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REPORT

Cooperation and Hamilton's rule in a simple synthetic microbial system

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A fundamental problem in biology is understanding the evolutionary emergence and maintenance of altruistic behaviors. A well-recognized conceptual insight is provided by a general mathematical relation, Hamilton's rule. This rule can in principle be invoked to explain natural examples of cooperation, but measuring the variables that it involves is a particularly challenging problem and controlling these variables experimentally an even more daunting task. Here, we overcome these difficulties by using a simple synthetic microbial system of producers and nonproducers of an extracellular growth-enhancing molecule, which acts as a 'common good.' For this system, we are able to manipulate the intrinsic growth difference between producers and nonproducers, as well as the impact of the common good on the growth rate of its recipients. Our synthetic system is thus uniquely suited for studying the relation between the parameters entering Hamilton's rule and the quantities governing the systems' behavior. The experimental results highlight a crucial effect of nonlinearities in the response to the common good, which in general tend to limit the predictive value of Hamilton's rule.
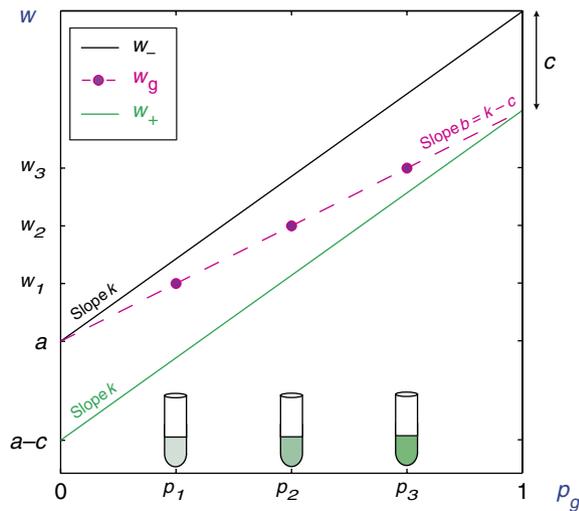
Introduction

The evolution and persistence of altruism, despite the direct fitness disadvantage associated with altruistic behaviors, have been intensely discussed since *The Origin of Species* (Darwin, 1872). The most influential contribution to this problem is Hamilton's rule (Hamilton, 1963, 1964, 1975), according to which cooperation in structured populations, and in particular altruism, requires that $br > c$, where b and c represent the 'benefit' and 'cost' of cooperation, whereas r corresponds to a measure of 'relatedness' between individuals (see Box 1). This formulation illustrates very generally how cooperation can be consistent with natural selection acting at the level of individuals.

A considerable number of formal extensions of Hamilton's rule have concurred to reinforce its importance (Queller, 1985, 1992; Lehmann and Keller, 2006). Precisely connecting the various theoretical frameworks to actual biological systems, however, faces two major difficulties: (i) theoretically, Hamilton's rule derives its genera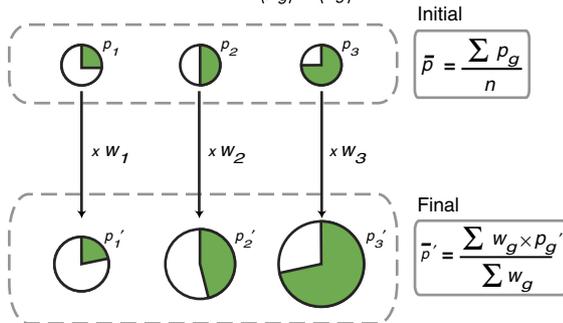lity from its purely mathematical basis (Box 1; Supplementary Text S1), but this comes at an expense: the definitions of the parameters b, r, c can be subtle and may subsume many intricate and idiosyncratic details (Frank, 1998; Gardner *et al*, 2007); (ii) experimentally, r can be measured and varied (Gilbert *et al*, 2007), but measuring quantitatively the variables determining the cost and benefit parameters b and c can be time consuming and tricky (Grafen, 1984; Emlen and Wrege, 1992; Bowles and Posel, 2005; Krakauer, 2005), and even more challenging is manipulating these parameters in natural settings (Field *et al*, 2006). In this work, we overcome some of the problems of (ii) by using a tractable synthetic microbial system that allows us to explore experimentally the nature of difficulty (i).

Microbes have been argued recently to be choice systems for bridging the gap between theoretical models and biological phenomena (Crespi, 2001; Rainey and Rainey, 2003; Velicer, 2003; Kerr *et al*, 2006; Mehdiabadi *et al*, 2006; West *et al*, 2006; Gilbert *et al*, 2007; MacLean and Brandon, 2008; Gore *et al*, 2009). In addition, the field of synthetic biology has illustrated how design principles of biological networks can be

Box 1 Hamilton's rule

$$r = \frac{\langle p_g^2 \rangle - \langle p_g \rangle^2}{\langle p_g \rangle - \langle p_g^2 \rangle}$$

Initial

$$\bar{p} = \frac{\sum p_g}{n}$$

Final

$$\bar{p}' = \frac{\sum w_g \times p_g'}{\sum w_g}$$

A basic insight underlies Hamilton's rule: an altruist trait can be maintained in a population provided it preferentially benefits individuals sharing the same heritable propensity to behave altruistically. Three levels of selection are generally relevant: the lower level of genes, at which the heritable determinants of altruism are defined; the intermediate level of individuals, at which the altruistic phenotype is defined; and the higher level of groups of interacting individuals, at which the effects of altruism are defined. For haploid and asexual microorganisms, there is, however, no fundamental distinction between the genetic and phenotypic levels and only the individual and group levels need to be considered. In such cases, the central parameter is the probability for two altruists to interact, which is dictated by the structure of the interactions between individuals.

An example of such structure is a population distributed in several groups, indexed by g, where individuals interact exclusively with members of their group. As a simple model for intra-group interactions (Hamilton, 1975), we may assume that a group consists of a fraction p_g of cooperators and $1-p_g$ of non-cooperators, and that non-cooperators grow by a factor $w_- = a + k p_g$ (black line in figure), whereas cooperators grow by the factor $w_+ = a + k p_g - c$ (green line). Overall, such a group, therefore, grows by a factor $w_g = p_g \, w_+ + (1-p_g) \, w_- = a + b p_g$ (dashed magenta line), where $b = k - c$. In this model, $b > 0$ represents the 'benefit' and $c > 0$ the 'cost' of cooperation. If several mixed groups are formed with different initial proportions p_g of cooperators (represented in the figure as pie charts whose green slice is proportional to p_g and area proportional to population size), a straightforward calculation, detailed in Supplementary Text S1, shows that the conditions for the global, population-wide proportion of cooperators to increase is

$$br - c > 0. \tag{1}$$

This inequality constitutes Hamilton's rule. The so-called relatedness coefficient, r, describes the bias for altruists to benefit from altruism and

Box 1 Continued

depends here only on the distribution (p_g) of initial proportions. In the simplest case of equal group sizes, it is given by

$$r = \frac{\langle p_g^2 \rangle - \langle p_g \rangle^2}{\langle p_g \rangle - \langle p_g^2 \rangle}. \tag{2}$$

This model thus illustrates how altruism may be maintained by showing that Equation (1) can be satisfied ($br - c > 0$), even if within each group g, non-altruistic individuals have the advantage ($c > 0$).

We previously showed how such a model could be implemented in an actual microbial system of common good producers and nonproducers (representing 'cooperators' and 'non-cooperators', respectively) (Chuang *et al*, 2009). Modifications of this original system, presented in this study, however, generically lead to situations where the linear relation $w_g = a + b p_g$ does not hold. The role of the population structure in maintaining cooperation may nevertheless still be described by relations (1) and (2) provided b and c are properly defined (see Supplementary Text S1). In particular, b should correspond to the regression coefficient β of w_g on p_g,

$$b = \beta \equiv \frac{\langle w_g p_g \rangle - \langle w_g \rangle \langle p_g \rangle}{\langle p_g^2 \rangle - \langle p_g \rangle^2}. \tag{3}$$

Hamilton's rule can, therefore, always be invoked, but the parameters b and c may lose their interpretation as phenotypic properties defined at the level of individuals.

investigated by forward engineering of novel behaviors in cells; the resulting systems, having been built from the ground up, are amenable to manipulation, thereby serving as simplified experimental systems for the study of more complex phenomena (Sprinzak and Elowitz, 2005; Mukherji and van Oudenaarden, 2009; Song *et al*, 2009; Tanouchi *et al*, 2009).

Thus, we previously built and analyzed a synthetic microbial system comprised of two genetically engineered 'cooperator' and 'noncooperator' strains of *Escherichia coli*, represented, respectively, by producers and nonproducers of a 'common good.' The common good, specifically the small molecule Rhl autoinducer (Miller and Bassler, 2001), is synthesized solely by producers, but activates expression of the antibiotic resistance gene chloramphenicol acetyltransferase (*cat*) in both strains, as Rhl autoinducer is membrane permeable. In mixed cultures of producers and nonproducers containing the antibiotic chloramphenicol (Cm), nonproducers—which do not bear the burden of production—outgrow producers and increase in proportion at the expense of producers (Chuang *et al*, 2009).

This synthetic system may be viewed as an experimental implementation of one of the simplest models originally introduced by Hamilton for conceptual purposes (Box 1). By forming mixed groups, indexed by g, with sufficiently different initial proportions (p_g) of producers, one can vary the parameter r characterizing the structure of the population, because r is here directly related to the distribution of the p_g's, and a higher variance of p_g results in higher r. It is thus possible to satisfy Hamilton's rule $br - c > 0$, and to obtain an increase in the overall fraction of producers, $\Delta \bar{p} > 0$, despite the fact that within each mixed subpopulation, the proportion of producers decreases ($\Delta p_g < 0$ for all g)—a situation that can be interpreted as an instantiation of the so-called

Simpson's paradox (Wagner, 1982). Experimentally, this is observed in the synthetic system (Chuang *et al*, 2009) when the variance of initial producer proportions, is sufficiently large (corresponding to high *r*).

An important feature of the synthetic approach is that both the structure of the population and the interactions between individuals can be experimentally manipulated. Whereas in previous work on Simpson's paradox we varied the population structure of a single-fixed system of producers and nonproducers (thus keeping the intrinsic properties of the system constant), here we hold constant the population structure while altering the nature of the interactions between producers and nonproducers. With the synthetic approach, we are able to modify with relative ease two intrinsic properties of the bacterial system: (i) the growth difference between producers and nonproducers, when having access to the same amount of common good autoinducer and (ii) the growth improvement, for both producers and nonproducers, because of the presence of autoinducer. Such modifications allow us to study quantitatively how varying the parameters associated with microbial biology affects the three parameters *b*, *r* and *c* entering Hamilton's rule.

Results and discussion

First, to control the growth difference between producers and nonproducers, we introduced an auxotrophic mutation, $argH\Delta$, solely into the synthetic producer strain (Figure 1A). As the *argH* gene remains intact in nonproducers, varying the level of arginine (in minimal media) should allow us to tune the relative growth of producers compared with nonproducers. A similar approach using histidine mutants was recently used in a study of yeast cooperation (Gore *et al*, 2009). As expected, when arginine was not limiting, the growth of Arg⁻ producers in the absence of antibiotic was equivalent to the original Arg⁺ producers (Figure 1B). As we lowered the arginine concentration, the overall growth of producers decreased (Figure 1B). This first modified system (Figure 1A) thus gives us the ability to test the effect of decreasing the growth of producers relative to nonproducers (see below).

Second, to control the growth rate improvement conferred by the autoinducer common good, we increased the dosage of the *rhlR* gene, which encodes the transcription factor to which Rhl autoinducer must bind to activate resistance to antibiotic (Figure 2A). This was accomplished by transforming into both producers and nonproducers a second compatible plasmid, which either provided extra copies of *rhlR* ('extra *rhlR*') or not ('control'). As extra *rhlR* cells contain more autoinducer-sensitive transcription factor, they should respond more readily to the autoinducer common good. Indeed, when we added to a population of cells (producers or nonproducers) with extra *rhlR* the same amount of autoinducer as before, we observed that their growth rate in the presence of antibiotic increased compared with the original system (Figure 2B). This second modified system (Figure 2A) thus gives us the ability to test the effect of varying the influence of the common good.

To assess how varying these two elements would affect the interactions between producers and nonproducers, we performed experiments in which we measured the change

in overall producer proportion ($\Delta\bar{p}$) when producer–nonproducer subgroups were formed and grown in selective conditions in antibiotic-containing media. In each experiment, we fixed the compositions of the subgroups (i.e. fixed the distribution of p_g) by creating mixtures containing 10, 20, 30, 40, 50, 60, 70, 80, 90, and 95% producers. In accordance with our previous study of Simpson's paradox, in large concentrations of arginine and without extra copies of *rhlR*, the change in total producer proportion $\Delta\bar{p}$ in the whole population (ensemble of all subgroups) was positive, that is producers were overall favored (Chuang *et al*, 2009). Next, we changed either the amount of arginine (Figure 1C) or the dosage of *rhlR* (Figure 2C).

Using the first modified system with Arg⁻ producers, we observed that the change in overall producer proportion $\Delta\bar{p}$ became negative at low arginine concentrations (Figure 1C). This can be easily understood from the perspective of Hamilton's rule. With the proper definition of the parameters *b*, *c* and *r* (Box 1; Supplementary Text S1), decreasing the level of arginine in the first modified system (Figure 1A) leads to an increase of the cost parameter *c*, as intuitively expected, as well as a moderate decrease in *b*. Thus, the difference $br-c$ can become negative, causing nonproducers to be favored in the overall population.

When using the second modified system with cells expressing extra copies of *rhlR*, we also observed a decrease in $\Delta\bar{p}$ (Figure 2C). From the perspective of Hamilton's rule, this means that increasing the dosage of *rhlR* transcription factor in the second modified system (Figure 2A), which makes the autoinducer common good more helpful (as the same amount of autoinducer stimulates faster growth in antibiotic), leads to a nonintuitive decrease of the benefit parameter *b*. The reason is that the parameter *b* entering Hamilton's equation, given by Equation (3) of Box 1, is a linear regression coefficient. In a linear system, *b* is independent of the distribution of initial producer proportions (p_g). However, the increased dosage of *rhlR* caused the relationship between growth and producer proportion to become nonlinear. As a result, *b* depends here both on the dosage of *rhlR* and on the distribution of the initial producer proportions p_g (Figure 3); it is thus a nonintuitive function dependent both on 'intrinsic' properties of the microbes and 'extrinsic' properties of the population structure (Box 1).

Our synthetic microbial system clearly illustrates the general problem in applying Hamilton's rule to real biological systems (Gardner *et al*, 2007). As Hamilton's rule is a demonstrated mathematical inequality, the problem in analyzing any particular system centers on the relation between *b*, *c* and *r* and the parameters of the system (Box 1; Supplementary Text S1). This generally leads to parameters *b* and *c* that are not necessarily related in a simple way to the intrinsic parameters describing the organisms only, but also involve the population structure.

Thus, even if one has measured the values of *b*, *c* and *r* for a particular system of producers and nonproducers, one cannot predict what will result from changing either the structure of population or the biochemistry of the individuals. In particular, the growth (w_g) of producer–nonproducer mixtures has to be a linear function of the fraction of producers (p_g), as in the model in Box 1, for the knowledge of the values of *b* and *c* to provide a
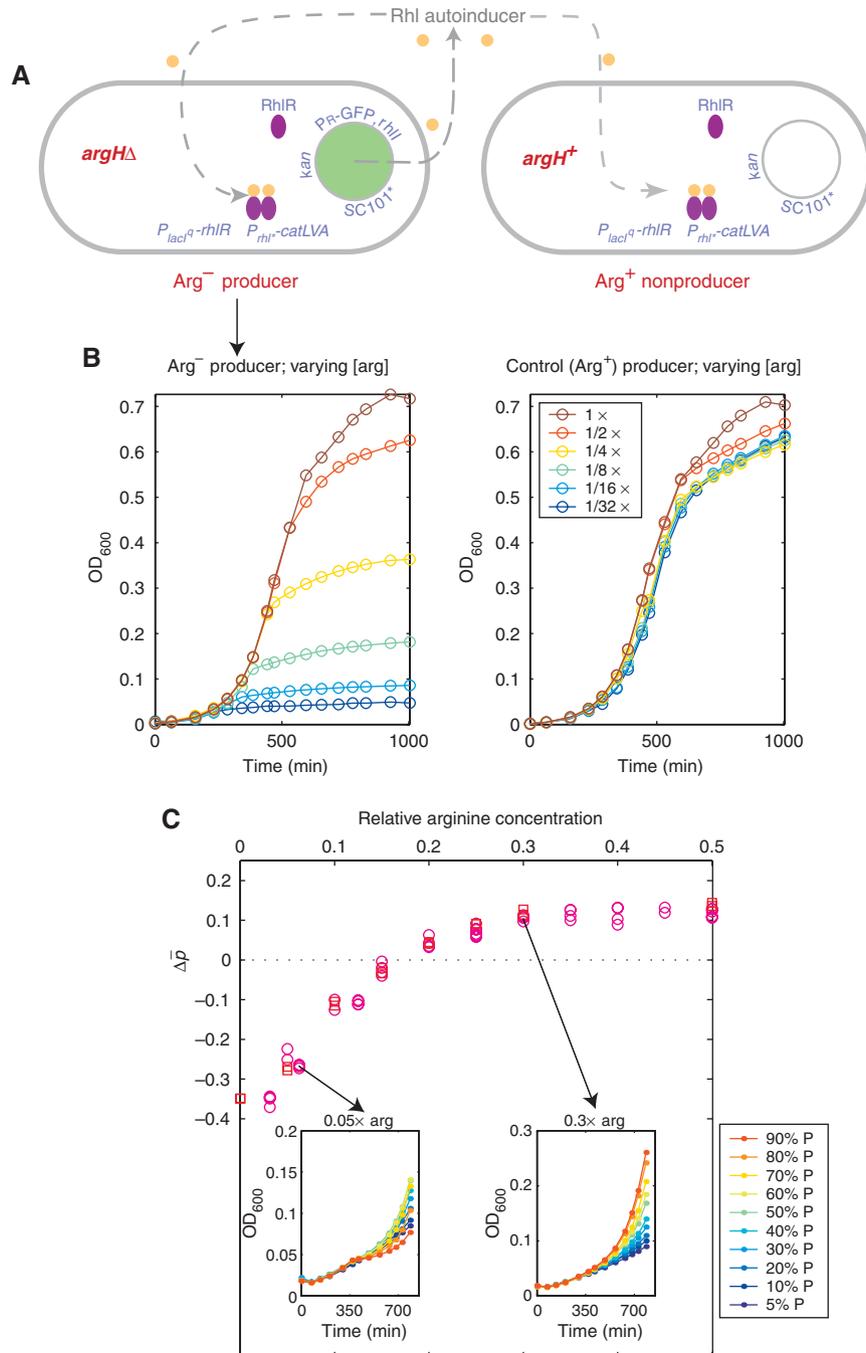
Figure 1 Decreasing the relative growth of producers versus nonproducers reverses the direction of selection. (**A**) Schematic of a modified producer–nonproducer system composed of Arg⁻ (arginine auxotroph) producers and Arg⁺ nonproducers (see text and Materials and methods for details). (**B**) Growth curves of Arg⁻ versus Arg⁺ producers grown in minimal media lacking antibiotic at 30°C with varying arginine concentrations (different colors; $1\times = 520\,\mu M$ arginine). (**C**) The overall proportion of producers, $\Delta\bar{p}$, decreases under selective conditions (in antibiotic; producer–nonproducer mixtures with p_g distributed uniformly) when the arginine concentration is decreased. (Insets) Growth curves of producer–nonproducer mixtures (differing in p_g) corresponding to two conditions of low ($0.05\times$) and intermediate ($0.3\times$) arginine concentrations. Note: With *argHΔ*, limiting arginine does not have large effects on growth *rates*, but rather overall growth levels—this does not affect our conclusions as what matters here is only how much growth occurred during the selection phase in antibiotic.

sufficient basis for predicting the evolution of the system upon changes of its population structure. Our original synthetic system (Chuang *et al*, 2009) was specifically designed to be in the linear regime. This property is, however, not generic and we found here that modifications of the system readily pushed it out of the linear regime.

A nonlinear growth response to a molecular signal (Figure 3) is a feature commonly found in biological systems and is probably inherent to common good-based cooperation in microbes. Hamilton's rule indicates that the maintenance of producers is typically destabilized relative to the ideal linear case. More precisely, the stronger the nonlinearities of the
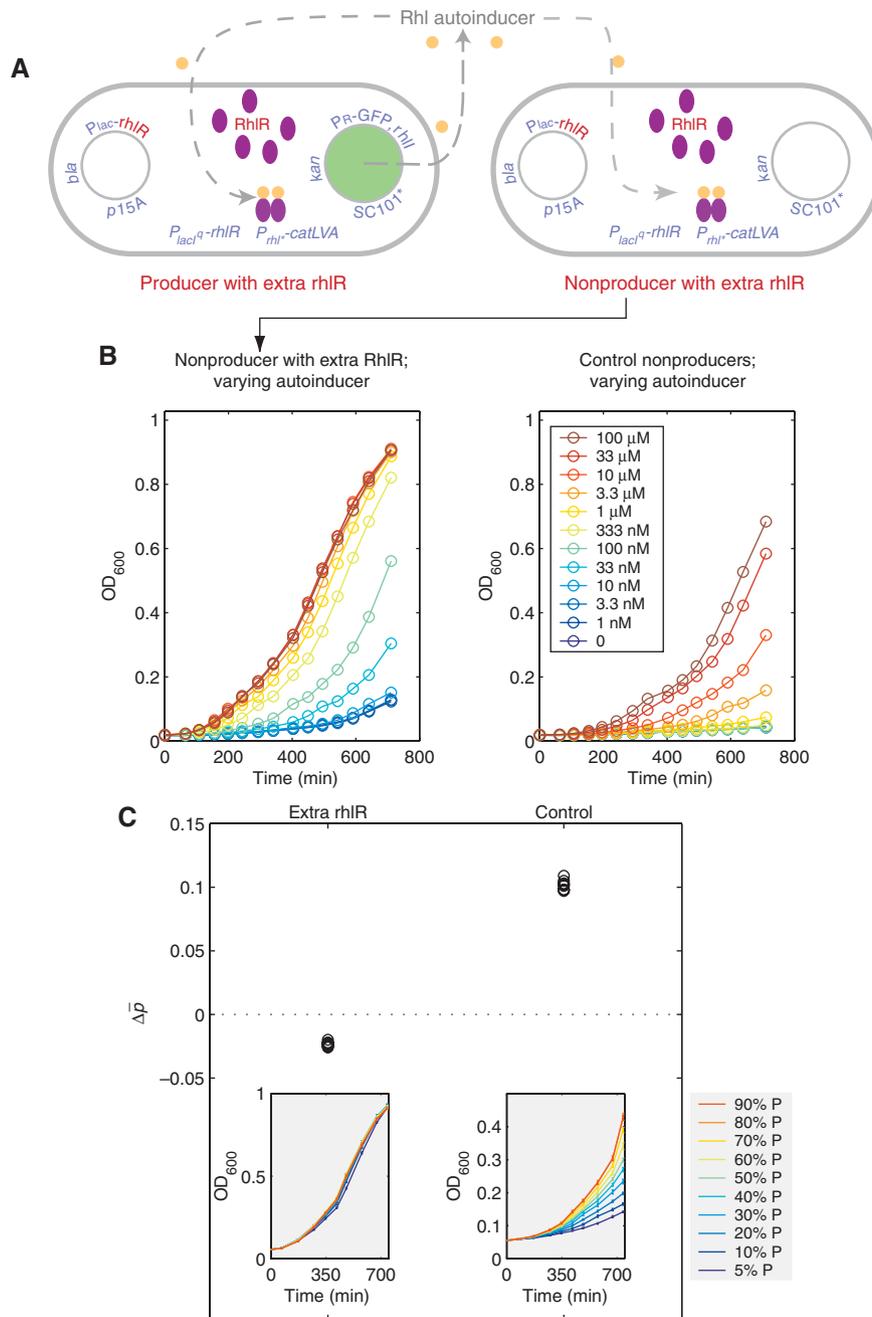
Figure 2 Increasing the antibiotic resistance response to autoinducer reverses the direction of selection. (**A**) Schematic of a modified producer–nonproducer system in which all autoinducer recipients have increased dosage ('extra *rhlR*') of the autoinducer-responsive transcription factor gene, *rhlR* (see text and Materials and methods for details). (**B**) Growth curves of extra *rhlR* nonproducers versus control nonproducers in antibiotic in the presence of varying amounts of exogenously added autoinducer. (**C**) The overall proportion of producers, $\Delta \bar{p}$, decreases under selective conditions (in antibiotic; producer–nonproducer mixtures with p_g distributed uniformly) when autoinducer recipient cells were made more responsive to autoinducer by increased *rhlR* dosage ('extra *rhlR*'). (Insets) Growth curves of producer–nonproducer mixtures (differing in p_g) from the extra *rhlR* system (left) versus the control system (right).

system, the stronger are the constraints on the population dynamics (e.g. constraints on the distribution into groups) implied by Hamilton's rule. This suggests that in each particular system under study, an important goal should be to characterize the nonlinearities controlling the dynamics of cooperating microorganisms. In a recent study of the problem

of cooperation in yeast sucrose metabolism (Gore *et al*, 2009), for instance, nonlinear benefits explain why both invertase producers and nonproducers could mutually persist instead of being taken over by nonproducers.

We developed here an approach based on synthetic biology for addressing the nature of the main biological constraints
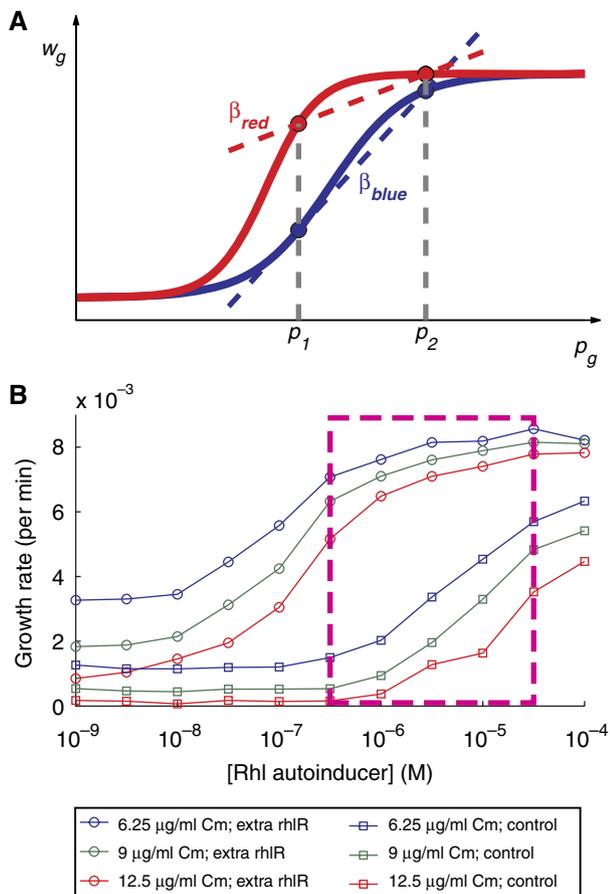
Figure 3 Interpretation of the 'benefit' parameter b in the presence of nonlinearities. (**A**) Two different systems (blue and red) have different nonlinear relations between the initial proportion of cooperators, p_g, in a mixed group, g, and the factor by which such a population is multiplied, w_g. When different groups of equal size are formed, here two groups with proportion of producers p_1 and p_2, the value of b entering Hamilton's rule, Equation (1) of Box 1, is given by the regression coefficient b defined in Equation (3) of Box 1. This regression coefficient corresponds here to the slope of the line joining the two points associated with p_1 and p_2 on the curve (in the presence of more than two groups, b would correspond to the slope of the best linear interpolation between the different points). In this example $b_{blue} > b_{red}$ in spite of the fact that the red system systematically outgrows the blue system with any given fraction of cooperators, i.e., $w_{blue}(p_g) < w_{red}(p_g)$ for all p_g. (**B**) Growth rates of extra *rhlR* (circles) and control (squares) nonproducers in the presence of varying amounts of Rhl autoinducer and antibiotic (Cm). Growth rates were determined by fitting growth curves to a logistic equation. The approximate range of autoinducer concentrations experienced by cells during an experiment of Figure 2C is outlined in the dashed magenta box.

underlying a general principle well known in evolutionary biology, Hamilton's rule. As a first step, we showed how a simple mathematical model (Box 1; Supplementary Text S1), which serves as a transparent illustration of Hamilton's rule, could be implemented in a microbial system (Chuang *et al*, 2009). We then took a further step by exploring the response of the synthetic system to different modifications, so as to understand how the properties of a generic biological system are most likely to deviate from those of our specifically designed system. As a result of this approach, we found that a major constraint for the maintenance of cooperation in microorganisms has the form of a nonlinear growth response to a molecular signal (Figure 3).

Beyond its specific conclusions, this study illustrates how synthetic biology can be used to develop and analyze mathematical models in a biological context, following an approach that could be extended to address other basic questions in biology beyond the maintenance of cooperation.

Materials and methods

Producer and nonproducer strains

The original system (Chuang *et al*, 2009) consists of a producer and nonproducer strain, both derived by plasmid transformation of *E. coli* strain JC1191, which has genotype *att::rhl-catLVA(Sp^R)* Δ*sdiA::FRT rrnB3* Δ*lacZ4787 hsdR514* Δ*(araBAD)567* Δ*(rhaBAD)568 rph-1. att::rhl-catLVA(Sp^R)*, which is the spectinomycin (Sp) resistance-marked integration (into the bacteriophage λ attachment site) of an Rhl autoinducer-responsive promoter (P_{rhl*}) driving an unstable (Andersen *et al*, 1998) version of *cat*. Specifically, the '*rhl-catLVA* cassette' carries both $P_{lacI}{}^q$-*rhlR* and P^*_{rhl}-*catLVA* (Chuang *et al*, 2009). Therefore, the growth of *rhl-catLVA* strains in Cm is strongly dependent on Rhl autoinducer. Producers carry in addition the low copy, GFP-marked production plasmid, pZS*2R-GFP,*rhlI*, whereas nonproducers carry a control plasmid lacking GFP and *rhlI*. *rhlI* encodes Rhl autoinducer (C$_4$-HSL) synthase (Parsek *et al*, 1999).

Plasmid and strain construction

An arginine-dependent producer was made by bacteriophage P1 transduction of *argHΔ::FRT(kan)* from the Keio reference strain JW3932 (Baba *et al*, 2006) into JC1191 to create JC1209. The *kan* marker was looped out using a transient source of Flp recombinase (Cherepanov and Wackernagel, 1995) and the resulting plasmid-free strain was transformed with the production plasmid to create JC1211. There is no significant cross-feeding of Arg$^-$ producers by Arg$^+$ nonproducers. A producer–nonproducer pair with increased dosage of *rhlR* was created by transforming the original producer and nonproducer strains with a second compatible plasmid, pZA12-*rhlR*. This plasmid has a medium copy origin of replication (p15A), the ampicillin resistance gene β-lactamase (*bla*), and *rhlR* under control of the P_{LacO1} promoter. pZA12-*rhlR* was constructed by subcloning the *rhlR* coding sequence into pZA12 (Lutz and Bujard, 1997). As the strains in this study are LacI$^+$, the increased copy number of *rhlR* only increases RhlR expression moderately through leakiness of the P_{LacO1} promoter. A control pair of producers and nonproducers with no further increase in *rhlR* dosage was created by transforming instead with the control vector pZA1R, which lacks *rhlR*. Strains carrying pZA1 vectors were maintained by selection with ampicillin (50 μg/ml).

Growth experiments and measurement of producer proportions

Growth media, growth conditions, and quantification of the relative growth of producers and nonproducers were performed as described (Chuang *et al*, 2009) except that for the experiments involving arginine limitation (Figure 1), the producer–nonproducer mixtures were subjected to an initial dilution of 1/50 into MOPS minimal media (Neidhardt *et al*, 1974) (supplemented with thiamine, uracil, dextrose, antibiotics, and arginine) containing 4.5 μg/ml Cm. 1X arginine is defined as 520 μM. In brief, a typical experiment involved setting up subpopulations with different proportions of producers (p_g=10, 20, 30, 40, 50, 60, 70, 80, 90, and 95% producers). Each mixture was first diluted and grown in the absence of Cm, and then at 'time 0,' each subpopulation was diluted into media containing Cm. Growth curves were obtained by periodic measurements of optical density at 600 nm (OD$_{600}$) in a plate reader (Wallac Victor 2). Before (i.e. at time 0) and after a period of limited growth (12–14 h at 30°C), the initial (p_g) and final (p_g') producer proportions within each group were determined by flow cytometry as described (Chuang *et al*, 2009) The initial ($\bar{p}$) and final ($\bar{p}'$) global producer proportions were measured by pooling equal volumes of each subpopulation and subjecting the global pool to flow

cytometry. The change in the global producer proportion, $\Delta\bar{p}$, is defined as $\bar{p}' - \bar{p}$.

Supplementary information

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

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Conflict of interest

The authors declare that they have no conflict of interest.

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