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Genetic Circuits in *Salmonella typhimurium*

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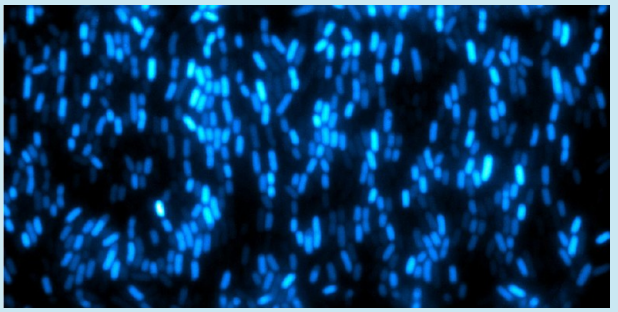
Supporting Information

ABSTRACT: Synthetic biology has rapidly progressed over the past decade and is now positioned to impact important problems in health and energy. In the clinical arena, the field has thus far focused primarily on the use of bacteria and bacteriophages to overexpress therapeutic gene products. The next generation of multigene circuits will control the triggering, amplitude, and duration of therapeutic activity *in vivo*. This will require a host organism that is easy to genetically modify, leverages existing successful circuit designs, and has the potential for use in humans. Here, we show that gene circuits that were originally constructed and tested in *Escherichia coli* translate to *Salmonella typhimurium*, a therapeutically relevant microbe with attenuated strains that have exhibited safety in several human clinical trials. These strains are essentially nonvirulent, easy to genetically program, and specifically grow in tumor environments. Developing gene circuits on this platform could enhance our ability to bring sophisticated genetic programming to cancer therapy, setting the stage for a new generation of synthetic biology in clinically relevant microbes.



KEYWORDS: *S. typhimurium, genetic circuits, microfluidics, clinical synthetic biology*

An explosion of DNA sequencing,[1] synthesis,[2] and manipulation[3] technologies has driven the development of synthetic genetic programs of increasing complexity in living cells.[4−6] Underlying this work is the hope that engineered biological systems will be used to solve important problems in energy and health over the coming years. Initially inspired by electronic circuits, researchers began by designing small transcriptional switches[7] and oscillators.[8] These early successes fostered a growing population of physicists, computer scientists, and engineers that aimed to apply an engineering-based methodology to the design of biological systems. In the past decade, substantial success has been achieved using this genetic circuits approach termed *synthetic biology*.[4,5,9]

Multigene logic gates capable of integrating environmental signals have been constructed in bacteria,[10] yeast,[11] and mammalian cells.[12] Electronics-inspired networks have included counters,[13] pulse generators,[14] filters,[15,16] and communication modules.[14,17] Sophisticated circuits can now be controlled by light, yielding genetic programs readily tunable both *in vitro*[18] and *in vivo* in live animals.[19] Dynamic genetic clocks have been constructed that function at the single-cell,[20] colony,[21] and multicolony[22] level in growing bacterial populations, and even in mammalian cells.[23] In a recent study, redox signaling mediated by H_2O_2 vapor permitted the synchronization of millions of oscillating bacteria across an LCD-like sensor array.[22]

Early efforts toward clinical applications have utilized bacteria[24−28] and bacteriophages[29,30] (viruses that infect bacteria) to perform therapeutic functions *in vivo*. Commensal bacteria have been engineered to fight diabetes,[26] HIV,[27] and cholera[28] by producing and delivering therapeutic agents directly in the human microbiome. Because certain bacteria grow preferentially in hypoxic environments, a number of studies have engineered cancer-fighting bacteria to selectively attack tumors.[24,25] Toward still another application, a pair of studies has engineered phages to produce foreign enzymes, making them far more potent than their unmodified counterparts at dispersing bacterial biofilms.[29,30]

In most of these cases, the genetic programs involved were responsible for overexpressing target genes, similar to traditional genetic engineering where genes are added, removed, or

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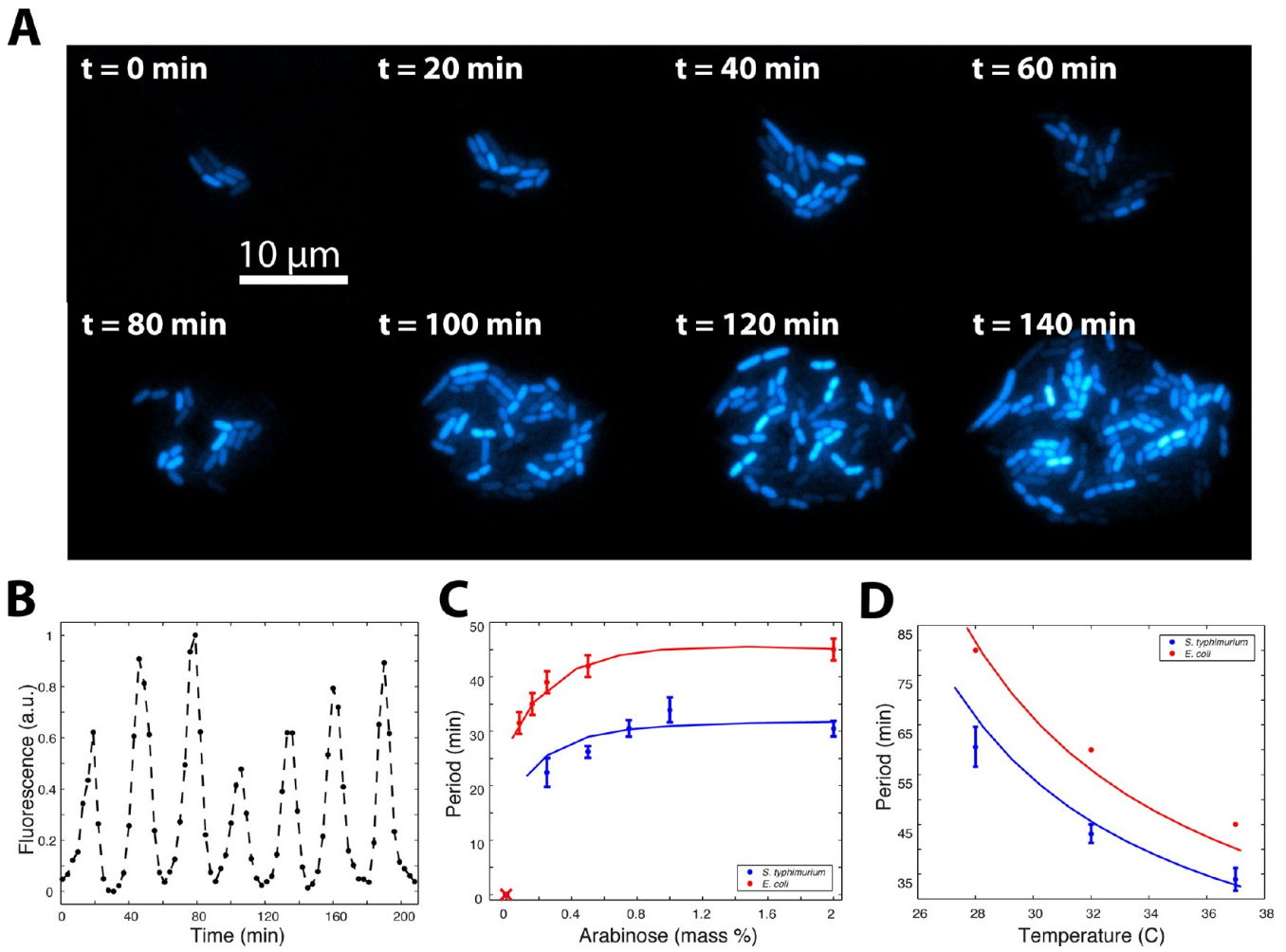

Figure 1. A fast, robust, and tunable genetic oscillator in *S. typhimurium*. (A) Timelapse fluorescence microscopy depicting asynchronous oscillations in a growing colony of *S. typhimurium*. (B) A single-cell trajectory extracted from image data. (C) Period vs inducer concentration for *S. typhimurium* compared to original data taken in *E. coli*. The trends are qualitatively similar, yet *S. typhimurium* is shifted toward shorter periods. Points are experimental measurements fit to a line generated by computational modeling. (D) Period vs temperature for *S. typhimurium* compared to original data taken in *E. coli* with similar trends.

modified one at a time in a stepwise fashion. To truly achieve its clinical potential, synthetic biology must continue to do what has made it successful: engineer progressively more complex, multi-input networks in which the triggering, amplitude, and duration of therapeutic activity is controllable. This will require using hosts that are easy to genetically modify and compatible with the clinical requirements regarding safety, immunogenicity, and drug resistance. While bacteriophage and adenovirus have their advantages, viruses have smaller genomes and therefore have a narrower range of genetic modifications, frequently induce host resistance, and are highly cell-type specific.[31,32]

As one potential bridge between organisms such as *Escherichia coli* and clinically relevant microbes, *Salmonella typhimurium* is a bacterial anticancer platform that is closely related to *E. coli*, has been extensively studied *in vivo* for therapeutic applications,[33−38] and has been shown to be safe in human clinical trials.[39−41] The development of attenuated strains has utilized auxotrophy and phoPQ deletions to suppress virulence cell invasion and virulence.[35] Lipid A mutations have been generated to reduce immunogenicity, stimulating a much weaker immune response than wild-type strains.[38] Despite this reduced potency, systemically injected *S. typhimurium* cells retain their ability to target and selectively replicate within tumors, displaying a thousand-fold growth preference relative to other organs.[33−38] Their motility allows them to follow chemical gradients and penetrate deep into the tumor vasculature,[42,43] much further than passively diffusing small molecules.[33] And many of these strains also display innate oncolytic activity, regressing tumors simply by growing in them.[34,38,44,45]

Perhaps the most important property of *S. typhimurium* for synthetic biology is the ease of genetic modification. It is a model organism whose genome is sequenced,[46] has knockout collections, and the genetic tools are almost identical to *E. coli*. *S. typhimurium* is capable of stably expressing recombinant DNA from plasmid-based circuits *in vivo*. This approach has already been used to produce a number of therapeutic compounds directly within tumors, but most often *via* "always on" expression of well-established genes.[35,47,48] This work has laid the foundation for more sophisticated functionality, such as programmed delivery profiles that take advantage of plasmid instability.[49] Such a focus will merge the dynamic sensing, production, and delivery capabilities of genetic circuits with the native tumor seeking and penetration of *S. typhimurium*.

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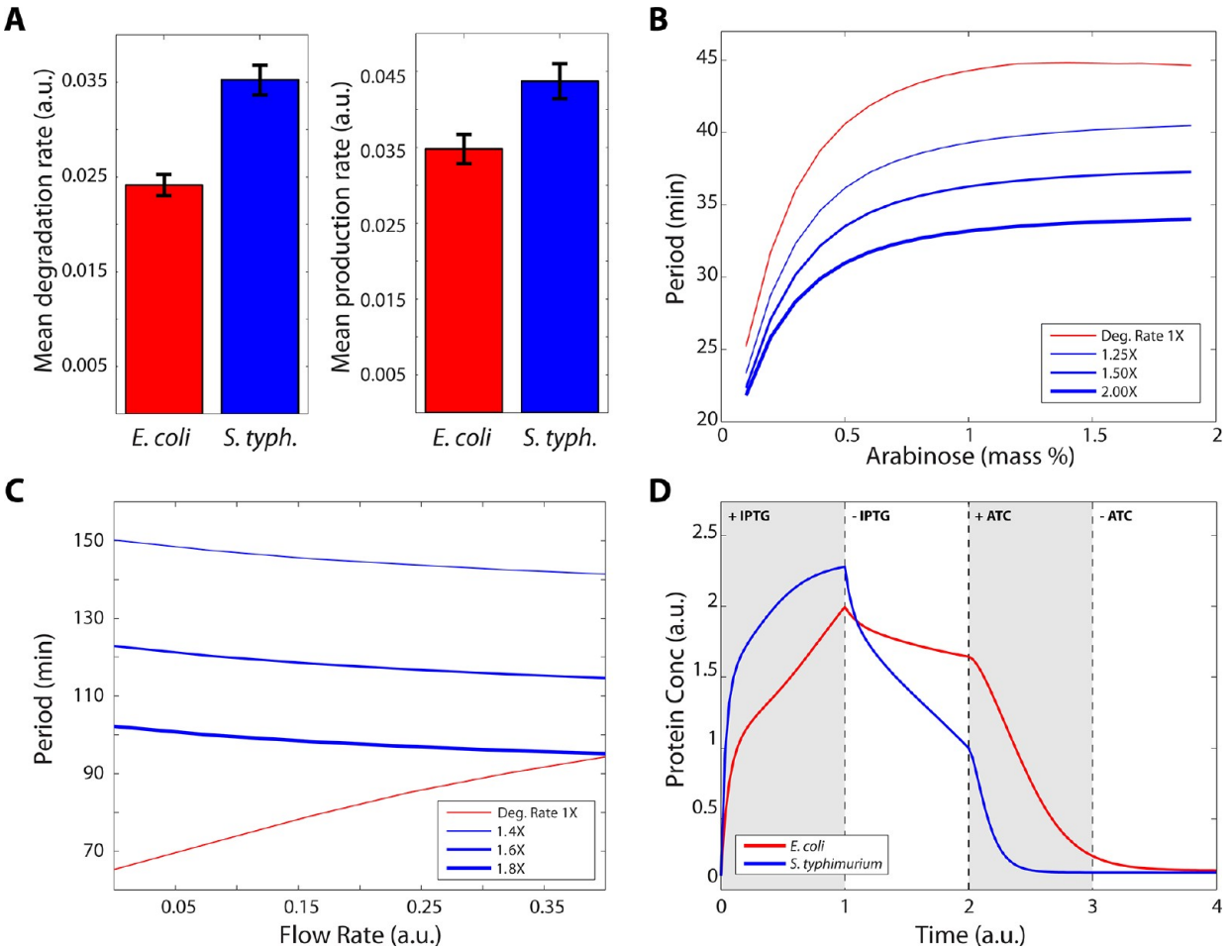

Figure 2. Computational modeling of *S. typhimurium* genetic circuits. (A) Comparison of enzymatic degradation rate between *S. typhimurium* and *E. coli* generated from automated single-cell tracking. Degradation rate is approximately 1.5× higher in *S. typhimurium*. (B) A higher degradation rate results in the shorter periods observed experimentally for the single-cell oscillator. (C) In contrast, increased degradation rate results in longer periods for the quorum-sensing oscillator that are comparatively unchanged with flow rate. (D) Increased degradation and expression rates produce the experimentally observed behavior for the *S. typhimurium* toggle switch.

EXPERIMENTAL RESULTS

In order to test the degree to which existing synthetic circuits function in *S. typhimurium*, we transformed the attenuated strain ELH430 (SL1344 ΔphoPQ, gift of Elizabeth Hohmann, MGH)[50] with several genetic oscillator constructs. First, we tested a single-plasmid variant of a published single-cell gene oscillator.[20] Using our microfluidic platform,[51,52] we observed robust oscillations for all *S. typhimurium* cells over many generations (Figure 1A,B). While the qualitative period-inducer relationship was similar to *E. coli*, the curve was shifted toward faster periods as compared to *E. coli* strain JS006 (MG1655 ΔaraC,lacI) (Figure 1C). In contrast, we initially expected *S. typhimurium* to oscillate slower since longer division times generally result in period lengthening.[20] When we measured the dependence of oscillatory period on temperature in *S. typhimurium*, we found the trend qualitatively similar to *E. coli*, where lower temperatures (and therefore longer doubling times) resulted in longer oscillatory periods (Figure 1D). We therefore hypothesized that the faster oscillations in *S. typhimurium* are not due to growth rate differences, but rather a strain-dependent factor such as mean promoter level, transcription rate, or enzymatic degradation rate.

To explore this quantitatively, we used automated single-cell tracking using a previously developed algorithm[53] to compare a large number of single-cell time courses from *S. typhimurium* and *E. coli* (Supporting Information). Oscillators are an ideal circuit to quantify strain-specific parameters such as transcription and degradation rates since they allow for hundreds of measurements in a single experiment. For each oscillatory period, the trough-to-peak and peak-to-trough slopes were measured. Since the ClpXP degradation machinery is likely saturated,[54] the peak-to-trough slope yields an estimate for the zeroth-order enzymatic degradation rate in degrade-and-fire oscillators.[55] Interestingly, we found that the apparent enzymatic degradation rate in *S. typhimurium* was roughly 1.5-fold that of *E. coli* (Figure 2A). In our computational model of the oscillator, this increase reproduced the experimentally observed period-inducer relationship (Figure 2B).

Next, we transformed *S. typhimurium* with a quorum-sensing oscillator that had been previously characterized in *E. coli*,[21] and observed coherent, colony-level oscillations for more than 48 h (Figure 3A,B). Here, we found that the period-flow rate dependence was markedly different in *S. typhimurium* than in the original study, where oscillatory period was much longer and changed very little across a wide range of flow rates (Figure 3C). Interestingly, while increased degradation rate resulted in faster oscillations for single cells (Figure 2B), our computational model correctly predicts the opposite trend for the

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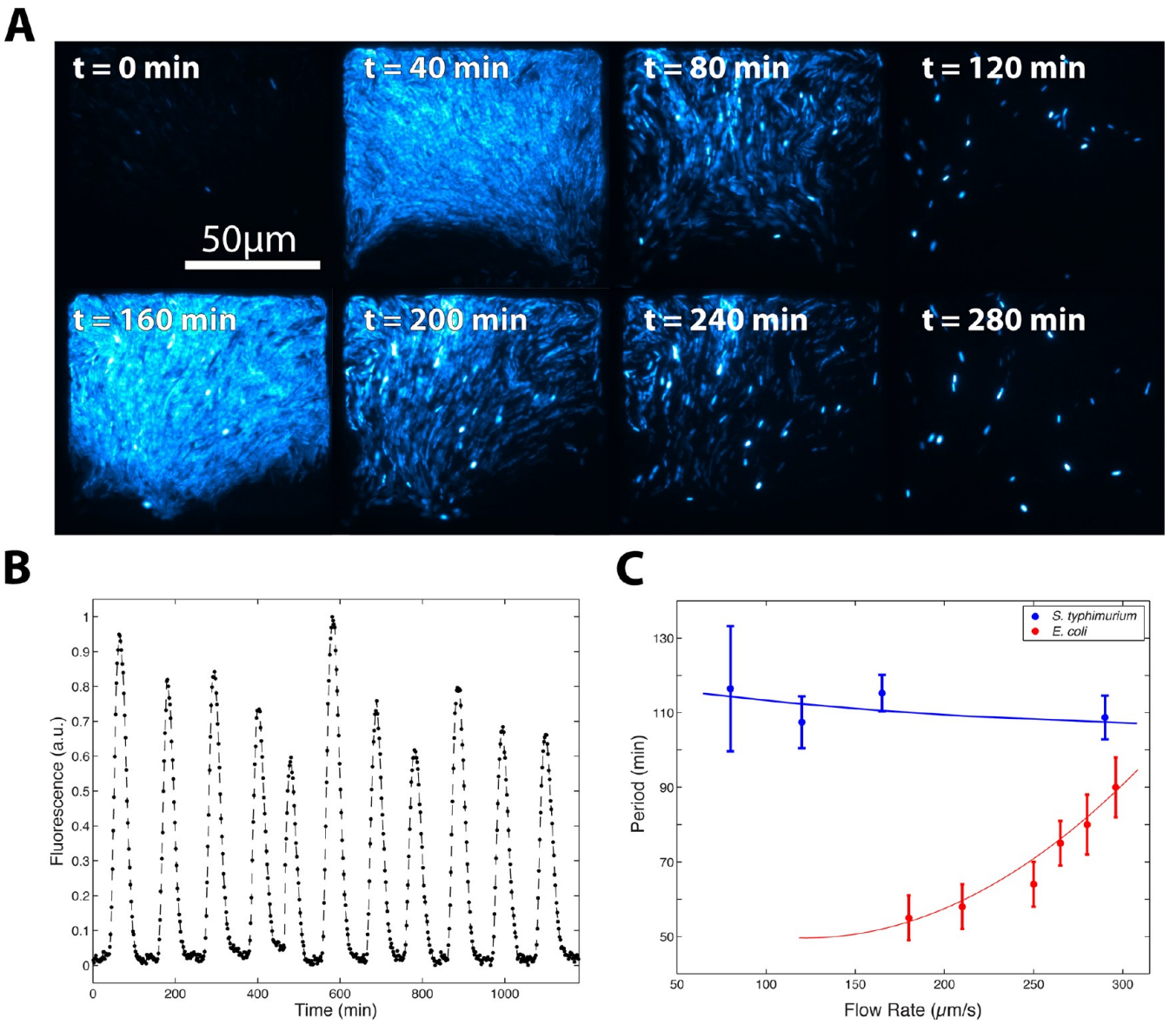

Figure 3. A synchronized quorum of genetic clocks in *S. typhimurium*. (A) Timelapse fluorescence microscopy depicting coherent oscillations at the colony-level for a growing colony of *S. typhimurium*. (B) A colony trajectory extracted from image data that illustrates the regularity of oscillations over time. (C) Period vs flow rate for *S. typhimurium* compared to original data taken in *E. coli*. *S. typhimurium* displays much higher periods that appear to be independent of flow rate.

quorum-sensing oscillator when degradation is increased (Figure 2C).

Finally, we tested the original genetic toggle switch, plasmid pIKE107.[7] In this circuit, a transient pulse of IPTG inducer turns the switch ON and reporter expression is maintained at a high level. A second pulse of ATC inducer turns the switch OFF, dropping reporter expression indefinitely. In periodically diluted batch culture experiments similar to the original study, we used flow cytometry to observe robust switching and bistability when inducing with either 2 mM IPTG or 500 ng/μL dox in cultures growing at 37 °C (Figure 4A–C). Interestingly, the fluorescence level at which *S. typhimurium* settled after we removed IPTG was lower than the same circuit in *E. coli* (Figure 4A). We suspected that the differences in apparent degradation and expression rates (Figure 2A) might explain this change, since the steady-state repressor balance would be adjusted.

To test this hypothesis, we used the original computational model of the toggle switch[7] and quantified the steady-state expression level over time for strain parameters measured in *E. coli* and *S. typhimurium*. We found that the *S. typhimurium* parameters reproduced the experimentally observed curves, where expression rises to a higher level when switched ON then decays to a lower steady-state when IPTG is removed (Figure 2D). While these parameters are particularly important for dynamic circuits, they can also impact the performance of stable switches since repressors are continuously being produced and degraded.

A central issue in the design of genetic circuits is the degree to which native and engineered networks should be integrated. Synthetic biology began by fully isolating itself from the strain background, using it solely to supply energy, enzymatic machinery, and a cellular volume in which to function. In contrast, industrial applications in medicine and energy have commonly utilized a variety of microbes for their native

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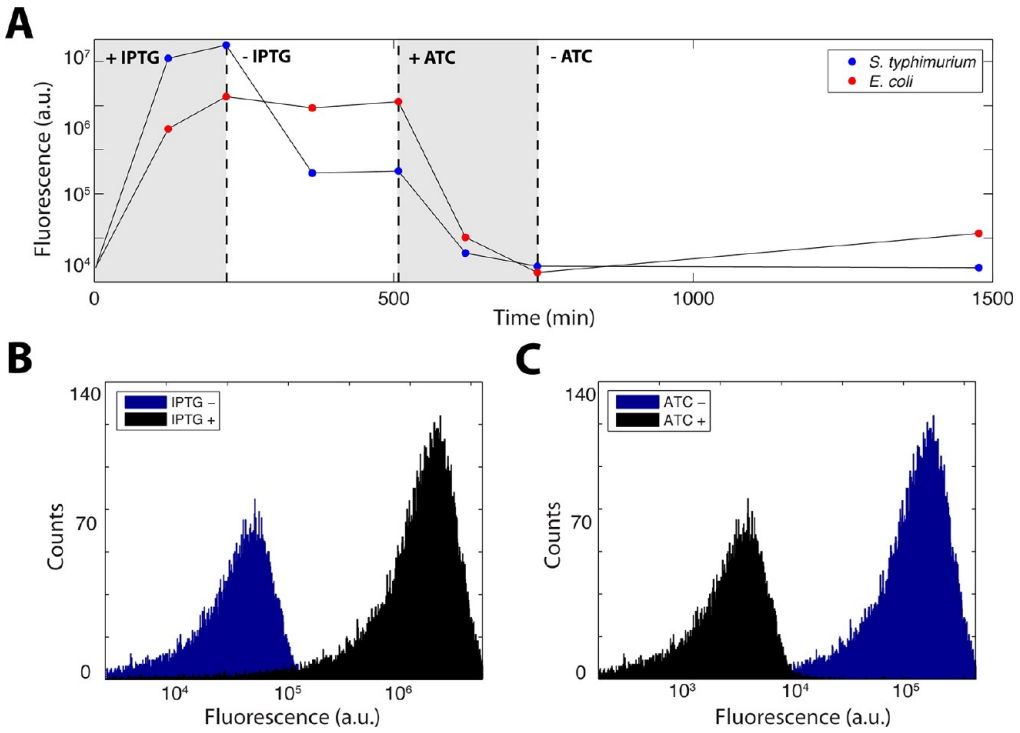

Figure 4. A genetic toggle switch in *S. typhimurium*. (A) A time course of fluorescence output that illustrates switching by both IPTG and ATC quantified by flow cytometry in periodically diluted batch culture experiments. (B) Raw flow cytometer data illustrating switching by 2 mM IPTG and (C) 500 ng/μL of ATC.

networks.[4,56,57] As our biological knowledge of native networks and our ability to engineer new circuits has improved, it has become increasingly possible to blend these two strategies.[5]

S. typhimurium is an ideal strain for clinical synthetic biology since it is closely related to *E. coli*, well studied *in vivo*, has safety precedence for clinical trials in humans, and displays a thousand-fold growth preference for tumor environments.[33−36]

Moving to other microbes for clinical and industrial purposes will require the determination of the critical strain parameters that define the space of bacteria capable of hosting genetic circuits. Next steps will involve measurement of these parameters and testing circuits in strains of interest that are further removed in the phylogenetic tree.[58] One such roadmap would begin with more distantly related gamma proteobacteria like *Pseudomonas aeruginosa* before moving outside the phylum to alpha proteobacteria such as *Calubacter crescentus*. Additionally, individual components and modules can also receive a "portability" score that estimate the degree to which they translate to other hosts. For example, while lacI- and tetR-based circuits are nearly universal, more generally the function of other components are likely to be more sensitive to strain-specific parameters. This work will enable synthetic biology to move beyond *E. coli* into a diverse range of microbes for clinical and industrial applications.

■ ASSOCIATED CONTENT

Supporting Information

Microscopy and microfluidics, degradation and production rate quantification, and modeling. This material is available free of charge *via* the Internet at http://pubs.acs.org/.

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Notes

The authors declare no competing financial interest.

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Synthetic biology in clinically relevant microbes

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Microscopy and Microfluidics

We used a microscopy system similar to our recent studies [1]. Fluorescent images were taken at 4X every 30 seconds using the EMCCD camera (20ms exposure, 97% attentuation) or 2 minutes (2s exposure, 90% attenuation) using a standard CCD camera to prevent photobleaching or phototoxicity.

In each device, *E. coli* cells are loaded from the cell port while keeping the media port at sufficiently higher pressure than the waste port below to prevent contamination (Suppl. Fig 8). Cells were loaded into the cell traps by manually applying pressure pulses to the lines to induce a momentary flow change. The flow was then reversed and allowed for cells to receive fresh media with 0.075% Tween which prevented cells from adhering to the main channels and waste ports.

To measure fluid flow rate before each experiment, we measured the streak length of fluorescent beads (1.0 μm) upon 100 ms exposure to fluorescent light. We averaged at least 1,000 data points for each.

We used several microfluidic devices over the course of the study. For single-cell oscillators (Fig. 1), we used a previously described device consisting of a trapping region and a dynamic switch[2]. Traps have dimensions 40 μm wide x 50 μm long x 0.95 μm high, with the long sides

open to media flow. Since *E. coli* and *S. typhimurium* cells have a 1 μm diameter, the trap maintains growing cells in a monolayer. For colony oscillators (Fig. 2), we used a previously described device consisting of arrays of square trapping regions[1, 3]. Trap dimensions were always 100 μm x 85 μm x 1.65 μm high and spacing between traps was 25 μm. This size allows cells to grow in a colony arrangement rather than a monolayer, while still allowing quantitative measurement of colony fluorescence.

Degradation and Production Rate Quantification

Single cell fluorescence trajectories were obtained from time-lapse movies using custom software previously developed in MATLAB [2]. Each cell fluorescence trajectory represents the median GFP fluorescence signal inside that cell over time. Using built-in MATLAB functions we identified the peaks and troughs for each trajectory. The degradation rate was calculated by taking the amplitude change from peak to the successive trough and dividing by the time change between the peak and the trough. These peak-to-trough sections of the trajectory represent the time when the production of GFP is repressed and the observed dynamics are solely driven by degradation of GFP. Similarly we calculated the net production rate, by calculating the amplitude change from trough to successive peak and dividing by the time change between the trough and the peak. The measurement gives the net production rate, which includes the degradation of the protein.

	Mean Degradation Rate (SE)	**Mean Net Production Rate (SE)**
E. coli	0.024 (0.001)	0.035 (0.002)
S. typhimurium	0.035 (0.002)	0.044 (0.002)

Modeling

To generate the plot in Figure 4D, we used previously described genetic toggle switch model [4]. We included three additional parameters to model the effects of IPTG (C_{IPTG}), ATC (C_{ATC}), and dilution (D) on the synthesis and degradation of proteins:

$$\begin{aligned}\frac{\partial u}{\partial t} &= \frac{C_{IPTG_{(0,1)}}\alpha_u}{1+v^n} - (\gamma_u + D)u \\ \frac{\partial v}{\partial t} &= \frac{C_{ATC_{(0,1)}}\alpha_v}{1+u^n} - (\gamma_v + D)v\end{aligned}$$

In this model, we set n=2 to allow for cooperativity of repression of both promoters. C_{IPTG_0} and C_{ATC_0} were set to 1 for the case of no inducers present. Next, we used metropolis algorithm to find the rest of the parameters to fit the qualitative nature of the curves from Figure 1A. The parameters found to generate the *E. coli* curve were: $C_{IPTG_1} = 1.25$,$C_{ATC_1} = 1.68$,$\alpha_u = 4.28$,$\alpha_v = 5.80$,$\gamma_u = 1.76$,$\gamma_v = 2.37$,$D = 0.11$. The parameters found to generate the *S. typhimurium* curve were: $C_{IPTG_1} = 1.25$, $C_{ATC_1} = 1.68$,$\alpha_u = 11.00$,$\alpha_v = 8.36$,$\gamma_u = 4.86$,$\gamma_v = 3.21$,$D = 0.08$. It is

interesting to note that the optimized parameters show higher production and degradation as well as lower dilution for *S. typhimurium* curve relative to *E. coli* curve, which correlates well with our experimental measurements.

The dynamics of single cell oscillator were modeled using previously described model for activator (a_2) and repressor (r_4) proteins [5]. The production and degradation of these proteins is described by the following set of reactions:

$$
\begin{aligned}
P_{0,0}^{a/r} &\xrightarrow{b_{a/r}} P_{0,0}^{a/r} + m_{a/r} \\
P_{1,0}^{a/r} &\xrightarrow{\alpha b_{a/r}} P_{1,0}^{a/r} + m_{a/r} \\
m_a &\xrightarrow{t_a} m_a + a_{uf} \\
m_r &\xrightarrow{t_r} m_r + r_{uf} \\
a_{uf} &\xrightarrow{k_{fa}} a \\
r_{uf} &\xrightarrow{k_{fr}} r \\
a + a &\underset{k_{-da}}{\overset{k_{da}}{\rightleftharpoons}} a_2 \\
r + r &\underset{k_{-dr}}{\overset{k_{dr}}{\rightleftharpoons}} r_2 \\
r_2 + r_2 &\underset{k_{-t}}{\overset{k_t}{\rightleftharpoons}} r_4 \\
a_{uf} &\xrightarrow{\lambda f(X)} \emptyset \\
r_{uf} &\xrightarrow{f(X)} \emptyset \\
a &\xrightarrow{\lambda f(X)} \emptyset \\
r &\xrightarrow{f(X)} \emptyset \\
a_2 &\xrightarrow{\lambda f(X)} \emptyset \\
r_2 &\xrightarrow{f(X)} \emptyset \\
r_4 &\xrightarrow{f(X)} \emptyset
\end{aligned}
$$

We updated the degradation function $F(X)$ to include dilution as follows:

$$f(X) = \frac{\gamma}{c_e + X} + DX$$

Here, X is the total number of *ssrA* tags in the system (one for each monomeric version, two for dimers, and four for tetramers, including proteins bound to operator sites). We varied the parameter γ from 1x to 2x to evaluate the effect of degradation difference between *E. coli* and *S. typhimurium* on the period of oscillation calculated from single cell model simulations. Dilu-

tion rate was calculated from experimentally measured cell half life as $\frac{ln(2)}{T_{\frac{1}{2}}}$.

To model the dynamics of the quorum-sensing oscillator, we used our previously described model for intracellular concentrations of LuxI (I), AiiA (A), internal AHL (H_i), and external AHL (H_e) [1],

$$\frac{\partial A}{\partial t} = C_A[1-(d/d_0)^4]\ G(\alpha,\tau) - \frac{\gamma_A A}{1+f(A+I)} - DA \quad (1)$$

$$\frac{\partial I}{\partial t} = C_I[1-(d/d_0)^4]\ G(\alpha,\tau) - \frac{\gamma_I I}{1+f(A+I)} - DI \quad (2)$$

$$\frac{\partial H_i}{\partial t} = \frac{bI}{1+kI} - \frac{\gamma_H A H_i}{1+gA} + D(H_e - H_i) - DH_i \quad (3)$$

$$\frac{\partial H_e}{\partial t} = -\frac{d}{1-d}D(H_e - H_i) - \mu H_e + D_1\frac{\partial^2 H_e}{\partial x^2} \quad (4)$$

To model the difference in periods of oscillation between *E. coli* and *S. typhimurium* we varied the degradation parameters γ_A and γ_I. We looked at the changes in the period over different values of the flow rate parameter μ, while varying the degradation prapameters from 1x to 2x of the original model value. To account for the difference in doubling time between the two strains, we introduce exponential decay terms into the model to account for dilution in addition to the enzymatic degradation terms. We add terms $-DI$, $-DH_i$, and $-DH$ to the first three equations respectively, with $D = \frac{ln(2)}{T_{\frac{1}{2}}}$. We then looked at how the change in doubling time affected the period of both strains.

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