SYNTHETIC BIOLOGY

Rock-paper-scissors: Engineered population dynamics increase genetic stability

Michael J. Liao[1,2], M. Omar Din[2], Lev Tsimring[2,3], Jeff Hasty[1,2,3,4]*

Advances in synthetic biology have led to an arsenal of proof-of-principle bacterial circuits that can be leveraged for applications ranging from therapeutics to bioproduction. A unifying challenge for most applications is the presence of selective pressures that lead to high mutation rates for engineered bacteria. A common strategy is to develop cloning technologies aimed at increasing the fixation time for deleterious mutations in single cells. We adopt a complementary approach that is guided by ecological interactions, whereby cyclical population control is engineered to stabilize the functionality of intracellular gene circuits. Three strains of *Escherichia coli* were designed such that each strain could kill or be killed by one of the other two strains. The resulting "rock-paper-scissors" dynamic demonstrates rapid cycling of strains in microfluidic devices and leads to an increase in the stability of gene circuit functionality in cell culture.

Over the past two decades, synthetic biologists have developed sophisticated molecular circuitry to control the activity of individual cells (*1*–*9*). Over time, such systems inevitably lose function due to evolutionary selection pressures that lead to runaway mutations (*10*). Approaches to this challenge include the integration of recombinant elements into the host genome (*11*) and the use of plasmid-stabilizing elements (*12*), synthetic "kill switches" (*13*), or synthetic amino acids (*14*–*17*). Although stabilizing elements can prolong the march to mutation, evolution will inevitably render stabilizing elements ineffective (*10*). This is particularly true in the case of in vivo applications where delivery of antibiotics is difficult (*18*–*23*) or where the interruption of plasmid function is particularly problematic (*24*). We show how a small ecosystem can be engineered to stabilize gene circuit functionality in a manner that complements genetic engineering at the single-cell level. Rather than housing stabilizing elements within a single strain, we decoupled the components of stability to different subpopulations of a community such that a runaway mutation in one strain would not cause the stabilizing element to fail.

We created three engineered *Escherichia coli* strains, each of which contains a specific toxin and antitoxin system (TA module). In monoclonal populations, TA modules aid in plasmid stabilization because mutation or loss of the antitoxin results in cell death due to either the presence of extracellular toxin produced by the healthy bacteria or killing of newborn plasmid-free cells through the prolonged persistence of toxin-producing mRNA (*12*). By expanding the mechanism of the TA module to multiple strains, each TA module gives its host strain the added ability to kill any other strain that does not have the corresponding antitoxin, enabling external control over displacement of the existing population (Fig. 1A). We used this strategy to create a "rock-paper-scissors" (RPS) dynamic by adding a secondary antitoxin into each strain so that each strain can be killed by a subsequent strain in a cycle (Fig. 1B). This RPS system can be coupled with any circuit of interest to increase stabilization. In this combined system, a circuit that has mutated in one strain can be replaced by the introduction of a fresh batch of the next RPS-housing strain. Thus, rather than attempting to "beat Darwin," we have developed an engineering strategy that enables external control over the evolution and composition of the community.

As an initial test of the efficacy of this approach, we created a three-strain RPS system in which each strain contains a distinct toxin-antitoxin pair, as well as a secondary antitoxin to one other strain (Fig. 1C). To achieve this, we used colicins, naturally occurring TA systems that are lethal to certain *E. coli* strains and are effective antagonistic agents within *E. coli* populations in vivo (fig. S1) (*25*–*28*). Strain R contains a plasmid producing colicin E7 and E7 immunity protein and a colicin V immunity protein. Strain P contains a plasmid producing colicin E3 and E3 immunity protein and an E7 immunity protein. Strain S contains a plasmid producing colicin V and V immunity protein and an E3 immunity protein. Thus, each pair of strains has one "dominant strain" that is immune to the toxin produced by the other strain and a "susceptible" strain that is vulnerable to the toxin produced by the other.

Because displacement within microbial cocultures is typically dominated by differences in growth rate, we first measured the growth rate of each RPS strain (Fig. 1D). We validated efficacy of the engineered colicin kill circuits in microfluidic devices by coculturing the strain pairs at a 1:2 ratio of dominant to susceptible. In each case, we observed a rapid increase of the dominant strain and decay of the susceptible strain, confirming that the colicin produced by the dominant strain was effective against the susceptible strain, but not vice versa (Fig. 1, E and F, and movies S1 to S3).

In any environment, once engineered cells have mutated, they cannot be reverted to the nonmutated state. They must be removed and replaced by healthy cells. Such a "system reboot" interrupts dynamic circuit function in the environment. A multistrain RPS system should in theory enable the removal of mutated or mutation-prone cells without interrupting the dynamics of the circuit of interest. After validating the expanded function of the TA module in an RPS system, we tested the utility of the stabilizing RPS elements with a genetic circuit that is subject to high selective pressure. We chose the quorum-driven synchronized lysis circuit (SLC), which has been investigated for microbial therapeutics such as drug delivery to tumors in vivo (*21*, *29*). With the SLC circuit, a quorum-sensing molecule (acyl-homoserine lactone) gradually accumulates in the growth environment in proportion to population density. When the population reaches a threshold density, synchronized lysis eliminates ~90% of the bacteria, leaving ~10% to reseed population growth. The resulting dynamics of the cell population are cycles of cell growth and synchronized lysis. However, when used in in vivo environments where selective media cannot be used, plasmid loss or mutations are expected to result in loss of function over long time periods.

We tested whether successful plasmid stabilization from the RPS system would result in strain takeover without any interruption of the oscillatory dynamics (Fig. 2A). We integrated the SLC and TA modules into a two-plasmid system, with one plasmid containing the lysis gene, E, from the phage ϕX174 (X174E); a TA module; immunity proteins; and an activator-reporter plasmid (Fig. 2B). Using this architecture, we created three strains that exhibited simultaneous cycles of synchronized lysis and constant RPS competition (Fig. 2C).

We characterized each RPS lysis strain individually (fig. S2) and validated each strain pair in microfluidic devices with initial seeding ratios of both 1:1 and 1:5, dominant to susceptible. We observed that in addition to successful strain displacement, the function of the SLC remained unaffected, as all strains lysed synchronously within each chamber (movie S4). At a 1:1 dominant to susceptible ratio, takeover occurred in 100% of cultures (n = 35) for each strain pair and a single lysis event was sufficient to complete strain takeover (Fig. S3A). For the three pairings at a 1:5 ratio (strains 1 and 3, 3 and 2, and 2 and 1) dominant strain takeover occurred

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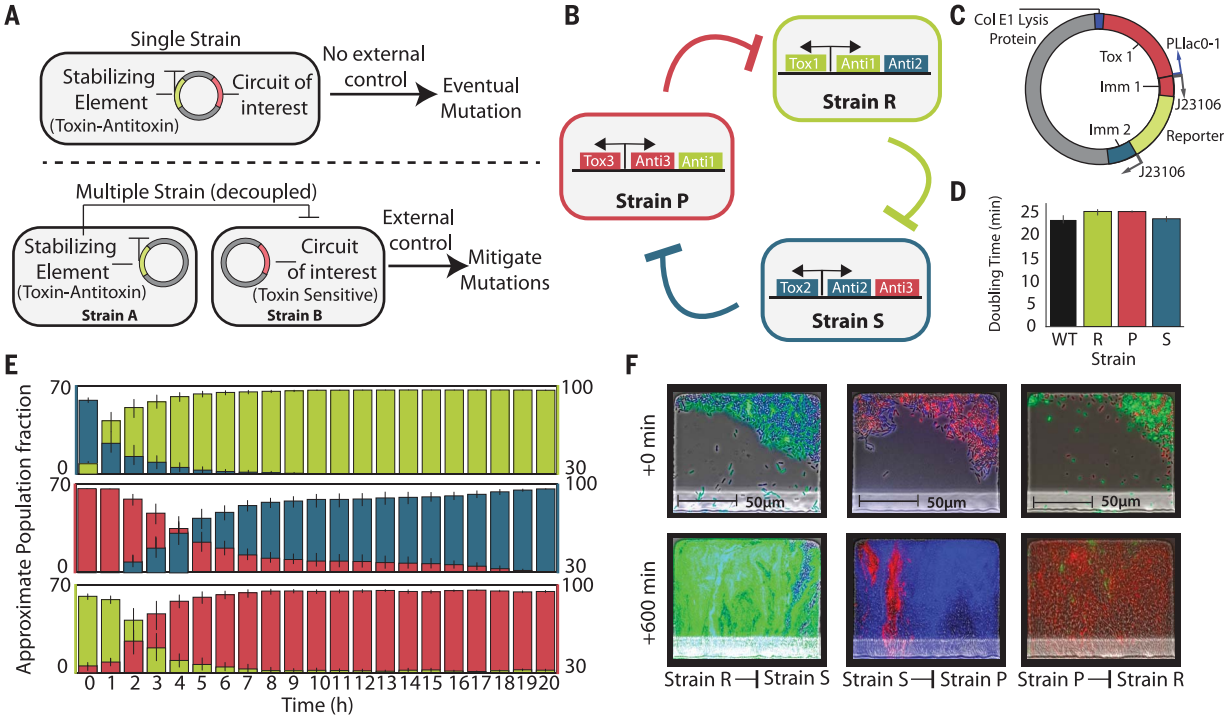

Fig. 1. Expanding the function of plasmid-stabilizing TA modules in multistrain populations. (**A**) In monoclonal populations, plasmid-stabilizing TA modules enable the killing of progeny cells that have mutated or lost the antitoxin gene. When expanded to a system of $n > 1$ strains, the strain that contains the TA module maintains a self-stabilizing function while gaining the added ability to kill any strain that does not have the antitoxin. (**B**) Each strain produced its own TA pair while also producing the antitoxin of the following strain, enabling sequential strain inhibition. Strain R contained toxin 1, antitoxin 1, and antitoxin 2. Strain P contained toxin 3, antitoxin 3, and antitoxin 1. Strain S contained toxin 2, antitoxin 2, and antitoxin 3. (**C**) General plasmid diagram of the RPS strains. (**D**) Batch culture growth rates of the engineered RPS and wild-type *E. coli* MG1655 strains ($n = 3$). All strains were started from the same diluted density and under the same growth conditions. (**E**) Bar plots depicting the approximate population fractions over time for strain cocultures seeded at an initial ratio of 1:2 dominant to susceptible ($n = 8$). From top to bottom: Strain R (green) inhibits strain S (blue); strain S (blue) inhibits strain P (red); and strain P (red) inhibits strain R (green) (bottom). (**F**) Fluorescence microscopy images showing a composite of phase-contrast, green fluorescent protein, cyan fluorescent protein, and red fluorescent protein fluorescence. From left to right: Strain R (green) inhibits strain S (blue); strain S (blue) inhibits strain P (red); and strain P (red) inhibits strain R (green).

in 100%, 100%, and 92% of the cultures ($n = 396$), respectively (fig. S3B and movies S5 to S7). In all cases, the function of the SLC circuit was not interrupted during strain changeovers (Fig. 2, D to F).

Although coculture of dominant-susceptible strain pairs enabled consistent strain displacement, an ecosystem consisting of three antagonistic strains may introduce novel emergent properties when cultured simultaneously. We first developed a mathematical model that described the switching population dynamics and reduced it to a discrete-time map that permitted analytical predictions for the switching frequency and interval duration (supplementary text). We found that when all three strains were present simultaneously, cycles of population dominance among them was an emergent property of the system. Under the assumption that the system was closed to the introduction of fresh cells, the strain that initially started at a higher concentration subsequently dominated the trap (fig. S4). Alternatively, given a small constant supply of each strain, the trajectory of the three-strain system converged to a stable limit cycle regardless of the initial ratios between the three strains (Fig. 2G).

To test this emergent property, we simultaneously loaded all three strains into a single microfluidic device. By applying a high flow rate after cell loading in the microfluidic device, we flushed a majority of cells out of the device, leaving ~10 cells in each culture region (resulting in widely varying ratios of three strains). We analyzed four separate microfluidic experiments totaling 1582 individual microfluidic culture regions and observed that the system exhibited cyclical behavior between the three strains, such as strain 2 to strain 1 to strain 3 (fig. S5). As predicted by the model, the three strains competed until two were eliminated and a single strain survived (Fig. 2H). The consistent convergence of the RPS ecosystem to the expected behavior supports the feasibility of engineering communities to exhibit predictable and precise dynamics.

The potential for synthetic microbial communities to enable new biotechnological applications has long been known (*30*–*33*). Engineered communities exhibit complex functions that can be difficult to engineer into single populations (*24*, *34*). An RPS dynamic strategy enables external control over the evolution and composition of the community, enabling displacement of undesired strains through manual input without interrupting circuit function. To demonstrate this concept and to prolong the functional stability of the SLC using the RPS system, we attempted to extend dynamic expression in the absence of the selective antibiotic (kanamycin). In this case, selective pressure against the SLC activator plasmid should result in rapid loss of function.

We investigated two scenarios (Fig. 3A). In scenario 1, we cultured each strain individually in LB medium lacking kanamycin. In the absence of selective antibiotic, loss of circuit function was universal and resulted in the loss of fluorescence expression and synchronized lysis dynamics. We recorded the elapsed time before synchronized lysis was lost ($n = 16$) and found that between 80 and 90% of plasmid loss occurred within 32 hours, after which the strains grew uncontrollably (Fig. 3, B and C). In scenario 2, we started the experiment with a coculture of strain 2 and strain 1, allowing strain

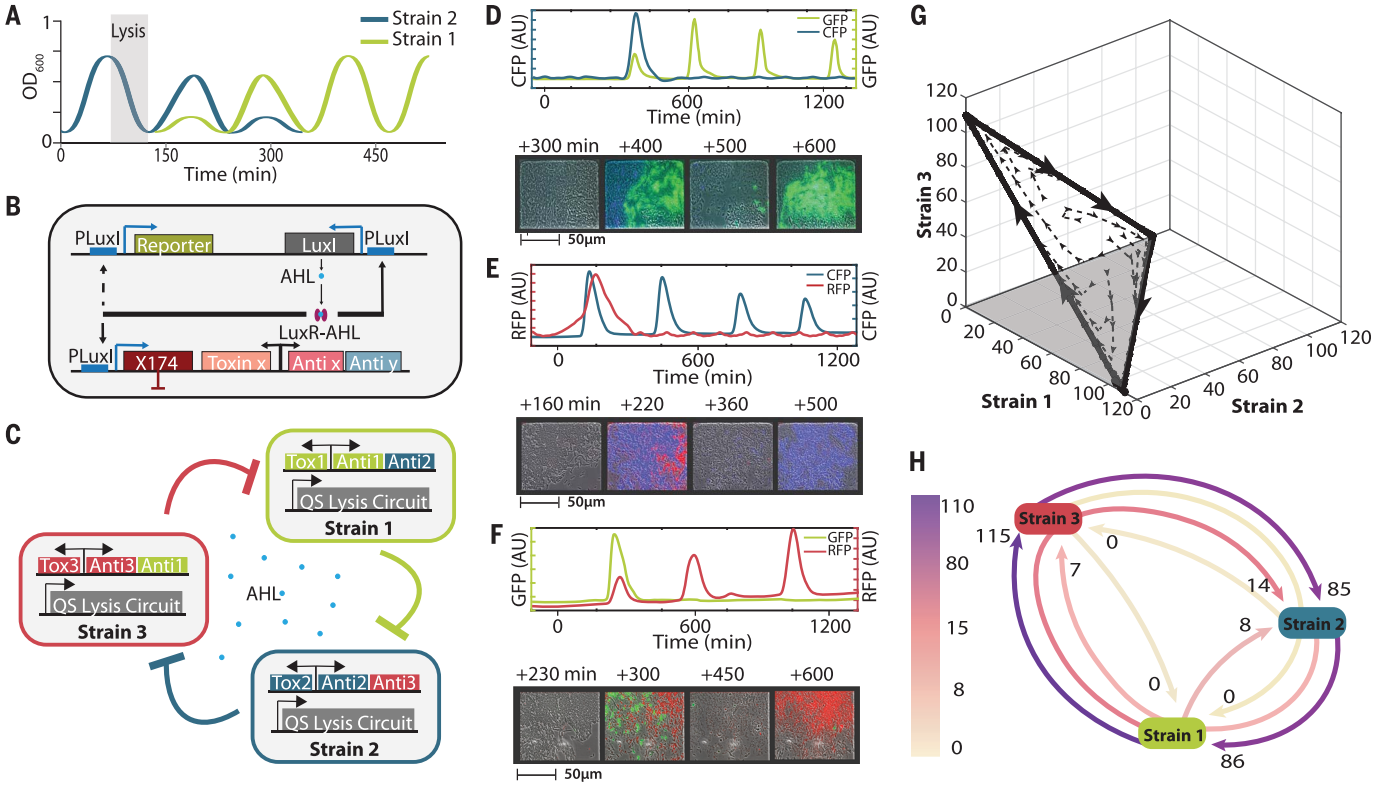

Fig. 2. Development of engineered RPS dynamics. (**A**) Schematic of the envisioned two-strain population dynamics with the integration of the SLC. Depicted is continuous synchronized population lysis during a strain-takeover event. (**B**) Genetic diagram of the quorum-sensing SLC and TA module. The first plasmid contains the X174E lysis protein driven by the *luxI* promotor and the corresponding toxin-antitoxin-antitoxin gene. A second, "activator plasmid" contains the *luxI* and *luxR* genes driven by their native promoter, *pLuxI*. (**C**) Each strain produced its own TA pair while also producing the antitoxin of the following strain. All three strains used the same Lux-AHL quorum-sensing system to drive fluorescent reporter protein expression and self-limiting synchronized lysis. (**D**) Time series of fluorescence expression and video stills of a coculture seeded at a 1:1 ratio of strain 1 to strain 2 exhibiting strain takeover by strain 1 in a microfluidic chamber. CFP, cyan fluorescent protein; GFP, green fluorescent protein. (**E**) Time series of fluorescence expression and video stills of a coculture seeded at a 1:1 ratio of strain 2 to strain 3 exhibiting strain takeover by strain 2 in a microfluidic chamber. RFP, red fluorescent protein. (**F**) Time series of fluorescence expression and video stills of a coculture seeded at a 1:1 ratio of strain 3 to strain 1 exhibiting strain takeover by strain 3 in a microfluidic chamber. (**G**) Limit cycle of the three-strain system demonstrating convergence to stable transitions between the three strains regardless of initial strain ratios. (**H**) Diagram representing strain-takeover events observed when all three engineered strains are cultured simultaneously. Arrows represent each possible transition event between the three strains; arrow colors indicate the number of observed transitions for each arrow.

1 to take over the trap. After 12 hours, strain 3 was added to displace strain 1, and at 30 hours, strain 2 was reintroduced to displace strain 3. Therefore, starting with strain 2, we completed a full cycle in a way that would likely be maintained over an indefinite period. By manually adding subsequent strains before the previous strain mutated, we were able to prolong the duration of circuit stability without interrupting circuit function (Fig. 3D).

To further explore the effect of the RPS system on a mutable genetic circuit, we conducted a batch passage experiment to compare two scenarios (Fig. 4A). In the first scenario, we inoculated culture medium containing antibiotics with strain 1 and passaged the culture into fresh growth medium every 12 hours. In scenario 2, we also inoculated a culture with strain 1 and passaged every 12 hours; however, every three passages, we also added the next strain in the RPS cycle.

We observed that in scenario 1, mutations began at passage 4 and once the loss of synchronized lysis occurred it was never recovered. However, in scenario 2, we observed a delay in the loss of synchronized lysis as well as recovery of lysis function even after it had been lost in previous passages (Fig. 4B). Sanger sequencing of a 1–kilobase pair region containing the X174E lysis gene and Lux cassette revealed that the RPS strategy reduced the occurrence of mutations over the same duration. Therefore, by shifting the challenge of eliminating individual mutated cells to the elimination of an entire population, we demonstrated the ability of the RPS system to prevent loss of function of a circuit, whether by mutation or plasmid loss.

As an expandable and modular platform to improve genetic stability, the RPS system provides an additional layer of control, allowing it to be combined with other traditional strategies to maintain plasmid stability. This approach may enable synthetic biologists to engineer systems that can be maintained long term in the absence of selective antibiotics, affecting applications ranging from therapeutics to bioremediation, production, and sensing technologies. Owing to the prevalence of colicin resistance, which can appear relatively quickly depending on environmental conditions, these applications will require the development of new RPS strains that are armed with different cell death systems. These systems may consist of other antimicrobial peptides, mechanisms that regulate toxin production such as orthogonal quorum systems, or perhaps even strains that naturally exhibit predator-prey relationships. Overall, the use of engineered ecological systems to circumvent selective pressure and improve the stability of genetic constructs may enable new possibilities for applied dynamical synthetic communities. One of these possibilities is the stabilization of genetic circuits for therapeutic delivery in vivo, where RPS strains not only

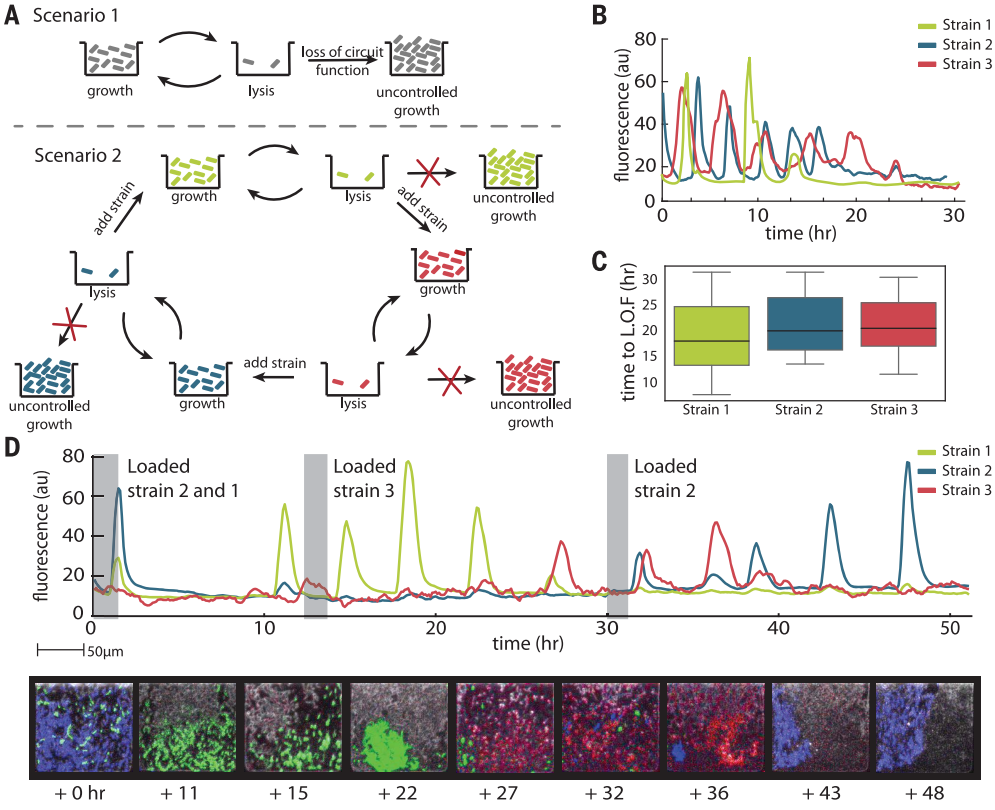

Fig. 3. Prolonging circuit function in the absence of antibiotics. (**A**) Scenario 1 depicts a system in which oscillations between growth phase and lysis transition to uncontrolled growth caused by loss of circuit function. Scenario 2 depicts a cycle composed of the three-strain system in which the transition to the uncontrolled growth is prevented through the addition of the next strain of the system. (**B**) Consistent with scenario 1 from (A), each strain was cultured in the absence of kanamycin for a duration of 32 hours. Time trace of fluorescence expression for each of the strains shows the loss of circuit function over time. (**C**) Boxplots depicting the time to loss of function (L.O.F.) for each strain (n = 16). (**D**) Consistent with scenario 2 from (A), strains were loaded sequentially, starting with strain 2 and strain 1. Time trace of GFP, CFP, and RFP fluorescence expression of a single trap that demonstrates cycling through all three strains in a single continuous uninterrupted run. Bottom, Video stills showing a composite of phase contrast and fluorescence at the indicated time points. AU, arbitrary fluorescence units (background subtracted).

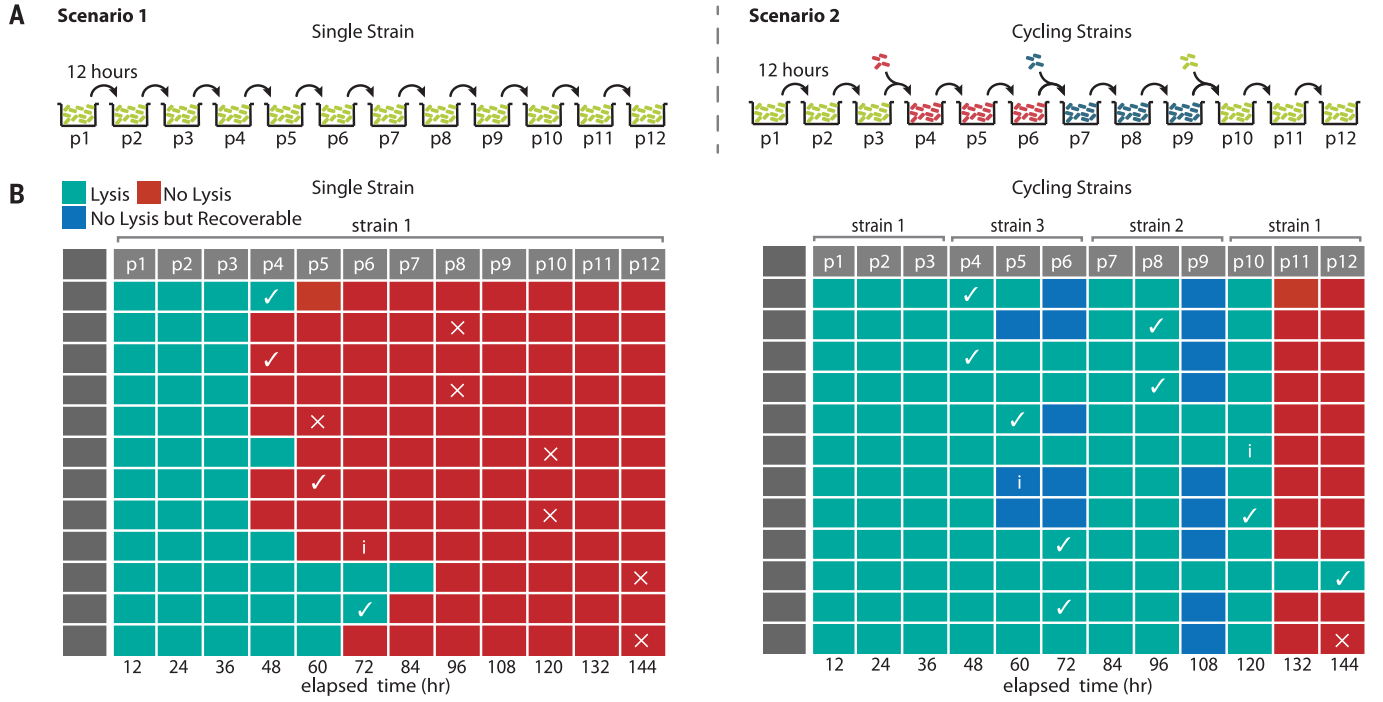

Fig. 4. RPS effect on a mutable genetic circuit. (**A**) Scenario 1 depicts a system in which strain 1 was cultured in a 96-well plate for the duration of a single lysis event and passaged every 12 hours. Scenario 2 depicts a system in which strain 1 was cultured in a 96-well plate for the duration of a single lysis event and passaged every 12 hours. On every third passage, the next strain of the RPS system was simultaneously added to the culture. (**B**) Side-by-side comparison of each scenario (n = 12) from (A) for a duration of 12 passages. Green squares represent functioning synchronized population lysis. Red squares represent loss of synchronized population lysis. Blue squares represent loss of synchronized population lysis that was later recovered. Additionally, 12 corresponding samples from each scenario were Sanger sequenced across a 1000–base pair region containing the X174E lysis protein and Lux cassette. Checkmark represents a correct sequence, "X" represents an incorrect sequence, and "i" represents an inconclusive sequencing read. All strains were started from the same diluted density and under the same growth conditions.

retain circuit function, but can also be leveraged as a platform to deliver multiple therapeutics over time.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S7
Tables S1 to S3
Supplementary Text
Movies S1 to S7

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Rock-paper-scissors: Engineered population dynamics increase genetic stability

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Stabilizing synthetic gene circuits

Making synthetic gene circuits in bacteria is one thing, but making them stable under selective pressure with high mutation rates is another. Liao *et al.* addressed this problem with an ecological strategy in which they created three strains of bacteria, each of which could kill or be killed by one of the other strains (see the Perspective by Johnston and Collins). Once the first strain of bacteria hosting the engineered circuit underwent mutations that decreased function, the system could be "rebooted" by addition of another strain that killed the first but also contained the desired synthetic circuit, allowing its function to proceed unperturbed. This strategy provides a way to control synthetic ecosystems and maintain synthetic gene circuits without using traditional selection to maintain plasmids with antibiotics.

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