SYNTHETIC BIOLOGY

Engineering microbial peer pressure

A distributed rock-paper-scissors network extends the lifetime of shared gene circuits

By **Chad W. Johnston** *and* **James J. Collins**

Synthetic biologists aspire to reengineer the molecular basis of life to perform new-to-nature functions. Their efforts have led to the development of increasingly complex genetic circuits that have been used to create programmable cells that can serve as living diagnostics (*1*) and living therapeutics (*2*). However, these engineered organisms benefit from mutating or disabling synthetic circuits because they often impose fitness costs, thus limiting the practical applications of designer cells (*3*). On page 1045 of this issue, Liao *et al.* (*4*) demonstrate that an engineered ecology can be used to maintain circuit fidelity and circumvent evolutionary interference. By overlaying a network of mutually exclusive gene pairs onto bacteria with a shared circuit, three engineered strains can be used to seamlessly displace each other after serial addition to a continuous culture. Iteratively removing older bacteria eliminates potential mutants, allowing the functionality of the shared circuit to be preserved.

Natural selection has guided the evolution of life and driven the development of biological parts that can be assembled to perform complex functions. Many of the components that control cellular programming—transcriptional repressors, activators, biomolecular sensors—often function in ways that are analogous to electronic components and, as such, can be used to create biological circuits, including oscillators (*5*), toggle switches (*6*), and logic gates (*7*). By assembling nonnative circuits, synthetic biologists can build designer organisms that respond to stimuli in a reliable manner (*8*), paving the way for engineered probiotics and designer cellular therapeutics, for example.

Ironically, the same evolutionary processes of mutation and selection also limit the efficacy of engineered circuits and programmable cells. Because synthetic gene circuits use resources that cells would otherwise devote to growth, engineered microbes replicate slower than their wild-type counterparts. Bacteria that evolve mutations to disable these circuits can undo this deficiency and quickly outcompete nonmutated engineered neighbors. In the laboratory, antibiotic-resistance genes give scientists a selectable marker that encourages retention of plasmids—the mobile DNA elements into which gene circuits are commonly encoded and delivered to cells—but this technique is not amenable to would-be engineered cells.

Previous efforts in this area have mostly focused on limiting the mutation of synthetic systems to ensure the retention of desired genes and behaviors. Recoding an organism's DNA en masse has proven to be an effective tool for limiting the mutational landscapes available to engineered bacteria (*9*) and viruses (*10*), and "kill switch" systems have been used to link the survival of modified organisms to signals detected by functional gene circuits (*11*). However, a general means to maintain gene circuit function has proven elusive, because individual cells that can overcome the imparted selective pressure will rapidly dominate the population.

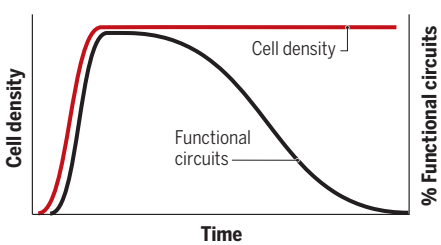
Instead of relying on individual bacteria to police themselves, Liao *et al.* opted to use peer pressure. They implemented a strategy for DNA retention that uses a toxin-antitoxin system, in which bacteria produce a selective antibacterial toxin to kill off microbes that lack the antitoxin encoded in the engineered gene circuit (*12*). This community control provides a considerable fitness advantage to toxin producers and ensures that cells maintain the plasmid to keep the life-saving antitoxin. Moreover, the bactericidal effects of the toxin maximize the probability that cells carrying the toxin-antitoxin circuit will quickly take over naïve populations, killing sensitive bacteria and removing mutants, effectively resetting the gene pool.

To engineer this system as a cyclical ecology, one additional antitoxin gene was added to each toxin-antitoxin pair, creating a synthetic three-part "rock-paper-scissors" (RPS) network in which each strain would be capable of replacing the one that preceded it. If all three components of the network share a second common circuit, this strategy of serial rebooting could be used to considerably elongate the second circuit's lifetime. As a proof-of-principle study, Liao *et al.* showed that a newly added strain from this RPS network could take over an existing culture if it was resistant to the toxin being produced by the bacteria that had been growing before. This second bacteria could then be replaced by the third, which could then be replaced by the first, demonstrating that this ecology could function as a distributed genetic network to prevent the rise of mutants that escape selection (see the figure).

One benefit of this distributed network is that it can be overlaid onto other, shared genetic circuits. If a genetic program receives an external signal as an input, new cells bearing this program should quickly synchronize to the existing gene expression pattern from the culture they are added to, while also displacing the old cells as a part of the RPS cycle. To demonstrate this behavior, Liao *et al.* combined their RPS network with a circuit for population-dependent lysis that their laboratory had previously developed (*13*). In this system, a sensor de-

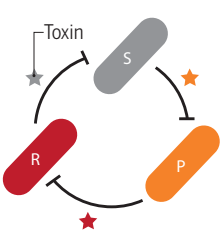
Harnessing peer pressure

Using cyclical ecology can preserve genetic integrity of engineered bacteria.

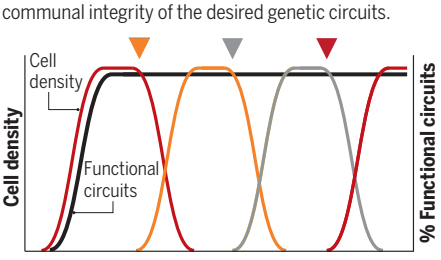


As engineered microbes grow, they accumulate mutations that can inactivate synthetic gene circuits.

In the engineered **"rock-paper-scissors"** (RPS) ecology, three bacterial strains produce both a toxin that can kill one of the other strains and the corresponding antitoxin to protect themselves.



Serial introduction of an RPS bacterial strain eliminates the evolving synthetic population that came before, removing potential mutants and retaining the communal integrity of the desired genetic circuits.



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Institute of Medical Engineering and Science and Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA, USA. Email: jimjc@mit.edu

tects the presence of a quorum-sensing molecule—a chemical produced by cells that corresponds with population density and controls the expression of specific genes. In this circuit, when the quorum-sensing molecule reaches sufficiently high concentrations, the cells are programmed to burst open, releasing their contents as a proposed means of drug delivery. This population-dependent lysis yields a stable oscillation in culture density, and because it relies on a diffusible signal to trigger lysis, any newly added cells carrying the same gene circuit are quickly synchronized to the established population, maintaining oscillations of cell density with similar periodicity.

Given the inherent fitness cost of the lysis circuit, growing these bacteria quickly results in the evolution of mutants, and the communities typically stop lysing after 2 days. By pairing this costly pathway with their RPS system, the authors were able to remove any potential mutants and reset the genetic integrity of continuous cultures through addition of the next strain in the RPS sequence, considerably elongating the longevity of their density-dependent lysis. Of note, this kind of RPS approach should be a generalizable means of continually rebooting communal gene pools of engineered microbiomes, which, if combined with previously established approaches, could considerably extend the lifetime of synthetic probiotics and other in vivo therapies.

The growing understanding of the connected nature of life underscores the impact ecological systems have on human health and that of the environment. The collective genetic network of the microbiome, for example, is now implicated in a number of diseases (*14*). As synthetic biology continues to build more complex devices, echoing these distributed genetic networks could unlock higher-order functions for the next generation of engineered microbes. ■

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CORALS

Coral spawning, unsynchronized

A breakdown in the coral spawning synchrony may threaten coral reef recovery

By **Nicole D. Fogarty**[1] *and* **Kristen L. Marhaver**[2]

During annual mass spawning events, hundreds of corals release millions of egg–sperm bundles in a coordinated manner. Underwater, the reef appears awash in a blizzard of pink snowflakes, but instead of falling, they rise to the surface, resulting in a slick of eggs that has even been seen from space. Mass spawning helps to overcome the dilution that is an ever-present challenge to fertilization for free-spawning marine species. It provides high gamete densities to ensure fertilization (*1*) while swamping gamete predators (*2*). However, the reproductive coordination of corals may be breaking down. On page 1002 of this issue, Shlesinger and Loya (*3*) compared four recent years of coral spawning observations to data collected from the same reef in the Red Sea 30 years before. They show that three of five species exhibited spawning asynchrony in recent years relative to earlier observations at the same site.

As Shlesinger and Loya report, not only did coral species at their study site spawn in different months from year to year, but colonies also spawned asynchronously over a broad range of days within each month. The authors further examined the potential consequences of spawning asynchrony on population demographics, finding a paucity of coral recruits and juveniles in the species with disrupted spawning. Even well-synchronized corals, such as Caribbean *Orbicella* spp. (*1*), can suffer recruitment failure (*4*) if mortality occurs after fertilization or during larval development, dispersal, and recruitment. However, Shlesinger and Loya demonstrate that the two Red Sea species that maintained spawning synchrony also maintained high recruitment, leading them to hypothesize that asynchronous spawning was a major contributor to recruitment failure in the other three species.

Spawning corals rely on a hierarchy of environmental cues to coordinate the months-long gametogenesis cycles that lead up to a narrow, minutes-long mass spawning window; therefore, they are particularly sensitive to changes in the environment (*2*). Resulting mistiming of coral spawning can be detrimental at multiple levels. Individual corals that deviate from the population's peak spawning time by even a few minutes can suffer reduced fertilization; individuals that miss the peak spawning time by hours or days often fail to achieve fertilization at all (*1*). Below a threshold density of spawning individuals, populations may fail to reproduce as a result of sperm limitation. Although fertilization failure is the main concern, low sperm densities and spawning discordance can also cause fertilization mistakes if the breakdown of temporal reproductive isolation between closely related species contributes to accidental hybridization (*1*).

Shlesinger and Loya warn that coral populations around the globe might appear healthy while suffering silently from these reproductive struggles. They posit that climate change, thermal stress, light pollution, and endocrine disruption are among the likely culprits. Yet now more than ever, the world's coral reefs need the genetic diversity generated by sexual reproduction to create new, stress-tolerant genotypes to adapt to global change.

How could human pressure cause so

How coral synchronize mass spawning

Reproductive stage and spawning times are correlated to a hierarchy of factors over multiple temporal scales.

TIME	FACTORS CORRELATED WITH CORAL SPAWNING	CORAL REPRODUCTIVE STAGE
Month	Solar irradiance, sea surface temperature, rainfall, wind, tides	Late-stage gametogenesis
Night	Lunar cycle	Gamete bundle formation
Hour	Sunset	Setting of gamete bundles
Minutes	Pheromones and coral genotype	Release of gamete bundles

[1]*Department of Biology and Marine Biology, Center for Marine Science, University of North Carolina Wilmington, 5600 Marvin K. Moss Lane, Wilmington, NC 28409, USA.* [2]*CARMABI Foundation, Piscaderabaai z/n, P.O. Box 2090, Willemstad, Curacao. Email: fogartyn@uncw.edu; kristen@marhaverlab.com*

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