A synthetic *Escherichia coli* predator–prey ecosystem

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We have constructed a synthetic ecosystem consisting of two *Escherichia coli* populations, which communicate bi-directionally through quorum sensing and regulate each other’s gene expression and survival via engineered gene circuits. Our synthetic ecosystem resembles canonical predator–prey systems in terms of logic and dynamics. The predator cells kill the prey by inducing expression of a killer protein in the prey, while the prey rescue the predators by eliciting expression of an antidote protein in the predator. Extinction, coexistence and oscillatory dynamics of the predator and prey populations are possible depending on the operating conditions as experimentally validated by long-term culturing of the system in microchemostats. A simple mathematical model is developed to capture these system dynamics. Coherent interplay between experiments and mathematical analysis enables exploration of the dynamics of interacting populations in a predictable manner.

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Subject Categories: synthetic biology
Keywords: microchemostat; microfluidics; quorum sensing; synthetic biology; systems biology

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Introduction

Since its recent emergence, synthetic biology has shown great potential for generating practically useful systems while serving as an approach to decoding biological ‘design principles’ (Hasty et al, 2002; Sprinzak and Elowitz, 2005; Andrianantoandro et al, 2006; Chang and Keasling, 2006; Chin, 2006; Haseltine and Arnold, 2007). A key challenge in this area is to identify general, scalable strategies that enable construction of increasingly complex gene circuits with reliable performance, as well as to develop novel technological platforms for quantitative circuit characterization (Simpson, 2004; Endy, 2005; Marguet et al, 2007). The predator–prey ecosystem serves as an excellent model for such a challenge. As an essential component of ecological dynamics, natural predator–prey systems have been analyzed extensively by modeling and experiments (May, 1974; Murray, 2002). In comparison with other types of ecological interactions (e.g. mutualism and competition), predation often generates richer dynamics and as such represents a greater challenge to engineer *de novo*. In this study, we constructed synthetic gene circuits that converted two *Escherichia coli* populations into a synthetic predator–prey system. Long-term quantification of the system dynamics, which required an improved version (Supplementary Figure S1) of the recently developed microchemostat (Balagadde et al, 2005), validated the designed function. In addition to pushing the limits of programming and characterization of complex cellular dynamics, our system can serve as a model system to address questions arising from its natural counterparts.

Results and discussion

As shown in Box 1A, two *E. coli* populations (‘predator’ and ‘prey’) communicate and regulate each other’s density through quorum sensing (QS), a mechanism by which many bacteria broadcast and respond to changes in their local concentration of autoinducing molecules. QS is a well-characterized phenomenon, which is utilized in a wide variety of applications such as bioluminescence and in the development of fluorescent reporters (Storz, 1999). In our study, we engineered QS circuits into synthetic gene circuits via the use of transcriptional activators and repressors, which are known to control gene expression in *E. coli*. The predator population constitutively expresses a QS receptor protein that responds to a uniquely constructed and engineered signal molecule, while the prey population expresses a QS receptor protein that responds to a second, distinct signal molecule produced by the predator. In our study, we used two different signal molecules, both of which are comprised of a non-native, foreign DNA sequence. This approach is designed to minimize the potential for interference and crosstalk between different synthetic circuits, which is a concern in the co-expression of multiple gene circuits in a single cell. Once the prey turn on the expression of the target antibacterial protein in response to the second signal molecule, the predator is released from inhibition and can kill the prey by expressing a killer protein, which is toxic to the prey (Balagadde et al, 2005). The prey are thus able to rescue the predators by expressing an antidote protein, which is toxic to the predator (Balagadde et al, 2005). This dynamic cycling between predator and prey populations is possible depending on the operating conditions as experimentally validated by long-term culturing of the system in microchemostats. A simple mathematical model is developed to capture these system dynamics. Coherent interplay between experiments and mathematical analysis enables exploration of the dynamics of interacting populations in a predictable manner.

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population density (Camilli and Bassler, 2006). Two QS modules, LuxI/LuxR from *Vibrio fischeri* and LasI/LasR from *Pseudomonas aeruginosa*, are used to enable two-way communication between the predator and prey populations. When the prey density is low, the predator cells die due to constitutive expression of a suicide gene (*ccdB*). In prey cells an acyl-homoserine lactone (AHL), 3OC6HSL, is synthesized by LuxI. As the prey density increases, 3OC6HSL accumulates in the culture and, at sufficiently high concentrations, it is bound by the transcriptional regulator LuxR in the predator cells leading to increased expression of an antidote gene (*ccdA*) to rescue the predators. The predator cells in turn produce another AHL, 3OC12HSL by LasI that permeates into the prey cells where it is bound by LasR, which activates expression of the killer *ccdB* gene, inducing ‘predation.’ This system satisfies the broader definition of predation for a two-species ecosystem, where one species (prey) suffers from the growth of the second (predator) and the latter benefits from the growth of the former (Taylor, 1984). However, it differs from the canonical predator–prey system in two aspects. First, instead of acting as a food source, the prey provides an ‘antidote’ to programmed death of the predator. Second, there is competition between predator and prey for nutrients in a co-culture, which is generally absent in natural predator–prey systems.

We employed ordinary differential equations to model the major kinetic events during the functioning of this circuit (see Supplementary information and Figure S5 for details). The model predicts extinction, coexistence or oscillatory dynamics (Box 1B) depending on rates of cell growth, cell death controlled by AHL, as well as production and degradation of AHL. According to bifurcation analysis (Box 1B, left panel), the larger the CcdB killing rate constant (*d_c*), the higher the likelihood of robust oscillatory behavior: increasing *d_c* significantly broadens the parameter region that gives rise to oscillation (region bound by the loci of Hopf bifurcation points). Our experiments indicated that the original LacZa-CcdB fusion protein, which was used in our population control circuit (You et al., 2004), might lack the necessary toxicity (data not shown). To overcome this limitation, we constructed a new LacZa0-CcdB protein by deleting 32 amino
acids in LacZa, which resulted in an eight-fold increase in potency compared to the full-length protein (Supplementary Figure S2). The LacZa-CcdB fusion protein is referred to as ‘CcdB’ in this paper.

It was previously shown that both active LasR and active LuxR can initiate gene expression at the luxI promoter (PluxI) (Gray et al., 1994) (Supplementary Figure S3). Therefore, we used PluxI to drive both the ccdA gene in the prey cells (regulated by LuxR) and the ccdB gene in the prey cells (regulated by LasR). To expedite rescue of the predator by its survival signal (3OC6HSL), the corresponding QS system was used in a synthetic ecosystem (Supplementary Figure S4). The ccdB expression in the predator by the QS system in the prey. Optimization of protein expression levels and synchronize the timing of the circuit components, we tested many combinations of promoters, origins of replication and bacterial host strains, and narrowed our focus to one configuration (Supplementary Figure S4A). Unless noted otherwise, the predator circuit was implemented in the E. coli strain MG1655. We verified the basic function of each population independent of IPTG and the corresponding AHL supplied exogenously (Figure 1). In the OFF culture containing neither IPTG nor 3OC6HSL, predator cells grew to a relatively high density in LBK medium (Figure 1A). Growth was significantly inhibited in the ON culture containing 1 mM IPTG, which fully induced ccdB expression. However, predator cells induced with IPTG were rescued by 3OC6HSL (100 nM), which when bound by LuxR can initiate ccdA expression. Rescued predators (with IPTG and 3OC6HSL) grew slightly slower than those in the OFF condition (Figure 1A), possibly due to the metabolic burden involved in expression of circuit components, incomplete neutralization of CcdB by CcdA, or both. As shown in Figure 1B, prey cells were able to grow in the OFF culture (without inducers) as well as medium containing only IPTG but perished when both IPTG and 3OC12HSL were present. The IPTG-induced prey population grew slower compared to that of the OFF culture. This minor growth retardation could be a result of the metabolic burden of the circuit components, basal level CcdB expression due to crosstalk between 3OC6HSL and LasR, or both.

This synthetic predator–prey system represents one of the most complicated synthetic circuits reported to date. Long-term characterization of such circuits, which is essential to the verification of the designed dynamics, presents a major technological challenge. Measurement of circuit performance in conventional macroscopic test tube cultures hinted at oscillatory dynamics (data not shown). But insufficient temporal resolution due to the arduous and time-consuming process involved in acquiring such data made these observations inconclusive. In addition, the short-lived stability of the circuit in the macro-sized populations (Desai et al., 2007) could only provide a fleeting glimpse of the programmed behavior: function was lost before the circuit behavior could be appreciably observed. To this end, we resorted to a recently developed microchemostat platform (Balagadde et al., 2005); a high-throughput screening technology that can inexpensively perform rapid characterization of synthetic circuits under a matrix of conditions with unprecedented capabilities including long-term, non-invasive measurements of microbial population properties under steady-state conditions with single cell resolution and advanced automation. Equally important, the microchemostat’s micro-sized population (~10^2–10^4 cells versus at least ~10^9 in macroscale cultures) reduces the number of cell division events per unit time and hence slows down microbial evolution (Desai et al., 2007). This aspect facilitates monitoring of programmed behavior of bacterial populations for hundreds of hours despite strong selection pressure to evade population control (Balagadde et al., 2005).

Furthermore, the challenge of real-time monitoring of community composition was met by an engineering revision to the microchemostat reactor channel to enable single-cell-resolved fluorescence quantification (Supplementary Figure S1). In addition, we reduced reactor volume from ~16 to ~9 nl to further reduce the bacterial population size in each reactor, which has been shown to help stabilize a population controller during long-term culturing (Balagadde et al., 2005). This strategy also allowed us to increase the number of parallel reactors per chip from 6–14 for higher throughput.

Figure 2A illustrates the typical oscillatory dynamics of predator and prey populations in the microchemostat with a period of ~180 h (~1.125 h^-1). Initially, when the prey density was low, predator growth was inhibited due to constitutive expression of CcdB. Conversely, the prey grew to a high density (~35,000 cells per reactor) in ~10 h. The high prey density led to rescue of the predator population, which recovered from ~5000 cells per reactor to >20,000 cells per reactor in ~10 h. The increased predator density in turn resulted in killing of the prey cells, leading to a
drastic reduction in the prey density (from ~35,000 cells per reactor to just a few cells per reactor). The decrease in the prey density led to insufficient rescue of predator cells and the subsequent decline in predator density at ~35h. The prey population recovered ~10h later. In summary, the system yielded oscillatory dynamics between the predator and prey populations that clearly resemble those observed in natural predator–prey ecosystems.

The parallel design of microchemostat reactors greatly facilitates the investigation of circuit response to changing circuit parameters. Figure 2B illustrates a typical set of dynamics of the synthetic predator–prey ecosystem for various circuit induction levels. Without induction (IPTG = 0), predator and prey had negligible circuit-driven interactions. Since the predator (MG1655 strain) grows faster than the prey (Top10F’ strain), the prey population eventually (after ~50h) driven to extinction (the fluctuations of the prey density around 0 cell per reactor were numerical artifacts of imaging processing; see Supplementary information for details). Higher IPTG levels ($\geq$5 $\mu$M) did elicit oscillatory dynamics between the predator and the prey populations.

These results suggest the crossing of a Hopf bifurcation point using the IPTG level as the control parameter (also see Supplementary Figures S6 and S7A). This aspect is illustrated with bifurcation analysis (Figure 2C) that accounts for the effects of IPTG on 3OC6HSL production by the prey and CcdB expression by the predator. At sufficiently low IPTG levels, there is minimal programmed predation between two populations and the primary interaction is competition. As a result, the predator population dominates due to its growth advantage, leading to the washout of the prey. Increasing IPTG beyond a critical level enables crossing of a Hopf bifurcation point and elicits oscillatory dynamics.

The model further predicts two salient features of the predator behavior at high IPTG induction levels. First, the response dynamics are much slower compared to those at low IPTG induction levels (Figure 2C, inset). Second, these dynamics involve minimal predator densities that are close to zero. Both aspects are qualitatively consistent with experimental data (top panels of Figure 2B; Supplementary Figure S7A), although the data do not exclude the possibility that the system may eventually approach steady state. Because of the miniaturized volume of the microchemostat reactor (~9 nl), the predator (or prey) population is prone to falling below the detection limit (~75 cells per reactor) during an oscillation trough (Figure 2B, row 1, columns 1 and 2; from ~10 to ~80h) and in severe cases, getting washed out (Figure 2B, row 1, column 3).
Similarly, we explored the impact of dilution rate ($D$) on the system dynamics. Figure 3A illustrates coexistence dynamics at $D=0.11 \text{ h}^{-1}$, suggestive of an initial phase of long-period oscillations. However, an increase in $D$ (to $0.2 \text{ h}^{-1}$) led to a phase of damped oscillatory dynamics with much shorter periods ($\sim 50 \text{ h}$). We observed similar response with a different circuit configuration: predator (Top10F') and prey (Top10F') (Figure 3B; also see Supplementary Figure S4B). These populations initially coexist at a low dilution rate ($D=0.15 \text{ h}^{-1}$). They switched to damped oscillations with short periods ($\sim 30 \text{ h}$) upon an increase in $D$ (to $0.23 \text{ h}^{-1}$). Further increase in $D$ (to $0.31 \text{ h}^{-1}$) at $\sim 250 \text{ h}$ led to predator washout. Bifurcation analysis qualitatively accounts for the experimental observations that an increase in the dilution rate can lead to a significant decrease in the period of oscillations (Figure 3C, inset). Simulations further show that an increase in $D$ can shift a slow, sustained oscillation (Figure 3C, bottom left panel) to a fast, damped oscillation (Figure 3C, bottom middle panel). Predator cells will be washed out if $D$ is too large (Figure 3C, bottom right panel).

These modeling results qualitatively account for the experimental observations of the dependence of systems dynamics on dilution rate. We note that the system’s response to a shift in $D$ is highly sensitive to the prevailing conditions of operation.

Upon switching $D$ from 0.11 to $0.2 \text{ h}^{-1}$, the system either exhibits a damped oscillation or approaches a steady state (the third row, Supplementary Figure S7A, where IPTG=50 μM). The sensitivity of the damped oscillation to perturbations of a parameter or initial conditions can qualitatively explain this phenomenon (Supplementary Figure S7C, insets). Since damped oscillations are transient dynamics, small variations in the control of the microchemostat dilution rate or different initial phases of the predator and prey populations can significantly impact the oscillation behavior.

Drawing inspiration from the nature, synthetic biologists have created circuits that operate in single cells (Elowitz and Leibler, 2000; Gardner et al, 2000; Atkinson et al, 2003; Fung et al, 2005; Levskaya et al, 2005), in individual populations (Kobayashi et al, 2004; You et al, 2004; Basu et al, 2005; Anderson et al, 2006) and between populations (Brenner et al, 2007). Our study represents a major advance beyond recent efforts in constructing synthetic ecosystems (Shou et al, 2007; Weber et al, 2007), in terms of system complexity and resolution of characterization. It provides a framework for exploring how naturally existing cellular components can be assembled to program and coordinate highly complex cellular behavior. In addition, synthetic ecosystems such as ours may serve as well-defined systems for exploring evolutionary and
ecological questions regarding, for example, the generation and maintenance of biodiversity (Chesson, 2000; Nowak and Sigmund, 2004). Conventionally, typical experiments involving microorganism systems are limited to using nutrient concentration and dilution rate as the sole control parameters (Fussmann et al., 2000; Becks et al., 2005). In comparison, our system allows direct manipulation of intrinsic parameters, such as growth rate, death rate and strength of cell–cell communication. In this system, there is a clear and explicit mapping between the genetic constructs and the corresponding population dynamics, which may facilitate future studies of how molecular interactions are manifested in the temporal patterns of population dynamics, a central theme in ecology (Bohannan and Lenski, 2000; Foster et al., 2007). The generic nature of our system design makes it portable to other ecological interactions, including mutualism, competition, commensalism and amensalism (May, 1974). The virtually unlimited configurations that are possible with these basic elements will allow us to further examine the interplay between the environment, gene regulation and population dynamics. With additional control over population mixing or segregation, it will be possible to program bacterial populations to mimic development and differentiation in multicellular organisms.

Materials and methods

Microchemostat design and fabrication

Microchemostat chips were fabricated out of silicone elastomer polydimethylsiloxane (General Electric RTV 615) using multi-layer soft lithography as described previously (Balagadde et al., 2005). In this chip version, the number of reactors per chip was increased from 6 to 14. In addition, a relatively thin imaging section 3 μm high (compared to ~10 μm high otherwise) was incorporated along a 150 μm stretch of the growth loop to constrain the entire fluorescence signal of the cells within a single focal plane (Supplementary Figure S1).

The microchemostat reader

The general assembly of the reader was described previously (Balagadde et al., 2005). The cells in the microchemostat were imaged through bright-field or fluorescence microscopy. Illumination in each et al. (Balagadde et al., 2005). The microchemostat reader

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The general assembly of the reader was described previously (Balagadde et al., 2005). The cells in the microchemostat were imaged through bright-field or fluorescence microscopy. Illumination in each reactor via bright-field microscopy, with rotary mixing of the culture in between consecutive snapshots. Image-processing algorithms in Matlab were used to determine the average number of cells in each picture set, from which

Microscopic cell counting

Within the 3 μm height of the imaging section (compared to the 10 μm height otherwise), imaged cells were constrained into a sufficiently thin horizontal plane so that they could all be in focus simultaneously. This also enhanced the single-cell resolvability by decreasing the cell–cell overlap in the horizontal plane (Supplementary Figure S1).

The total number of cells in each continuous reactor was determined directly through automated bright-field microscopy by counting the numbers of cells present in a growth chamber section of known volume. Typically, a set of eight phase-contrast images was taken at the imaging section of each reactor via bright-field microscopy, with rotary mixing of the culture in between consecutive snapshots. Image-processing algorithms in Matlab were used to determine the average number of cells in each picture set, from which

the total cell count was determined as described previously (Balagadde et al., 2005).

To determine community composition of mixed populations, one of the species (predator or prey) was tagged with a Gfpuv fluorescent label. The fluorescent signal was determined by directly counting the fluorescent cells in a set of typically four images acquired through automated fluorescence microscopy, using the appropriate Gfpuv filter set: excitation 395/40 and emission 510/40. Thus, the non-fluorescent density, obtained by subtracting the fluorescent from the total cell count, represented the density of the second population. According to this approach, members of a ‘fluorescent’ species that are not bright enough to be captured by the image-processing algorithms get counted as non-fluorescent species cells. This could be a source of noise in the number of cells reported for each species. In the future, this problem could be addressed by assigning a different fluorescent marker to each species involved in the experiment.

A motorized stage system (Prior Scientific, Rockland, MA, USA) enabled simultaneous documentation of multiple microchemostat experiments that operated in parallel on a single chip.

Plasmids

Plasmids used for this study are shown in Supplementary Figure S4. To construct placcCdBs (p15A origin, KanR), 96 base pairs between two NstI sites in the coding region of LacZa-CcdB (pZEO2-2) were deleted, and the resulting gene lacZa’-ccdB, along with its upstream lac promoter, was cloned into pPROLar.A122 (BD Biosciences Clontech). ptetLuxRLasI-lacZa (SC101 origin, CmR) was constructed in several steps. First, the lasR gene along with its ribosome-binding site was cloned from the P. aeruginosa (PAO1) chromosome into plasmid pLasR (Collins et al., 2005), where it was placed downstream of the luxR gene. Second, the luxR-lasR cassette was cloned into plasmid pPROTet.E132 (Clontech) to generate ptetLuxRLasI, where the cassette is under control of a PLasR-I promoter (Lutz and Bujard, 1997). Next, the ccdA gene was cloned from the F plasmid, PCR-fused with a PLasR-I promoter from pluxGFPuv (Collins et al., 2005), and inserted into ptetLuxRLasI, in opposite direction from the PLasR-I-lasR-luxR cassette. Finally, the ColE1 replication origin of this plasmid was replaced with the SC101 origin, which was cloned from the repressor plasmid (Elowitz and Leibler, 2000). PLasR-LuxI-lacZa-CcdBs (p15A origin, KanR) was also constructed in several steps. First, the lasR gene was cloned from the P. aeruginosa (PAO1) chromosome into pPROLar.A122 (Clontech) placing it under control of the PlacZa-I promoter to generate pLasR. Second, the luxR gene was cloned from plasmid pSND1 (Weiss and Knight, 2000) into pLasR where it was positioned directly downstream of the lasR gene, generating pLasRLuxI. Third, a pluxI-lacZa’-ccdB cassette was cloned from plasmid pluxCdBs, which was derived from pluxCdB by removing 96 base pairs between two NstI sites (see above). Plasmid ptetGFPuv (LVA) (ColE1 origin, CmR) was constructed by inserting an N-terminal portion of GFPuv amplified by PCR with a primer, including a KpnI site upstream of the start codon and a primer annealing to the internal AvaII site, and a C-terminal portion of GFPuv (LVA) amplified by PCR from pINV-4 (Weiss, 2001) with a primer annealing to the internal AvaII site and a primer including BamHI downstream of the LVA tag into KpnI- and BamHI-digested pPROTet.E132 (BD Biosciences Clontech).

Strains, growth conditions and macro-scale data acquisition

MG1655 and Top10F E. coli cell strains (Invitrogen) were used unless otherwise noted. For IPTG/AHL response tests in Figure 1, we used 3OC6HSL (Sigma Aldrich) and 3OC12HSL synthesized by E Toone (Department of Chemistry, Duke University), which were prepared and stored as reference (Collins et al., 2005).

PH-buffered LB media (containing 10 g tryptone, 5 g yeast extract and 7 g KCl 1⁻¹) were used for liquid culture. The media were buffered with 100 mM weak acids (PIPEs was used for pH 6.2 and 6.6, and MOPS for pH 7.0), and pH was adjusted by adding 5M KOH. Antibiotics (50 μg ml⁻¹ chloramphenicol and 50 μg ml⁻¹ kanamycin)
and anhydrotetracycline were added. IPTG plus 0.05% arabinose (1 mM) was used to activate the circuit. Cultures were shaken in a 12 ml culture tube at 37 °C and 250 r.p.m. A starter culture of the predator cells or the prey cells was inoculated from a single colony and grown separately for at least 10 h, and then diluted 1000-fold into 4 ml of fresh medium.

**Microchemostat medium, preculture preparation and growth conditions**

LBK medium was used in all microchemostat experiments unless otherwise stated. Predator and prey populations were independently tested for circuit function according to the procedure illustrated in Figure 1. Glycerol stock solutions (30 μl) were created using the tested predator and prey cultures and were placed in a −80 °C freezer for long-term storage. These mini-stocks were used to prepare pre-cultures for subsequent microchemostat experiments.

Precultures were prepared by inoculating a 2 ml sterile medium sample with 10 μl of previously prepared mini-glycerol stock cell solution and shaking at 280 r.p.m. for ~9 h at 37 °C (VWR bench top incubator, model 1575R). The precultures were then used to seed microchemostat reactors with ~20 cells ml⁻¹. To measure predator−prey circuit dynamics in the microchemostat, cells were grown in pH 7.0-buffered LBK medium. All microchemostat media were supplemented with 5 g l⁻¹ bovine serum albumin as an anti-adhesion adjuvant. The predator−prey circuit plasmids were maintained with 50 μg ml⁻¹ of kanamycin and 15 μg ml⁻¹ chloramphenicol. All experiments were performed within a microscope plexi-glass incubator to control growth temperature at 37 °C.

**Supplementary information**

Supplementary information is available at the Molecular Systems Biology website (www.nature.com/msb).

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