Killing in response to competition stabilises synthetic microbial consortia

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The scale of the synthetic biological systems we can produce is limited by the burden that host microbes can bear. Division-of-labour can spread that burden across a community of cells but competitive exclusion inevitably leads to the removal of less fit community members over time. Here, we leverage competitive exclusion to develop a novel system to stabilise multi-species communities by engineering the dynamic secretion of a toxin. We show mathematically that such a system can produce stable populations with a composition that is tunable by easily controllable parameters. We implement the system in *Escherichia coli* and demonstrate community dynamics in a chemostat. This is the first system to use competitive exclusion to create a stable two-species consortia and the first to only require the engineering of a single strain.

Techniques for the assembly (*1*) or synthesis (*2*) of DNA sequences has enabled the construction of very large, chromosome scale (*3, 4*), synthetic biological systems. Further, recent

efforts to systematically characterise "parts" (*5, 6*) and develop software tools to produce DNA sequences from function specification (*7*), are leading us towards synthetic biological systems in which large numbers of regulatory proteins and promoters are involved. The production of each of these proteins sequesters resources that would otherwise be used by the host organism for growth and therefore leads to a reduction in growth rate (*8, 9*). This provides a selection advantage to loss-of-function mutants that arise, which suggests that the functional period for our increasingly complex synthetic biological systems of the future will become ever more ephemeral.

Attempts have been made to select system designs with minimal burden (*9*) or use burden driven feedback (*10*) to reduce selection pressure for mutants. More recently a method for the "entanglement" of coding sequences with essential genes has been demonstrated in which mutations lead to cell death (*11*). However, each of these approaches are limited to systems with very few genes. Another approach has been the development of a population replacement system, in which a population with degrading functionality can be displaced by a new, functioning population (*12*). However, this system does not prevent or even reduce mutation and so is still limited to systems that can function stably for a "reasonable" length of time before needing to be replaced.

Over the past decade there have been attempts at division-of-labour, in which a system is split into subcomponents and distributed into specialised subpopulations of cells (*13, 14*). This minimises the burden that is placed on individual cells which reduces, but does not remove, the selective advantage of loss-of-function mutations. In addition, the creation of synthetic communities allows the diversification and compartmentalisation of functions, modularisation, spatio-temporal control and mechanisms for biosafety (*15*).

The fundamental challenge with constructing such heterogeneous communities is the principle of competitive exclusion, which states that two species competing within the same niche

cannot coexist (*16*). The principle should, perhaps, include the caveat: "in stable environments and in the absence of other interactions". This caveat may help to explain supposed problems with the competitive exclusion principle such as the "paradox of the plankton" (*17*).

Wild bacteria live in complex communities (*18*) with mutualistic and competitive interactions producing complex dynamics (*19*). Previous attempts to design synthetic microbial communities have relied on spatial segregation (*20*) or mutualism (*21*) to maintain multiple sub-populations. The control of the density of single species populations has been achieved through the use of quorum sensing to control self-killing (*22*) and more recently this has been extended to a two species system (*23*). More complex predator-prey systems have also been developed which produce oscillatory populations of two strains (*24*). These systems involve the engineering of all strains within the community. However, this requirement may not be desirable in industrial settings and is clearly not possible when working in natural environments such as the human gastrointestinal tract.

In order to control a community through a single constituent, we require a mechanism that allows control of the growth rate of one or more competitors at a distance. Here we suggest bacteriocins, secreted anti-microbial peptides, as such a mechanism and detail the construction and characterisation of a control system that uses them, Figure 1. We have previously demonstrated the ability of the bacteriocin microcin-V to improve plasmid maintenance (*25*); a challenge which includes preventing competitive exclusion. Further, gram-positive bacteriocins have been used to produce commensal and amensal interactions along with all pairwise combinations of the two (*26*).

Using a simple model, Supplementary Information 1, we were able to simulate growth dynamics, Figure 2B, which show that without bacteriocin production ($k_B = 0$) both populations grow exponentially until substrate limitation leads to the gradual removal of the slower growing strain. However, when bacteriocin production is turned on, the susceptible competitor strain be-

gins to grow but then dies off as the bacteriocin concentration builds up, allowing the engineered strain to take over.

We used two engineered strains (EcN:pUC-GFP and EcN:pUC-GFP-MCC (*25*)) carrying the same burdensome plasmid, expressing high levels of GFP, but one was able to produce microcin-V. Each strain was competed against a faster growing competitor, the same host strain but without any plasmid, at a variety of initial ratios, Figure 2C. After 24 hours the community composition was determined using flow cytometry. The non-bacteriocin producing strain was out-competed by the faster growing competitor, leading to a reduction in the ratio of engineered strain to competitor. However, the bacteriocin producing strain was able to kill the competitor, leading to its near complete removal. Our fitted model is able to describe these dynamics well, as shown by the solid and dashed lines on the plot.

Analysis of our model shows that at lower chemostat dilution rates there are two stable steady states, in which one or the other of the two strains goes extinct, and an unstable coexistence steady state, Figure 2D. At higher dilution rates, only the competitor strain is able to dominate and with no dilution only the engineered strain will survive. The green region shows the area in which the community will tend towards engineered strain dominance over time; the blue region shows the area in which the competitor will dominate over time. As such, with control of dilution rate one could switch between states in which the engineered strain or the competitor strain dominates based on the current community composition.

To stabilise communities in which we have no control of environmental parameters, such as dilution rate, we need another mechanism for switching state from one strain to the other dominating. We can achieve this through control of the production rate of the bacteriocin, Figure 3A. At lower rates of bacteriocin production, the killing of the competitor is reduced, allowing it to dominate, whereas at higher bacteriocin production rates the engineered strain dominates, 3B. To implement such a system, we constructed a biological circuit in which expression of

microcin-V is repressed by TetR, the expression of which is induced by *N*-3-oxohexanoyl-homo-serine lactone (3OC6-HSL), Figure 3C. This allows us to exogenously control the killing of a competitor in a dose dependent manner, Figures 2D & E; which to our knowledge is the first demonstration of dose dependent killing. Communities of the engineered and competitor strains were produced with different initial ratios of the two strains. The communities are grown with or without 3OC6-HSL, creating high and low bacteriocin expression conditions, and diluted 1:100 into fresh media every 12 hours. The population ratio at which the engineered or competitor strain dominates moves dependent on 3OC6-HSL concentration, Figure 3F, as predicted by the model and visualised in Figure 3B.

The use of the quorum sensing molecule 3OC6-HSL to control expression of the bacteriocin allows us to build a system in which the density of the engineered strain dictates the expression level. Using cell density to control system response affords the creation of an autonomous population control system in which actions are taken by cells dependent on the state of the community rather than through external control. This approach has previously been taken to control homogeneous (*27*) and heterogeneous populations (*23*) with intracellular toxins. Alongside our intercellular toxin mechanism for controlling the fitness of a population at a distance, we can also incorporate intracellular toxins which have been used before for population control. In addition to the expression of these toxins, it has also been suggested that their respective immunity genes can be regulated to change the viability of the engineered strain (*28*). One could additionally include the production of a growth enhancing substrate, but we neglect this as it would either require engineering, or careful selection, of the competitor strain. The expression of all of these molecules can be constitutive or under control of a quorum molecule also expressed by the engineered strain, Figure 4A.

By defining a set of expressible parts that can be assigned to a strain, we can produce a model space with the stipulation that only a single strain is engineered. In this case the space consists

of 168 unique systems for which we can compare their ability to produce stable co-existing communities. From a relatively small number of available parts, we have a large number of possible models to choose from. In order to narrow our search to a smaller set of candidate models we perform model selection using approximate Bayesian computation sequential Monte Carlo (ABC SMC), Figure 4B. This approach allows us to efficiently approximate model and parameter posterior probabilities by random sampling and weight assignment through a series of intermediate distributions (*29, 30*). The output of ABC SMC is an approximation of the marginal posterior distribution of models and the marginal posterior distribution of parameters for each model. The final marginal posterior distribution indicates which models have the highest probability of producing the objective behaviour; in this case coexisting communities at steady state, Figure 4C. All of the top performing systems use bacteriocin. Indeed, the best system without bacteriocins is ranked 75. The best system produced by our model space exploration requires the control of all four of the possible genes; the bacteriocin, immunity and antitoxin are repressed by the quorum molecule, while the intracellular toxin is induced by it, Figure 4Di.

The systems without the intracellular toxin and antitoxin perform particularly well when one considers their relative simplicity. The best of these uses the quorum molecule to turn off expression of both the bacteriocin and immunity, Figure 4Dii. Intuitively, this leads to less killing of the competitor at higher densities, but also increased susceptibility of the engineered strain. A slightly simpler system uses the quorum molecule only to repress the immunity gene, Figure 4Dii. The simplest system to robustly achieve stable coexisting populations, requires just the bacteriocin to be repressed by the quorum molecule, Figure 4Div. Simulation of this system demonstrates that there are stable steady states at which there is coexistence of the engineered and competitor strains, Figure 4E. A more complete steady state analysis shows the steady state coexistence manifold with varying dilution rate and quorum molecule production

rate, Figure 4F. This shows that across a range of environmental dilution rates, we can produce any population ratio by tuning the rate of quorum molecule production. Intuitively one can understand that if the engineered strain produces the quorum molecule faster, its concentration will be higher at lower population densities. Therefore, the bacteriocin production will be turned off at lower engineered strain densities, leading to a lower ratio of engineered strain to competitor strain at steady state.

In the construction of the system, we add a "tuning knob" to the model suggested in Figure 4Div which allows some control over the rate of production of the quorum molecule using arabinose induction. This system was constructed using a modular approach which allowed us to test the function of each component as we progressed, Figure 5A. This enabled us to use modelling during the construction and characterisation to determine whether the system, as constructed, could achieve the stable co-existence that we desired. Fluorescent proteins were cloned downstream of each promoter for characterisation and flow cytometry was used to quantify expression levels. We used a Bayesian approach to fit Hill functions to the flow cytometry data, though this could not be done for the cell density dependent characterisation.

The full system was tested in a chemostat in order to determine whether we could reproduce the dynamics predicted by the modelling, with defined arabinose concentration and dilution rate, Figure 5B. The engineered and competitor strains were inoculated at equal concentrations and grown at a constant dilution rate of 0.05 h^{-1} with 0.2% arabinose in the media. Samples were taken periodically and run through a flow cytometry analysis pipeline. The model, predominantly parametrised from the system characterisation, was fitted to the population composition data. The results show an initial phase in which the competitor strain, growing faster than the engineered strain, begins to dominate, Figure 5C. As the bacteriocin concentration builds up, the competitor is killed off and the engineered strain starts to take over. The bacteriocin production is turned off as the engineered population density increases and bacteriocin in

the environment is removed through dilution, allowing the competitor strain to grow up again. The model predictions for the initial dynamics, for which we had no flow cytometry samples, looked somewhat dramatic. As such, we ran the chemostat again and sampled every hour for the first 12 hours, Figure 5D. The dynamics show a similar pattern, though with less sharp transitions. This demonstrates that our model is able to predict population dynamics which may not be intuitive and could be missed by some experimental protocols.

We have demonstrated that bacteriocins can be used to control the relative fitness of strains in competition with one another. The dynamic control of bacteriocins through quorum sensing, as has been demonstrated with intracellular toxins (*22,23*), can be used to extend the coexistence of, and even stabilise, microbial communities. Uniquely, this allows the construction of synthetic communities while only requiring the engineering of a single strain. This feature enables applications of synthetic biological systems in which native microbiomes will be competing against the engineered population. In the shorter term, this system can be used in industrial biotechnology for the control of communities in bioreactors in which division-of-labour is desirable to prevent burden. Our ABC SMC model exploration has suggested a reasonably simple upgrade to the system we have constructed that should make the community control more robust. This method of model exploration could easily be extended to explore a model space in which the requirement for single strain engineering is dropped or other control mechanisms, such as substrate cross-feeding, are introduced.

The last decade has seen synthetic biology recognise the importance of context, be it compositional, intracellular or environmental (*31, 32*). However, community context has largely been neglected, with the consequence that we have limited ourselves to building systems of single, homogeneous populations that are only capable of functioning in controlled environments. Contending with intracellular context has required the use of feedback to take into account the cell's response to our demands (*10, 33*). This work is the first to demonstrate working with

community context rather than against it. We embrace competitive exclusion and demonstrate that feedback is key to the construction of stable synthetic microbial communities.

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Fig 1

A model for mixed community co-existence using bacteriocins and competitive exclusion requiring the engineering of only one strain. The engineered strain secretes a quorum molecule and a bacteriocin which can kill the competitor strain. When the engineered strain is at high cell density, there is a high concentration of quorum molecule which represses the expression of the bacteriocin. This allows the competitor to out-compete the engineered strain through competitive exclusion. As the engineered strain's density decreases, the concentration of quorum molecule decreases and the bacteriocin is expressed. This kills off the competitor leading to formation of a niche into which the engineered strain can grow.

Fig 2

Bacteriocins can controllably be used to overcome competitive exclusion. (A) In an environment in which an engineered strain competes with a competitor for a substrate, bacteriocins can be used to combat competitive exclusion (B). (C) We compete two engineered strains against a competitor, at different initial ratios, for 24 hours. If the engineered strain cannot produce bacteriocin (triangles and dashed line) it is outcompeted. However, when bacteriocin is produced (circles and solid line), the competitor is killed. (D) By changing the dilution rate of the chemostat environment, we can control whether the competitor or engineered strain dominates.

Fig 3

Exogenous control of bacteriocin production. (A) Adding the ability to alter the production rate of the bacteriocin allows another dimension of control over the relative fitness of the two strains. (B) Lower bacteriocin production rates leads the community to tend towards competitor strain dominance (blue area). Increasing the bacteriocin production rate improves the relative

fitness of the engineered strain, allowing it to dominate (green area). (C) Here we have used the *luxR*-AHL system to induce the expression of TetR which represses bacteriocin expression. (D) As the concentration of AHL is reduced, the repression of the bacteriocin is released, leading to larger zones of inhibition on a lawn of a susceptible strain. (E) Image processing on these images enables us to produce a dose response curve to which we fit a Hill function. (F) In a passaged batch competition assay, we test a high and low bacteriocin production rate. The strain that dominates depends on the ratio of engineered to competitor strain but the ratio at which the dominance changes is defined by the bacteriocin production rate, as predicted by the model (B).

Fig 4

Exploring the space of possible models of population control to find the best system. (A) The engineered strain can produce an intercellular toxin and its antitoxin, an intracellular toxin and its antitoxin, and a substrate to enhance growth. All of these can be positively or negatively regulated by a self-produced quorum molecule, allowing density dependent control of the engineered cell's actions. (B) Once the model space has been defined, we explore it using ABC SMC to determine which models with which parameters are capable of producing stable coexisting populations. (C) The ordered model marginal posterior probabilities. Most models can theoretically produce stable coexistence but require very tight control of model parameters making their posterior probability very low. The Boxes for each model are coloured according to the model's complexity; models with a larger number of parameters are closer to the yellow and fewer parameters are close to blue. (D) The best system (i) requires control over the expression of four genes. Systems that don't require intracellular toxin or immunity perform well for their level of complexity (ii - iv). In fact the simplest system able to produce stable coexistence requires only the control of bacteriocin production by the quorum molecule (iv). (E) This simple system is simulated to show how the system behaves at different rates of quo-

rum molecule production and chemostat dilution rate. These simulations demonstrate that the system can achieve stable coexisting steady states. (Initial conditions: solid line = steady state cultures mixed 1:1, dashed line = steady state cultures mixed 1:1 and diluted 1/100, dashed line = steady state cultures mixed 1:1 and diluted 1/1000). (F) The ratio of engineered strain to competitor strain at steady state can be controlled by both chemostat dilution rate quorum molecule production rate.The red points show the regions for the simulations in (F).

Fig 5

Construction and characterisation of the community control system. (A) The system was constructed in three parts: arabinose inducible expression of LuxI, AHL inducible expression of TetR, and TetR repressible expression of microcin-V. Each part was characterised by cloning a fluorescent reporter protein (GFP or BFP) downstream of the relevant promoter and measuring expression using flow cytometry. Hill functions were fitted to the data using a Bayesian method (solid line = mean prediction, dashed lines = 95% confidence in the mean). (B) A schematic of the system and environment as implemented. The concentration of arabinose and the dilution rates are parameters defined by the experimental set up. (C) The engineered strain, carrying the system above, was competed against a competitor strain. The experiment was run in a chemostat with 0.2% arabinose in the media, enabling the density dependent suppression of bacteriocin expression and allowing the competitor to re-establish as the bacteriocin is diluted (0.05 hr^{-1} dilution rate). (D) The chemostat was run again with sampling every hour for the initial growth period in order to check the model prediction from (C).

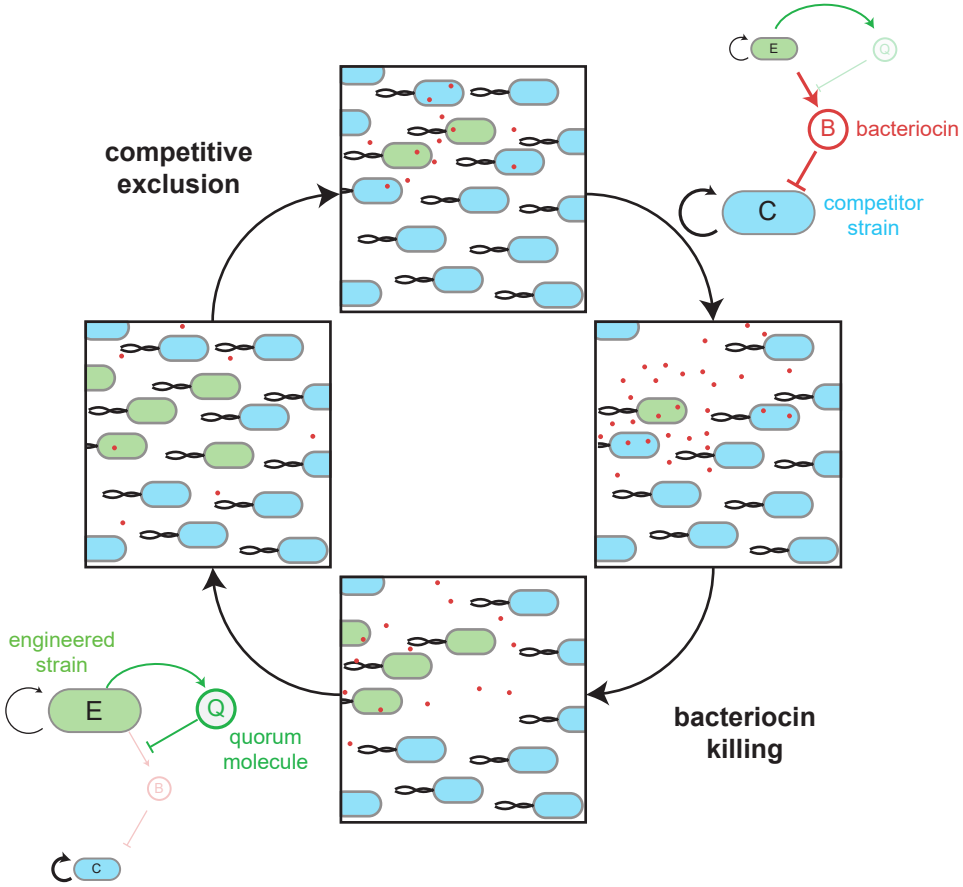
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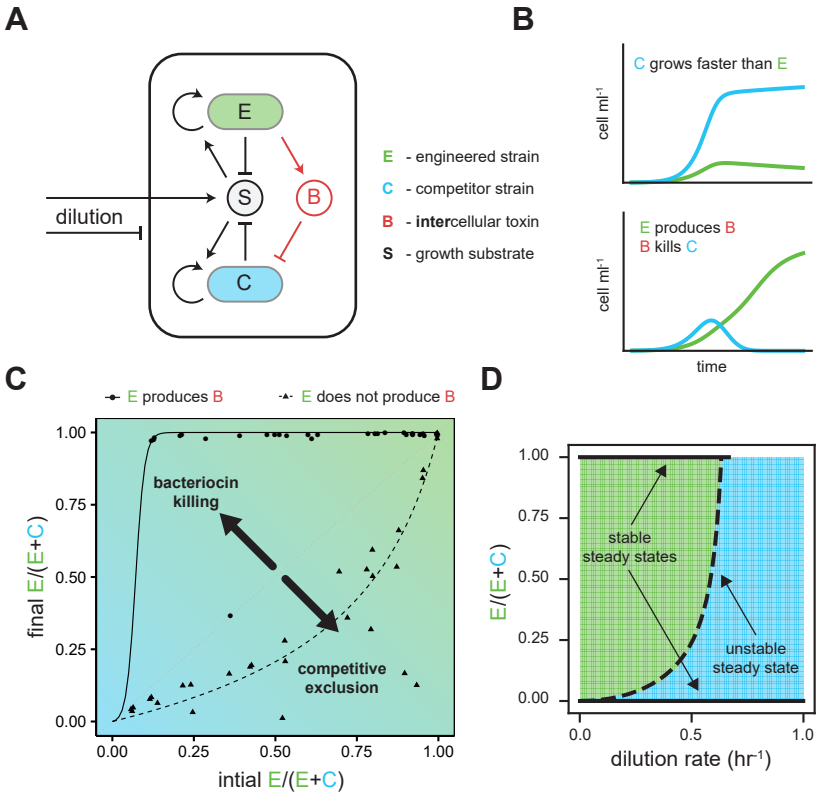
Figure 1: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C - competitor strain
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B kills C
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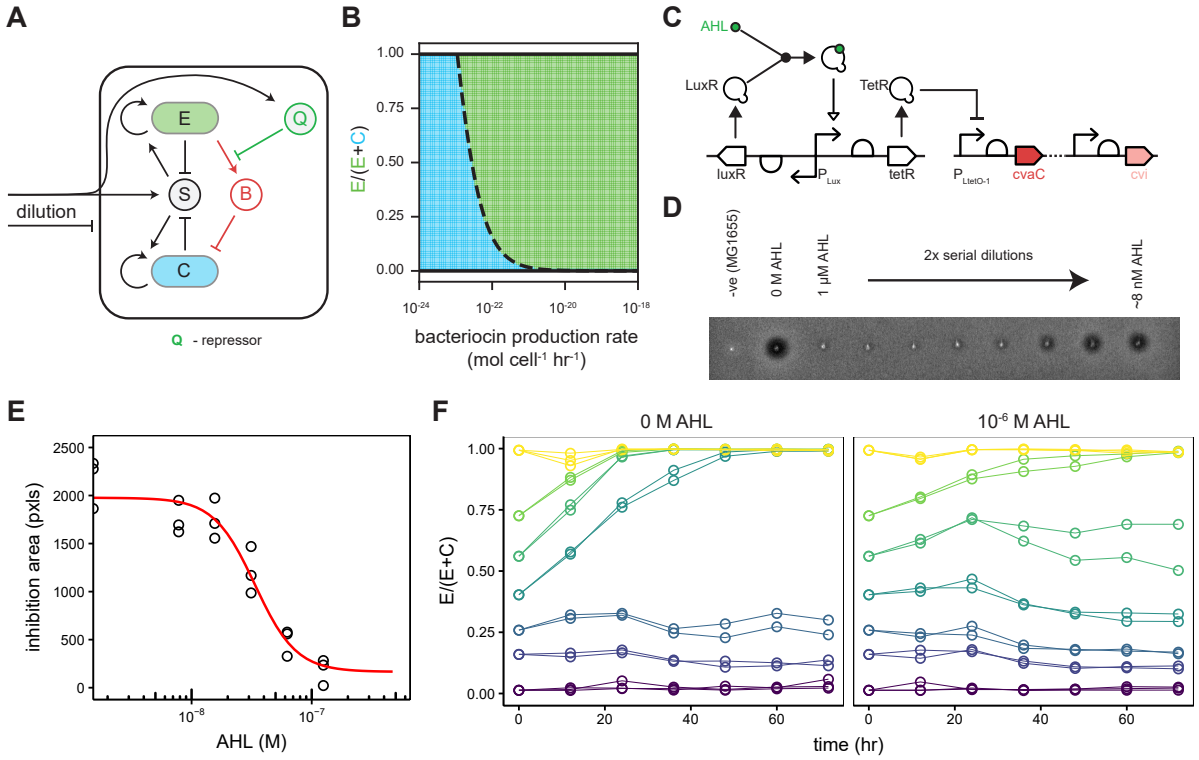
Figure 2: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bacteriocin production rate
(mol cell-1 hr-1)
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2x serial dilutions
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inhibition area (pxls)
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1500
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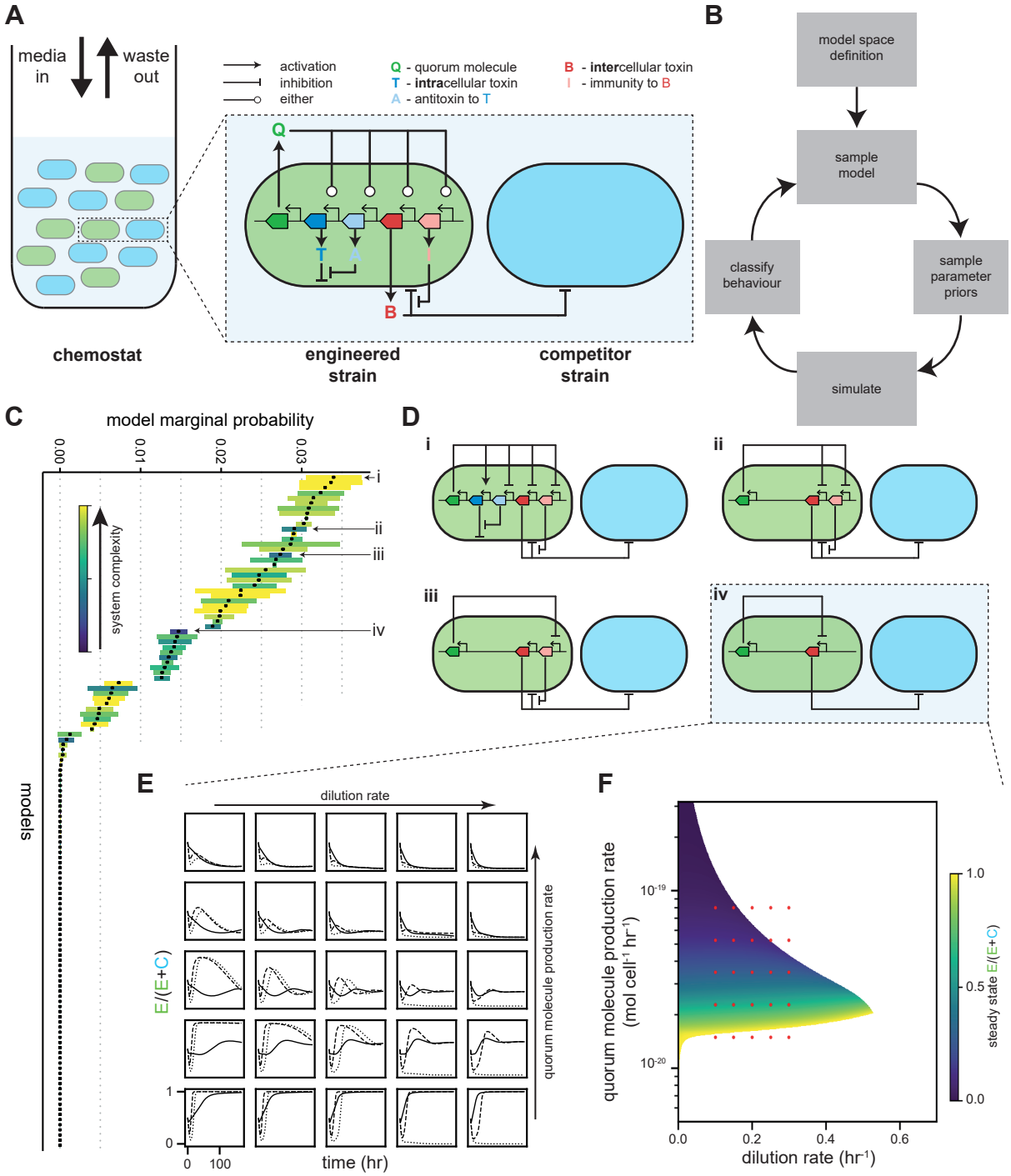
Figure 3: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sample model
sample parameter priors
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Figure 4:

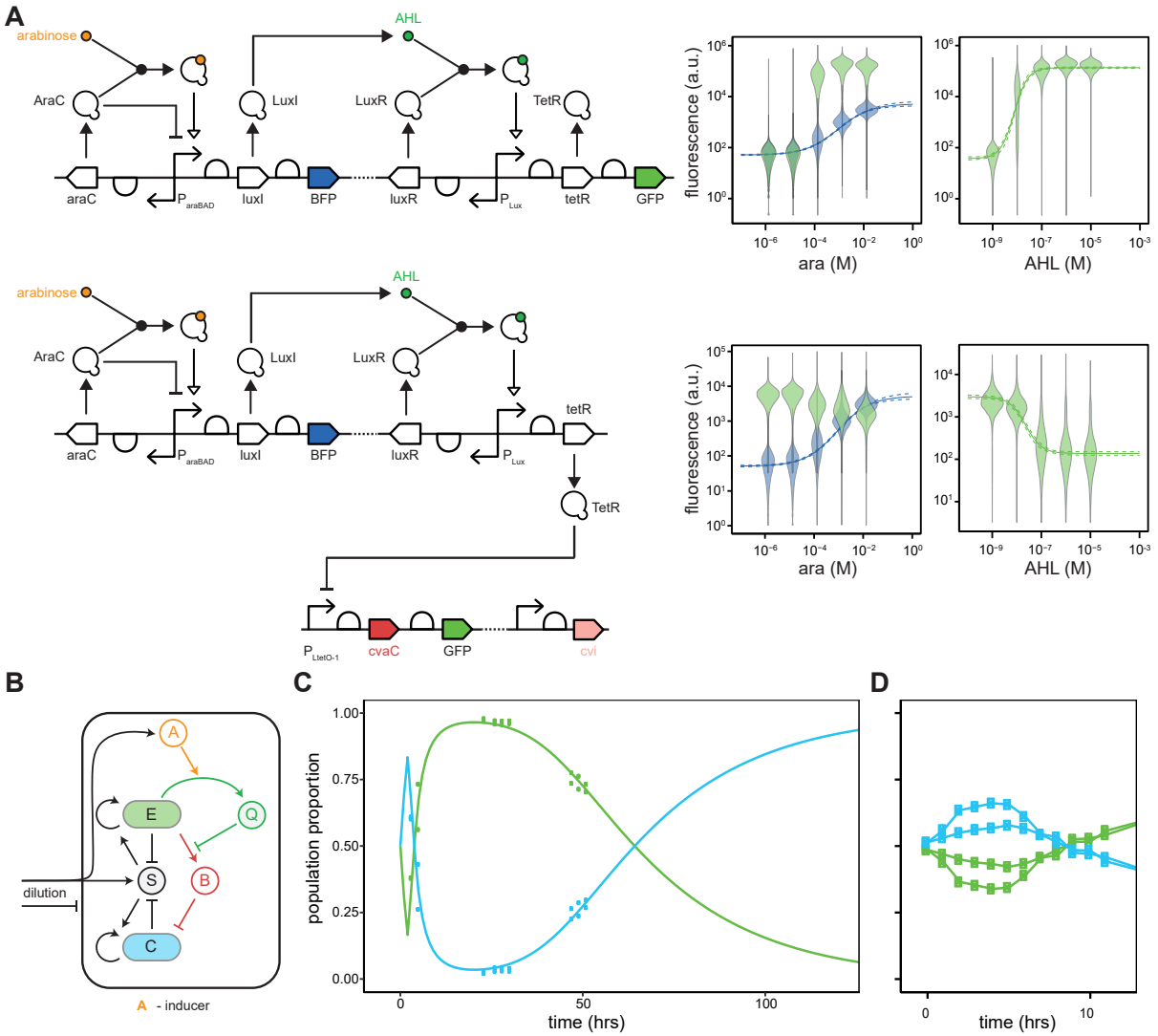
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Figure 5: