of the competing sensitive strain and a monotonic decrease with producer density (Fig. 2.1b). These results are qualitatively in accordance with our experimental data (Fig. 1.3a), Because the model does not account for any direct costs of production, the value of $\eta$ (selection for production) does not fall below 1 in the simulations. To simulate changing the colicin induction level, we varied the radius of inhibition $r_i$ in the model. As in the experimental system (Fig. 1.4), we observed a non-monotonic dependence: selection for producers peaked at an intermediate level of production $r_i^*$ (Fig. 2.1c). Interestingly, this peak arose solely from the competitive interactions accounted for by the model, and not from direct costs of production, which were not included in the model but can also lead to trade-offs in the net benefit of production. Because selection for production in this model is driven by competitive interactions, the peak selection decreased in magnitude and shifted to lower production levels with increased producer density, which benefits cheaters (Fig. 1.3c,d). An upper bound on the optimal radius of inhibition $r_i^*$ is given by $2r_g$: at this value, there is no overlap between the grazing zones of producer and sensitive colonies. Increasing $r_i$ above $2r_g$ gives no additional benefit to producers, but instead confers the additional benefit to cheaters. At the lowest producer density we simulated, $r_i^*$ approached this upper bound (Fig. 2.1c).

Simulations of the model were implemented in MATLAB. Colonies were seeded in a square environment of area $A$ by sampling location coordinates from a uniform random distribution. Sensitive colonies within $r_i$ of any producer colony were removed. Resource allocation was determined by partitioning the environment into square grid-cells with side length $dx$. For each grid-cell, resources equal to the area of the grid-cell were partitioned equally among all colonies within $r_g$ of the grid-cell. For visualization of colony growth, colony areas were set to the total resources obtained by each colony, scaled by a constant $k$. The full parameter set and values for the simulations in Fig. 2.1 are shown in Table 2.1.
Figure 2.1: A simple model of competition and inhibition recapitulates experimental results. a, Steps in simulating model: (1) Random seeding of producers (red), sensitive (blue) and cheater (green) colonies at given densities; (2) killing sensitive colonies inside inhibition zones of radius $r_i$ around producer colonies; (3) final growth of each colony is determined by the amount of resource available to it in a grazing zone of radius $r_g$ around it. Resources in overlapping grazing zones of two or more colonies are equally shared among them. b, Selection for production increases monotonically with the density of sensitive species and decreases with density of producers (average of 20 simulations per parameter set). c, Selection for production $\eta$ is maximized at an intermediate level of production $r_i$ (n=50 simulations per parameter set, error bars are standard error). Panels show sample simulations at indicated points. Parameter values are given in Table 2.1; see Fig. B.4 for effects of adding cost to the model.
Table 2.1: Static model simulation parameters.

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<th>Value</th>
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<tr>
<td>$S$</td>
<td>Number of sensitive colonies seeded</td>
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<tr>
<td>$k$</td>
<td>Colony area scale factor</td>
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2.2 Dynamic Model of Microbial Growth

We developed a dynamic model, based on a set of 2-dimensional partial differential equations, in order to investigate how underlying physical parameters affect the advantage producers can gain from secreting a diffusible antibiotic. This model tracks three variables: the concentrations of each bacterial strain $b_i(x, y, t)$, the concentration of a single limiting diffusible nutrient $n(x, y, t)$, and the concentration of an antibiotic $c(x, y, t)$. We used the Monod equation \(^{59}\) to account for the dependence of microbial growth on nutrient concentration:

$$ \frac{db_i}{dt} = \gamma_{\text{max}} \frac{n}{K_n + n} b_i. $$

To model an antibiotic-sensitive strain, we set the growth rate to zero if the antibiotic concentration exceeded the MIC. As in the static model described above, nutrient consumption is equal to the growth of the bacteria:

$$ \frac{dn}{dt} = - \sum_i \gamma_{\text{max}} \frac{n}{K_n + n} b_i - D_n \nabla^2 n. $$

Although natural antibiotic producers often regulate synthesis in response to growth phase \(^{36}\), nutrients \(^{16}\), or signals from neighboring species \(^{70}\), we chose to simply have the rate of antibiotic production be proportional to the growth of the producers:

$$ \frac{dc}{dt} = p \gamma_{\text{max}} \frac{n}{K_n + n} b_p - D_x \nabla^2 c. $$

We ran simulations of this model, varying the diffusion rates of the nutrient and the antibiotic.
Toxin diffusion rate, $D_x$ (cm$^2$/s)  
Nutrient diffusion rate, $D_n$ (cm$^2$/s)  

Number of producer cells: 4, 6, 8, 10

Figure 2.2: A dynamic, physics-based model predicts an optimal combination of diffusion rates. a, Growth of a single producer colony competing against a sensitive strain has a single maximum when varying nutrient and antibiotic diffusion rates (initial bacterial concentration $b_i(t = 0) = 1$ bac/mm$^2$, initial nutrient concentration $n(t = 0) = 10^8$ bac/cm$^2$, run time = 50 hr). See Table 2.2 for parameter values. b, Final simulation grid at the peak diffusion rates ($D_n = 10^{-5.75}$ cm$^2$/s, $D_x = 10^{-6.5}$ cm$^2$/s). Red: producer colony; blue: sensitive strain.

These physical parameters underlie the grazing zone radius $r_g$ and inhibition zone radius $r_i$, respectively, in the static model. In simulations of a single producer colony at the center of a lawn of sensitive bacteria, the final size of the producer colony was non-monotonic with both the nutrient and the antibiotic diffusion rates; too low or too high diffusion rates decreased the growth of the producers. Where the optimality in the size of the inhibition zone observed in the static model (Fig. 2.1) resulted from competitive trade-offs due to cheating, this non-monotonic behavior arose in the absence of cheating. We can make sense of this result by considering that to gain a benefit from toxicity, a producer colony must inhibit neighboring competitors to free up resources, then collect these freed resources. Fast diffusion rates cause the system to approach a well-mixed environment, where antibiotic dilution prevents localized inhibition. High nutrient diffusion rates allow freed nutrients to escape the zone of inhibition and be captured by competitors. However, if diffusion is too slow, the chemicals are effectively ‘stuck’ in place. Intermediate antibiotic and nutrient diffusion rates thus maximize producer growth.

We applied our model to explore the behavior of producer colonies in a range expansion scenario, where cells are inoculated in a spot or a line across an otherwise empty environment containing uniformly distributed resources (Fig. 2.3). As the cells grow and divide, they spread out from...
the initial site of inoculation. These conditions can allow for a very high local density of sensitive competitors, which promotes the selective advantage of production (Fig. 1.3) over many generations as the growing front expands into the unoccupied regions. To account for colonies spreading as cells divide, we added a non-linear diffusion term to the bacterial growth equation:

\[
\frac{db_i}{dt} = \gamma_{\text{max}} \frac{n}{K_n + n} b_i - k(\gamma_{\text{max}} \frac{n}{K_n + n} b_i)^a \nabla^2 b_i.
\]

The ‘diffusion rate’ of the cells increases with their growth, and there is no colony spreading if the cells are not growing. We ran simulations of our model using this growth equation, starting from a line-like initial condition. While at short time scales the simulations looked qualitatively similar to experiments, they did not result in the striking advantage of producers over sensitive competitors observed in the experiments at longer times. This could be due to the choice of parameters, or because our model of colony spreading does not account for factors in a real 3-dimensional biofilm, such as the physical forces between bacterial cells, or phenotypic variability due to differential oxygen penetration and nutrient depletion.

Simulations of this model were implemented in MATLAB on a two-dimensional lattice consisting of square grid-cells. On each time step, bacterial growth was first calculated by linearizing the growth equation. Based on this growth, colony spreading, nutrient consumption, nutrient dif-
Table 2.2: Dynamic model variables and parameters.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Value</th>
<th>Fig. 2.2</th>
<th>Fig. 2.3</th>
</tr>
</thead>
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<tr>
<td>$L$</td>
<td>Length of environment (cm)</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$W$</td>
<td>Width of environment (cm)</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$dx$</td>
<td>Grid-cell length (cm)</td>
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<td></td>
<td>0.1</td>
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<tr>
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<td>Bacterial strain $i$ concentration (bacteria/cm$^2$)</td>
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<td></td>
<td>var.</td>
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<td>$c$</td>
<td>Antibiotic concentration ($\mu g$/cm$^2$)</td>
<td>var.</td>
<td></td>
<td>var.</td>
</tr>
<tr>
<td>$\gamma_{max}$</td>
<td>Maximum growth rate (s$^{-1}$)</td>
<td>$5 \cdot 10^{-4}$</td>
<td>$10^{-3}$</td>
<td></td>
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<tr>
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<td>Nutrient concentration for half-max growth (bacteria/cm$^2$)</td>
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<td>$M$</td>
<td>MIC of sensitive strain ($\mu g$/cm$^2$)</td>
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<td>1</td>
</tr>
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<td>$p$</td>
<td>Antibiotic production rate ($\mu g$/bacteria)</td>
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<td>10</td>
</tr>
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<td>$10^{-6}$</td>
<td></td>
</tr>
<tr>
<td>$a$</td>
<td>Colony spreading power</td>
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<td></td>
<td>1</td>
</tr>
<tr>
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<td>Diffusion rate of nutrients (cm$^2$/s)</td>
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<td></td>
<td>$10^{-4}$</td>
</tr>
<tr>
<td>$D_x$</td>
<td>Diffusion rate of antibiotic (cm$^2$/s)</td>
<td>var.</td>
<td></td>
<td>$10^{-4}$</td>
</tr>
</tbody>
</table>

Diffusion, antibiotic production, and antibiotic diffusion were successively accounted for. Diffusion was simulated by distributing the concentration in a grid-cell to each of its four neighbors according to the length of the time-step and the respective diffusion rate. The length of each time-step was dynamically calculated based on the current fastest diffusion rate so that at most $1/5^{th}$ of the concentration in a grid-cell was distributed to each neighbor. See Table 2.2 for a list of variables and parameters.
3

Selecting for Producers in a Natural Environment

Microbial production of antibiotics can be a beneficial strategy for reducing competition under certain circumstances. We previously showed how the benefit of this strategy varies in a simple experimental system and in mathematical models. However, it is still unknown how antibiotics benefit their producers in natural environments. Although some have proposed alternate functions for antibiotics in nature\(^25\), the complexity of natural environments – in particular, their highly structured nature\(^80\) – may allow toxicity towards competing microorganisms to be a selective benefit that favors antibiotic producers. We designed an *in situ* competition experiment in order to measure selection for antibiotic production in a natural environment.

Inspired by a historic experiment\(^73\), we attempted to enrich for antibiotic producers in their natural habitat by adding competitor bacteria to otherwise unperturbed soil samples (Figure 3.1). If toxicity against competitors is important to the fitness of antibiotic producers present in the soil, then the growth of different types of producers should be affected by the antibiotic resistance profiles of each producers’ neighboring strains. We hypothesized that soils ‘fed’ with added competitor bacteria would be specifically enriched for producers able to inhibit the particular feeder strain. To that end, we performed an experiment in which feeder strains differing only at a single antibiotic-resistance locus (tetracycline-sensitive or resistant *E. coli*) were added to separate aliquots of the same
Figure 3.1: In-situ soil feeding experiment. a, Schematic of experimental procedure. Antibiotic-sensitive or resistant feeder cells are added to soil samples. Samples are incubated for three weeks, with feeder cells added again halfway through. Quantification of antibiotic producers is performed via DNA-based or culture-based assays. b, Standard 6-well tissue-culture plate adapted for the soil feeding experiment, with wells filled with soil or empty to show drainage holes.
soil sample. As controls, sets of soil aliquots were fed with either dead *E. coli*, water, saline solution, or left unfed. Each treatment was performed in triplicate, for a total of 18 treated soil aliquots.

Quantifying the results of the soil selection experiment requires measuring the abundances of antibiotic producers. We expected that the soil samples to which live bacteria were added would have more antibiotic producers than the control samples. In addition, we hypothesized that the samples fed with tetracycline-sensitive *E. coli* would be relatively enriched for tetracycline producers relative to the samples fed with the resistant *E. coli*. We designed two orthogonal assays to quantify the relative abundances of different types of producers: a phenotypic assay based on colonies’ ability to inhibit indicator bacteria, and a molecular assay of DNA content (Fig. 3.2).

For the phenotypic assay, we adapted a procedure using two colors of fluorescently-marked indicator bacteria 15 (Fig. 3.2a). In this assay, a dilute soil suspension is mixed with equal amounts of the indicator bacteria, which differ only at an antibiotic resistance locus and the color of the fluorescent reporter, and spread on agar plates. The principle of this assay relies on antibiotics diffusing out from the producing colony in a limited radius, the zone of inhibition. Counting the number of such zones of inhibition against both indicator strains and zones against only the antibiotic-sensitive strain, as well as the total number of colonies growing on the plate, should yield a quantitative indicator of the antibiotic production capabilities of the soil sample. However, we found that these plates were difficult to quantify: colonies took many weeks to develop and form zones of inhibition, the types of colonies growing varied depending on the plate media, filamentous strains grew over the surface of the plates and covered other colonies, and the number of colonies we were able to assay per plate were limited.

We purified DNA from each soil sample in order to quantify the amounts of antibiotic synthesis genes. Starting from annotated biosynthesis pathways, we designed a set of primers targeting several genes involved in antibiotic production in soil-dwelling bacteria (Table 3.1). Using these primers, we ran quantitative PCR on the purified soil DNA: sample traces are shown in Fig. 3.2b. We expected tetracycline-synthesis genes to be relatively enriched in the soil fed with tetracycline-sensitive *E. coli*. There were two concerning features of the qPCR results. First, there was a lower signal (later amplification) in the more diluted samples than in the more concentrated (Figure B.5), making quantification impossible and indicating the presence of PCR inhibitors in the soil that remained through the DNA purification. Second, comparison of the melt curves of the qPCR products shows multiple peaks that do not align with the expected product size (97 bp, see Table A.1). The observed products from the soil samples are therefore likely to be the result of non-specific amplification.

Non-specific amplification can occur if the amounts of the target genes in the soil samples are too small to quantify using conventional qPCR. Even for a relatively commonly produced antibiotic like
Figure 3.2: Assays for quantifying antibiotic producers in soil samples. 

a. Demonstration of culture-based fluorescence assay on raw soil. Soil suspension is diluted, mixed with fluorescent antibiotic-sensitive and resistant indicator strains (here, green tetracycline-sensitive and red tetracycline-resistant E. coli), and spread on agar plates. Top: bright-field image of growth after several weeks (scale bar = 1 cm). Bottom: fluorescence composite image of same plate. Arrows indicate colonies producing antibiotics selectively inhibiting the antibiotic-sensitive indicator strain (solid arrow) or equally inhibiting both strains (dashed arrow).

b. Quantitative PCR analysis of soil from feeding experiments. Primers specific for the oxytetracycline synthesis gene oxyD were used to quantify DNA extracted from soil fed with either tetracycline-sensitive or resistant E. coli; genomic DNA isolated from pure culture of an oxytetracycline producer was used as a positive control. Top: sample qPCR traces, showing higher measured DNA content from 8x diluted DNA. Bottom: Melting curves for reactions shown in top plot reveal non-specific amplification. See Fig. B.5 for full results.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Source</th>
<th>Antibiotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>gtfE</td>
<td><em>Amycolatopsis orientalis</em></td>
<td>vancomycin</td>
</tr>
<tr>
<td>neoN</td>
<td><em>Streptomyces fradiae</em></td>
<td>neomycin B</td>
</tr>
<tr>
<td>oxyD</td>
<td><em>Streptomyces rimosus</em></td>
<td>oxytetracycline</td>
</tr>
<tr>
<td>oxyS</td>
<td><em>Streptomyces rimosus</em></td>
<td>oxytetracycline</td>
</tr>
<tr>
<td>strK</td>
<td><em>Streptomyces griseus</em></td>
<td>streptomycin</td>
</tr>
</tbody>
</table>

tetracycline, a single gram of soil may contain only a few hundred copies of the biosynthesis gene\(^5\). At this low concentration, qPCR may not be accurate enough to quantify changes in gene copy number between the differently-treated soil samples. It may be better to instead target conserved regions of antibiotic biosynthesis genes, broadening the target space but losing the specificity to a particular antibiotic. Another solution could involve first enriching for the target genes prior to qPCR, e.g. through hybrid-capture\(^{14}\), as long as this enrichment process does not introduce more variability than the expected differences between the treatments. Finally, a non-targeted approach using next-generation metagenomic sequencing\(^{28}\), which could be costly at the depth required to resolve changes in abundances of specific genes, would also provide more information on the composition of the soil communities than qPCR.
Conclusion

The selective advantage of antibiotic production depends on the ability of producers to gain a benefit from inhibiting competitors even in the presence of resistant cheaters. Using an engineered ecosystem consisting of three strains of *E. coli*, we found that selection for antibiotic production, even in a structured environment, is only possible under a limited set of conditions (Fig. 1.3). These conditions include having of a high density of sensitive competitors so that producers can derive a benefit from freeing resources from nearby competitors. In addition, producers themselves must be present at a low density. The higher the producer density, the greater the probability that non-producing cheaters can benefit from the inhibition of sensitive competitors. This effect could explain why previous attempts to evolve antibiotic production resulted in loss rather than enhancement of toxicity 48.

With this experimental system, we showed that intermediate levels of antibiotic production maximize selection for production in structured environments (Figure 1.4), a result we recapitulated with a simple mathematical model (Fig. 2.1). This peak in selection occurs under varying initial conditions, with low producer densities yielding a higher peak shifted towards a larger optimal extent of production. The peak arises from a trade-off between inhibiting sensitive competitors while limiting the advantage of inevitably appearing cheaters. Higher producer densities benefit cheaters, decreasing the selective advantage of production and thus shifting the peak towards lowered production. It would be interesting to see if the direction of selection, when competing two strains that have differ-
ent non-zero production levels in the presence of sensitive non-producers, can be predicted by this single-producer selection curve.

We also found trade-offs in the diffusion rates of antibiotic and nutrients in a dynamic model of antibiotic production and competition (Fig. 2.2). While the simple model mentioned previously combines antibiotic production and diffusion into a single parameter (the radius of inhibition), the dynamic model explicitly accounts for antibiotic diffusion and production as a function of producer growth. Growth of a producer colony surrounded by sensitive competitors was maximized for intermediate values of these diffusion constants. Testing this prediction experimentally could involve comparing growth of producers either making antibiotics of varying sizes, or competing for nutrient molecules with different diffusibilities. Alternatively, the viscosity of the growth substrate could be varied, e.g. by changing the agar concentration, which may affect both the nutrient and antibiotic diffusion rates.

Adding a term to the model accounting for colonies spreading as they grow allowed us to use this model to simulate range expansion scenarios (Fig. 2.3). Range expansion, as opposed to growth more uniformly distributed in space, should enhance selection for antibiotic production. Inoculating cells in a localized area allows for high local densities of sensitive competitors and continual growth over long times as nutrients from farther away from the inoculation site become available to the expanding front. More simulations and experiments are necessary in order to determine how selection for antibiotic production quantitatively depends on seeding geometries, and the relative importance of total nutrient availability versus local densities.

While the work summarized thus far takes a reductionist approach to understanding selection for antibiotic production, we also wanted to investigate the role of antibiotics in a natural environment. To that end, we designed an experiment to measure selection for antibiotic producers within soil samples (Fig. 3.1). The results of this experiment proved difficult to quantify using phenotypic assays and qPCR (Fig. 3.2). Metagenomic sequencing may instead provide a richer, more quantitative data set. Alternatively, introducing known producers to the soil samples could boost the measurable signal (the abundance of producers) while maintaining other features of the natural environment. Adding both soil-dwelling producers and otherwise identical non-producing cheaters would allow selection for production to be measured as the relative growth of the two strains in situ. Such an experiment could be performed with *B. subtilis* strains that naturally reside in soil and are amenable to genetic manipulations to modify antibiotic production and insert marker genes.

There are two main implications of this work. The first is in helping to understand the limited quantities of antibiotics produced in nature. The low level of antibiotic production observed in natural producers may reflect inherent trade-offs between inhibiting sensitive competitors and be-
coming vulnerable to non-producing cheaters. However, antibiotic biosynthesis has been shown to be regulated by a variety of factors, and this regulation likely further optimizes the benefits of producing antibiotics. These factors include growth phase, nutrient availability, and secreted chemicals from neighboring microbial species. When extrapolating our results to antibiotic production in nature, it is important to remember that natural environments are more highly structured and heterogeneous than a 2-dimensional agar plate, and that interactions between strains are more complex than the single-antibiotic inhibition and nutrient competition that we considered in our models.

This study also provides a framework for designing improved selection schemes for antibiotic drug discovery. Antibiotics have been chiefly found by screening microbial isolates or natural product libraries. Given that the most common drugs have already been discovered and the need for new antibiotics to combat antibiotic resistance, researchers have turned to laboratory evolution to harness the power of selection. Our findings that high densities of producers decrease both the strength of selection for antibiotic production and the optimal amount of antibiotic produced imply that selection must be performed at a low producer density. Assuming that the desired mutations which increase antibiotic production are rare, the selection scheme would require a large number of plates to achieve a population size necessary for increased production mutants to arise. In addition, the requirement that selection for production requires high densities of competitors means that resources will be depleted in a small number of generations, allowing less time for fitness differences to become apparent.

A way to circumvent these problems could be to perform selection in small volumes, culturing antibiotic producers in microtiter plates, microfluidic chambers, or droplets. Small volumes restrict diffusion, preventing the problem of dilution that arises in a well-mixed environment. Ideally, each volume would initially contain a single producer cell, so that producers have a low likelihood of encountering non-producing cheaters. In addition, the volumes should also contain a high enough density of sensitive cells so that producers have a large advantage from inhibiting these competitors. The principles presented in this study – consideration of cell densities, physical parameters, and measuring selection for antibiotic production relative to resistant non-producers – would still apply to the design of such experiments.
Experimental Methods

Colicin strains and media

The original *E. coli* producer, resistant, and sensitive strains derived from BZB1011 are from a previously published study\(^4\). Plasmids expressing CFP, YFP, or mCherry under the PR promoter\(^5\) were constructed from the pZ vector system\(^9\) and transformed into the base strains. Cells were cultured on M9 minimal media supplemented with 0.4% glucose, 50 µg/L kanamycin, and mitomycin C. Media was made by autoclaving 11.26 g/L M9 minimal salts (Sigma) and 13 g/L Difco agar, then adding 20 mL/L 20% w/v glucose, 2 mL/L 1M MgSO\(_4\), 100 µL/L 1M CaCl\(_2\), 1 mL/L 50 mg/mL kanamycin, and mitomycin C (variable concentration) from sterile solutions. Mitomycin C (VWR) stock solution (1 mg/mL DMSO) was stored at -20°C in 100 µL aliquots in a light-proof container. A fresh aliquot was thawed for each experiment, and aliquots were discarded after one month of storage. After pouring, agar plates were cooled in the dark at room temperature overnight, and then dried in a sterile hood for 10 minutes to ensure even and consistent plating density before adding cells.

Competition experiments on agar plates

Overnight cultures of individual strains were diluted 1:100 and grown to log phase, OD\(_{600}\)=0.1-0.7. Cultures were mixed at specified ratios, immediately added to plates (100 µL/plate) and spread using
glass beads. Plates were incubated for 7 days at 37°C (varying density experiments) or at 27°C (varying mitomycin C experiments) in order to limit colicin expression at low mitomycin C induction (see Fig. B.6); temperature-dependent expression has also been observed for other colicins. To create uniform lawns used for image processing, 2-3 mL of turbid culture (OD600=0.1-0.5) was added to the surface of an agar plate. The plate was gently shaken to fully cover the surface with liquid, and excess liquid was removed by tilting the plate and aspirating from the edge. Plates were allowed to dry before inverting and incubating. For range expansion experiments (Fig. 2.3a), the edge of an autoclaved glass coverslip was dipped into the mixed culture, blotted on a clean agar plate, and pressed gently on the agar surface of the experimental plate.

**Single-strain growth measurements**

Overnight cultures were diluted 1:2·10^4 and grown in 200 µL cultures in a 96-well plate (Corning 3370) for 42 hours at 27°C with shaking. Growth yield was measured using a VICTOR3 plate reader (Perkin Elmer).

**Image processing**

1. **Image acquisition.** Bright-field and fluorescence images were acquired in raw format (CR2) using a Canon EOS T3i digital SLR mounted on a custom scaffold. Fluorescence images were obtained using LED lights for illumination in combination with excitation and emission filters (the latter placed on a rotating wheel directly in front of the camera lens). Each plate was imaged twice, with the plate rotated approximately 180° between imaging. Consistent exposures for each type of illumination were used for all images within an experiment. Dark (no illumination) and bright (uniform fluorescent bacterial lawns) images were acquired for background correction and normalization.

2. **Basic processing.** DCRAW was used to convert images from CR2 format to 16-bit TIFFs. We developed a semi-automated MATLAB processing pipeline to extract colony information from the TIFF images. Hot pixels were removed by averaging nearest neighbor pixel values. MATLAB’s built-in demosaic function (gradient-corrected linear interpolation) was used to convert the Bayer pattern encoded images to RGB format. Values below dark and above saturation for each channel were trimmed to enable visualization. For each fluorescence image, we only extracted data from a single RGB channel: red for mCherry, green for YFP, and...
blue for CFP. Combining these channels from the three fluorescence images yielded a composite image used in downstream processing.

3. **Normalization.** Background values were subtracted from each image channel based on the dark image controls for each exposure. Flat-field frames for each fluorescence channel were obtained by averaging and smoothing the corresponding bright images (8 images per channel, 2D Wiener filter with a 144 pixel window). Illumination correction was performed by dividing each image channel by the flat-field frame. This flat-field correction also served to normalize the intensities of each channel with respect to one another.

4. **Segmentation.** Global thresholds for each channel were manually selected to segment images into bright (colonies) and dark (no colonies) regions. The same thresholds were consistently used for all plates within an experiment. Areas within 0.5 cm of plate edges were masked out. Contiguous bright regions with area greater than 2 pixels were identified as putative colonies. We tabulated the centroid, area, total intensity and circularity \((\text{perimeter}^2 / 4\pi \cdot \text{area})\) of each such object.

5. **Colony recognition.** Segmented objects consisted of real colonies as well as artifacts caused by light reflections, in particular in the red channel (Fig. B.7). To remove these artifacts, we aligned the two rotated images of each plate and discarded segmented objects only present in one of the two images. Alignment in each channel was first performed automatically, by minimizing nearest-neighbor distances between centroids of round objects (circularity < 1.5) in the two images across three rigid transformation parameters (translation in two dimensions and angle of rotation). Disagreement in the transformation parameters between channels was resolved by setting the parameters in the failed channel equal to the successful channel parameters (alignment success was defined by the nearest-neighbor distances distribution peaking at less than 10 pixels). Manual setting of alignment parameters was used only when automated alignment failed in all channels. Unaligned objects (no corresponding object within 3 pixels) with area less than 65 pixels \((0.05 \text{ mm}^2)\) were discarded.

6. **Calculating selection for production.** Growth of each colony was calculated as the total intensity minus the mean intensity of the segmented background multiplied by the colony area. Selection for production \(\eta\) was defined as the relative growth of producers to cheaters, normalized to the initial ratio. For the variable-density experiments (Fig. 1.3), we calculated growth of each strain as the summed growth of identified colonies divided by the mean seeding density, since colonies were impossible to separate at high seeding densities. For the
colicin induction experiments, we calculated growth of each strain as the mean growth per colony, which yields a more precise value when the number of plated colonies is variable due to low-number fluctuations. Applying the summed-growth analysis to the Fig. 1.4 data yielded similar, but noisier, results (Fig. B.6).

**Soil Selection Experiment**

Soil was collected from Harvard Forest, Massachusetts and refrigerated during transport. The experiments were performed in standard 6-well tissue culture plates with one-eighth inch drainage holes drilled through the center of each well. 5 grams of soil were aliquoted into each well and plates were placed over water-filled trays (OmniTray, Nunc). Tetracycline-sensitive or resistant *E. coli* (derived from MC4100\(^{15}\)) were grown overnight in shaking liquid culture, back-diluted 1:100 and grown to mid log phase. Cultures were spun down and resuspended in PBS to a density of OD600=0.5. For the dead-cell control, 20 μL of tetracycline-sensitive cell suspension was boiled for 10 minutes, then cooled. 5 mL of liquid were added to wells in triplicate: sensitive or resistant feeder cells, dead cells, PBS, or water; there was also a set of no-liquid controls. Soil plates were incubated at room temperature for 3 weeks, and fed again halfway through.

**Phenotypic Assay for Antibiotic Producers**

We used YFP-marked tetracycline-sensitive or CFP-marked tetracycline-resistant *E. coli*\(^{15,14}\). 1 gram of soil was vortexed in 10 mL water. The suspension was allowed to settle, and an aliquot of the supernatant was taken and diluted 1:10 in water. This diluted solution was mixed with log-phase cultures of the sensitive and resistant indicator bacteria in a 2:1:1 ratio and diluted again 1:5 in PBS. 100 μL of the soil and cells mixture was spread onto ISP-2 agar plates supplemented with 100 μg/mL cycloheximide to prevent excessive mold growth. Growth was imaged periodically under brightfield and fluorescence channels until soil colonies were well-developed (up to several weeks). Antibiotic producers were identified by cleared zones of inhibition surrounding colonies. Producers of compounds (e.g., tetracycline-related antibiotics) selecting for the resistant indicator strain were identified in the composite fluorescence images as colonies surrounded by only resistant *E. coli*.

**Quantitative PCR for Antibiotic Production Genes**

DNA was isolated from 1 g of each soil plate well using the PowerSoil DNA Isolation kit from MO BIO Laboratories. Primers were designed to target antibiotic biosynthesis genes of soil-dwelling
<table>
<thead>
<tr>
<th>Target gene</th>
<th>Source species</th>
<th>Primer sequences</th>
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<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>ACCAGATACCAACGCTAC, ACCGAGTAGCAGTCGCATT</td>
<td>76 bp</td>
</tr>
</tbody>
</table>

bacterial species (Table A.1). SsoFast EvaGreen Supermix was used to perform qPCR on the purified soil DNA. Each 10 μL reaction contained 5 μL Supermix, 0.5 μL left primer, 0.5 μL right primer, 1 μL BSA, 1 μL water, and 2 μL template DNA. For the soil-purified DNA, the template DNA used were a 1:2, 1:4, 1:8, and 1:16 dilution series in duplicate. Genomic DNA was isolated using the PowerSoil kit from the source species (see Table A.1) as a positive control, and water was used for a negative control. For the reactions shown in Fig. 3.2b and Figure B.5, an annealing temperature of 58°C and extension time of 3 s were used on a Bio-Rad CFX96 thermal cycler.
Supplemental Figures
Figure B.1: Selection for antibiotic production depends on seeding densities. **a**, Mean selection as a function of sensitive competitor density. Increasing the seeding density of sensitive competitors enhances selection for antibiotic production. **b**, Mean selection as a function of producer density. Increasing the seeding density of the producers themselves decreases selection for antibiotic production. Error bars show s.d. (n=4).
Figure B.2: Growth of single strains in liquid culture with varying concentrations of mitomycin C. Above 10 ng/mL, mitomycin C drastically reduces producer growth at concentrations where non-producing strains are not significantly affected (n=4 wells per condition, error bars show standard deviation).
Figure B.3: Growth of individual cheater colonies close to producer colonies increased with colicin induction. Each series is combined data from 4 replicates where [P]=[C]=2 CFU/cm²; solid lines are smoothened averages calculated by local linear regression.
Figure B.4: Fixed and variable-cost static model simulations. 

**a.** Varying-density simulations (as in Fig. 2.1b) with an additional 10% cost imposed on the final producer growth.

**b.** Varying-inhibition simulations (as in Fig. 2.1c). Left, simulations with a 10% fixed cost on producer growth. Right, simulations with cost as a function of inhibition radius \( r_i \) (cost = 0.7\( b^2 \)).
Figure B.5: Quantitative PCR analysis of soil from feeding experiments. Primers specific for the oxytetracycline synthesis gene oxyD were used to quantify DNA extracted from soil fed with either tetracycline-sensitive or resistant *E. coli*; genomic DNA isolated from pure culture of an oxytetracycline producer was used as a positive control. The relationship between the amount of DNA added to each reaction and the threshold cycle (measured DNA concentration) is inverted. Positive control traces are shown in gray, negative controls in black.
Figure B.6: Colicin expression is temperature-dependent. Mean growth of producer colonies in competition with sensitive or resistant competitors at varying temperatures. [P]=8 CFU/cm², [S] or [R]=2000 CFU/cm², [mitomycin C]=0, n=2 replicate plates for producer versus sensitive and n=1 for producer versus resistant.
Figure B.7: Alignment of colonies in two images removes artifacts. 

a, Reflections in the red channel cause artifacts (dotted arrow) that can be mistaken for real colonies (solid arrow, scale bar = 1 mm). These artifacts tend to be small and located in the upper left corner of real colonies. 

b, Colonies identified from two images of the same plate. 

c, Colonies after alignment, with paired colonies circled in red. 

d, Histogram of putative colony sizes before and after alignment. Aligning images and pairing colonies discards smaller artifacts and preserves the larger real colonies.
Figure B.8: Normalization of varying-MMC experiment data by average seeding density. Instead of measuring growth as the mean colony intensity (as in Fig. 1.4), we applied the same normalization of the total intensity as in the Fig. 1.3a analysis, yielding similar but noisier results.
References


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