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Supporting Online Material for

Simpson's Paradox in a Synthetic Microbial System

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Supporting Online Material

Materials and Methods

Producer and Non-Producer Strains

Producer and non-producer strains (Fig. S1) were derived by transformation of JC1191, an *E. coli* BW25113 (*1*) derivative modified to allow growth in Cm in the presence of C_4-HSL. The production plasmid, pZS*2R-GFP,rhlI contains an artificial operon of *GFP* and *rhlI* (separated by a Shine-Dalgarno translation signal) under control of the strong promoter P_R (*2*) in a low copy, kanamycin-resistant plasmid. A control plasmid lacking *GFP* and *rhlI* was used to create the non-producer strain. The inherent fitness advantage of non-producers relative to producers, as judged by the *per generation* increase in non-producer proportion when both strains were competed in antibiotic-free medium, was between 1.04 and 1.05 for different transformants.

Plasmid and Strain Construction

Plasmid constructs were constructed using standard molecular biology methods and backbones were based on the pZ vector series (*3*). *rhlR* and *rhlI* were PCR-amplified from *P. aeruginosa* PAO1-LAC (ATCC #47085; American Type Culture Collection). pZS4Int-rhl-catLVA contains two diverging transcription units: 1) P_{lacI}^{q} *–rhlR*, constructed by subcloning *rhlR* behind the weak constitutive P_{lacI}^{q} promoter, and 2) P_{rhl}^{*}-*catLVA*, in which an unstable LVA variant of *cat* is expressed from an artificial *rhl* box-containing promoter. Note that the constitutive promoter P_{lacI}^{q} used to express *rhlR* is distinct from the well known IPTG (isopropyl β-D-1-thiogalactopyranoside) inducible P_{lac} promoter. P_{rhl}^{*} was constructed by amplifying a 159 bp P_{lux} promoter (coordinates 1028-1186 of GenBank AF170104) from *Vibrio fischeri* MJ-1 (Fotodyne) and replacing the 20 bp *lux* box sequence `acctgtaggatcgtacaggt` with the 20 bp sequence `tcctgtgaaatctggcagtt` from the *P. aeruginosa rhlA* promoter using fusion PCR (*4*). *catLVA*, which encodes an additional 13 amino acid C-terminal *ssrA* tag with sequence RPAANDENYALVA was made by PCR (*5*). pZS4Int-rhl-catLVA also contains spectinomycin-resistance as well as a bacteriophage λ attachment site (*attP*) which was used to integrate *rhl-catLVA* into the *E. coli* genome (see below).

JC1191 was constructed as follows. *E. coli* does not naturally produce AHL-based autoinducers, but its genome contains a *rhlR* family member, *sdiA*. Our puzzling observations of moderate basal expression of several *rhl* promoters in *E. coli* and the FACS-based isolation of a chromosomal *non-promoter* mutation which reduced leakiness (data not shown) was clarified by work explicitly addressing the effects of *sdiA* (*6*). Therefore, an *sdiA* null background was used to reduce C_4-HSL independent expression of the P_{rhl}^{*} promoter. JW1901_5 (Keio Collection; National BioResource Project), containing a *sdiA* knockout marked with kanamycin resistance (*kan*) flanked by two FRT sites (*7*), was used for P1 transduction (*8*) of BW25113. Subsequent excision of *kan*, induced by expression of FLP recombinase from plasmid pCP20 (*9*), was used to derive JC1080 (BW25113 Δ*sdiA::FRT*). The spectinomycin-marked *rhl-catLVA* construct was

then integrated into the lambda attachment site of the JC1080 genome using pLDR8 (*10*), a plasmid encoding bacteriophage λ integrase. The resulting strain, JC1191, has the full genotype: Δ*sdiA::FRT att::rhl-catLVA*(Sp^R) *rrnB3* Δ*lacZ4787 hsdR514* Δ(*araBAD*)*567* Δ(*rhaBAD)568 rph-1* (relevant genotype is underlined).

Media for Growth Experiments

Cells were grown in Luria-Bertani (LB; MP Biochemicals) medium, with or without 6.25 μg/mL chloramphenicol (Cm; Sigma-Aldrich). 50 μg/mL kanamycin (Kn; Sigma-Aldrich) and 25 μg/mL spectinomycin (Sp; Sigma-Aldrich) were also included in the medium to ensure maintenance of the Kn-marked production / control plasmids and to prevent growth of any possible contaminating strains not containing the integrated Sp-marked *rhl-catLVA* construct. Where indicated, 10 μM C_4-HSL (CAS# 67605-85-0; Cayman Chemical) was included. Cell-free producer-conditioned media was prepared by 0.22 μm filtration of liquid cultures containing producer cells.

Growth Experiments

All growth experiments were conducted at 30 °C in 96-well plates (BD Falcon 351177) under well-mixed conditions in 180 μL media. For experiments involving mixtures of producers and non-producers, the two strains were mixed at various ratios (vol/vol) and grown from a 1/100 dilution into media lacking Cm. Following this pre-incubation phase, the cultures were analyzed for initial producer proportions by flow cytometry (see below) and then diluted 1/100 into media containing 6.25 μg/mL Cm. After 12-13 h of growth, the final producer proportions were determined by flow cytometry and the optical density (OD_{600}) of each culture was measured and converted to relative growth (see below).

For experiments involving Poisson dilution conditions, an appropriate culture dilution was aliquoted into three 96 well plates (288 wells per treatment). Since it was not possible to observe directly the exact number of founder cells deposited in each well, λ, the mean number of founder cells per well, was estimated from the observed frequency f of empty wells as $\lambda = -\ln(f)$. Since this procedure is reliable only for small λ (because f approaches zero for larger values of λ), the means for other treatments, in which k times more culture was distributed to each well, were inferred as $k\lambda$. For example, in Fig. 4A, the λ=9.6 treatment (which had no empty wells) had four times more culture aliquoted relative to the λ=2.4 culture. For each treatment (Fig. 4A) or round (Fig. 4B), the 96 wells in each of the three plates were first individually pooled ("96-well pools"), and then the three 96-well pools were combined ("288-well pool"). The producer proportion was measured for all three 96-well pools and the 288-well pool.

Measurement of Producer Proportions

Since producers are GFP-labeled (Fig. 2A), producer proportions were measured by flow cytometry of 10,000 cells (Fig. 3) or 50,000 cells (Fig. 4) using a FACSCalibur (BD

Biosciences) equipped with a 488 nm laser line. The total cell count was measured by gating on forward and side scattering, and the number of GFP-positive and GFP-negative events gave an estimate of producer proportion. Although this method is rapid, allowing for high event counts, one concern was that flow cytometry, which may count dead and live cells, would lead to inaccuracies in producer proportion measurements. Therefore, a series of control platings was performed in which samples were also spread on LB agar plates and the proportion of producer clones was counted after imaging fluorescent colonies with a Typhoon 9400 Variable Mode Imager (Amersham Biosciences). These measurements (n=86 platings) ruled out a bias in producer or non-producer counts and validated the flow cytometry method. A second concern was that GFP fluorescence could be lost from producers in LB media which contains relatively high (171 mM) NaCl concentrations. However, no loss of GFP fluorescence occurred during the course of the experiment, because non-fluorescent events were not detected after growth of pure producer cultures ($p_i = 1$ in Fig. 3A). In addition, equivalent results were observed when the experiments of Fig. 3 were conducted in MOPS Minimal Medium (*11*), which contains 50 mM NaCl.

Measurement of Relative Growth

The relative growth (w_i) of each culture in Fig. 3A was estimated as follows. OD_{600} measurements made using a Wallac Victor2 plate reader (PerkinElmer) were converted into cell-forming units (cfu) by means of a calibration curve prepared from plate counts of mixtures grown under the same conditions. The correlation between OD_{600} and cfu (r=0.967; n=95 platings) was sufficient for our purposes, and the remaining noise likely accounts for the minor differences in observed versus predicted p' values in the 10-fold pools of Fig. 3B. For each trial in Fig. 3B, the cfu of each culture was normalized so that the maximum was 1.0. Since the 12 mixtures in each trial started from similar population sizes, the normalized cfu count is equivalent to the relative growth of the culture.

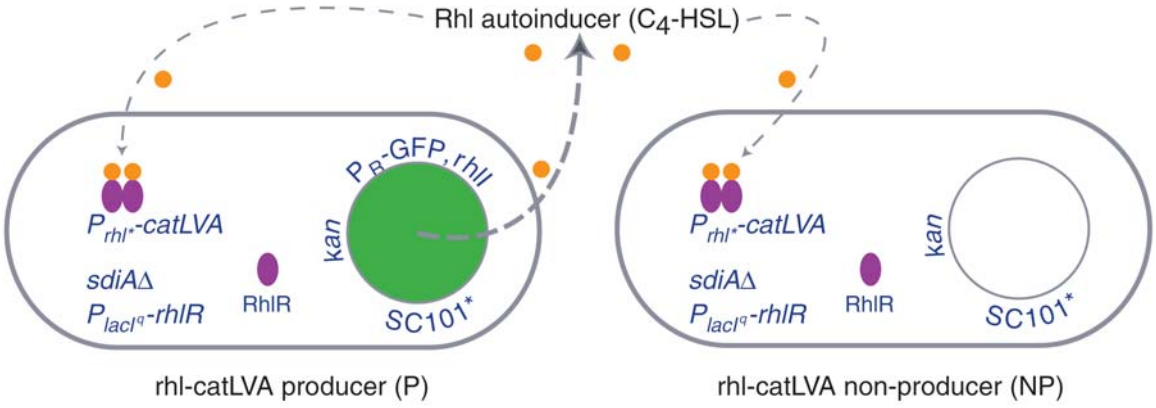

Fig. S1. A synthetic microbial system consisting of *rhl-catLVA* producers and non-producers. Our network design used signal production and response components from Gram-negative bacterial quorum sensing systems (*12-14*), in which membrane-permeable "autoinducer" molecules – N-acylated homoserine lactones (AHLs) – regulate target gene expression. Since *E. coli* naturally does not produce AHL autoinducers, genes involved in the production of and response to these molecules have been used by synthetic biologists to construct novel genetic networks with functions such as a "population control circuit" (*15*). Similarly, our synthetic system, consisting of two engineered producer and non-producer strains of *E. coli,* co-opted components from the *Pseudomonas aeruginosa* Rhl quorum signaling pathway. Isogenic producers (P) and non-producers (NP) differ by the presence of a GFP-marked production plasmid (green circle) in producers which constitutively expresses autoinducer synthase (*rhlI*) versus a control plasmid (white circle) in non-producers. When Rhl autoinducer (C_4-HSL; orange circles), which diffuses rapidly across membranes (*16*), binds the constitutively expressed transcription factor RhlR (purple ovals), the complex activates the artificial P_{rhl}^* promoter (see Methods), driving expression of an unstable variant of the chloramphenicol acetyltransferase gene, *catLVA*. The *sdiA* null background reduces Rhl autoinducer-independent expression (*6*). The resulting producer and non-producer strains exhibit Rhl autoinducer dependent chloramphenicol (Cm) resistance. Note: In the microbial context, to which our study belongs, the phenomena under consideration can be fully described in terms of the population dynamics of producers and non-producers, and we therefore find it preferable to avoid anthropomorphic terms, such as cooperation, altruism, cheating, kinship or game-playing.

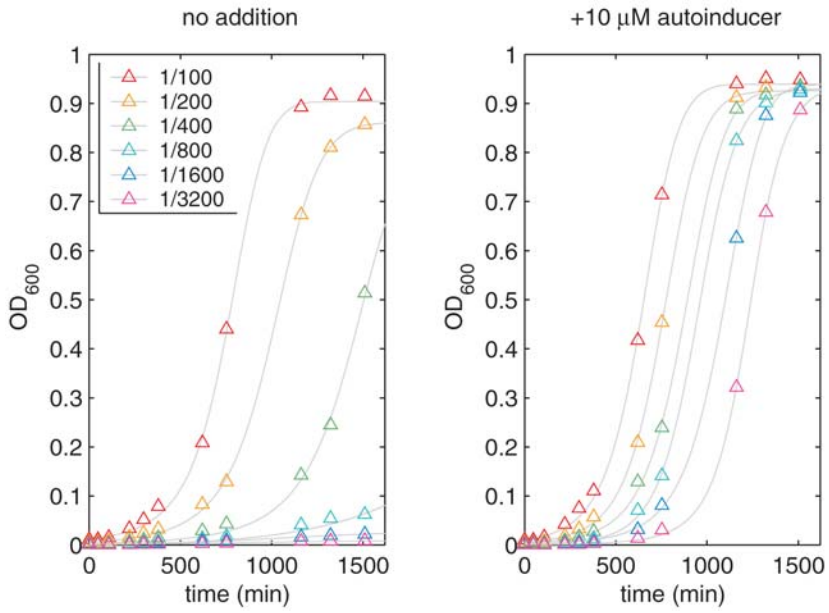

Fig. S2. Density dependence of producers. Growth curves of producers at 30 °C initially diluted to six different starting densities (colored triangles) into media containing 6.25 μg/mL Cm in the absence (left) or presence (right) of additional 10 μM purified autoinducer. Lines connecting points are visual guides derived from fits to generalized logistic functions. The divergence of the curves at higher dilutions shows that density dependent producer growth in Cm is due to limiting amounts of autoinducer.

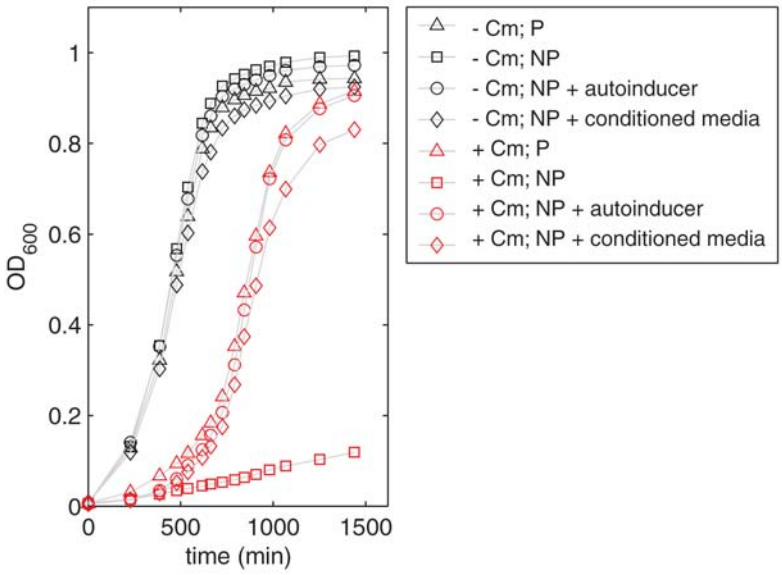

Fig. S3. Rescue of non-producers by either purified autoinducer or conditioned media from producers. Growth curves in the absence (– Cm, black symbols) or presence (+ Cm, red symbols) of 6.25 μg/mL Cm following 1/100 dilution of producers (triangles) and non-producers cultured at 30 °C with no further addition (squares), 10 μM autoinducer (circles), or 1/5 volume of producer-conditioned media (diamonds). The conditioned media curves saturate at lower densities because 1/5 of the input media is spent / used.

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