Experimental and Theoretical Studies of Artificial Microbial Symbiosis

Nikos Reppas[*]*, Xiaoxia Lin*[*] *and George M. Church*[†]

Department of Genetics, Harvard Medical School
77 Avenue Louis Pasteur, NRB-238, Boston, MA 02115

Introduction

Mutualistic symbiosis describes a cooperative relationship between two or more different organisms from which each partner benefits [4]. Symbiotic relationships exist widely in nature, for example, between plants and fungi in nutrient-poor soils, and between aphids and their endosymbiotic bacteria [3]. In this work, we are interested in investigating the evolutionary adaptation of a cross-feeding *E. coli* co-culture comprised of two different auxotrophic strains grown in serial batch culture on abundant glucose. We view this as a simplified experimental model of the much more complicated mutualistic ecosystems observed in nature that are often refractory to straightforward experimental analysis. One of the simplest manifestations of mutualistic growth is metabolite cross-feeding, whereby species A provides metabolite M required for the growth of species B, which in turn provides metabolite N required for the growth of species A. We artificially engineered such a system in vitro by co-culturing pairs of E. coli amino acid auxotrophs, each of which on its own is incapable of growth in minimal medium due to the absence of the required amino acid, but when grown with a partner can thereby obtain the needed nutrient. We hypothesized that upon evolving such a cross-feeding system by repeatedly passaging to fresh minimal medium, we could select for adaptive mutations in each strain that would increase the growth of the system as a whole, e.g. those enhancing amino acid export/import and/or biosynthesis. Apart from its significance to ecology and evolution, syntrophy (cross-feeding) is also of potential use for the engineering of microorganisms, for example, as an approach to select and/or maintain secretion of valuable biochemical products or biosensors.

Construction of *E. coli* auxotrophs through homologous recombination

We deleted genes (glyA, lysA, serA, tyrA) or operons (hisLGDCBHAFI, ilvEDAYC, proBA, thrBC, trpLEDCBA) encoding key enzymes in nine different amino acid biosynthesis pathways. These genes or operons were replaced separately by kan and cat genes, conferring resistance to kanamycin and chloramphenicol, respectively. We performed these genome engineering operations by recombinogenically substituting the desired genes with PCR-generated antibiotic-resistance cassettes flanked on either end by short (i.e. 45 bp) terminal regions of homology to the desired locus. For this purpose, we used a "wild-type" MG1655 *E. coli* strain, EcNR1, that carries a prophage expressing all the required proteins required for this type of linear recombination under the control of a temperature-inducible promoter [5]. Eighteen strains were thus constructed, all of which apart from EcNR1$\Delta glyA$ and EcNR1$\Delta lysA$ were indeed auxotrophic for the predicted amino acids.

Co-culturing of auxotroph pairs

[*]Equal contributions

[†]Corresponding author; URL: http://arep.med.harvard.edu

We then co-cultured all 21 possible pairs of differentially marked auxotrophic mutants in M9 minimal medium (without added amino acids). After 3 days growth, the optical density of the cultures was measured (Figure 1A); any cultures that had achieved a threshold turbidity by this time were passaged into fresh minimal medium. Four auxotrophic co-cultures were found to have grown relatively well after this second round of growth (Figure 1B). The greatest cross-feeding effect was seen for the EcNR1$\Delta tyrA$ :: kan + EcNR1$\Delta trpLEDCBA$ :: cat co-culture.

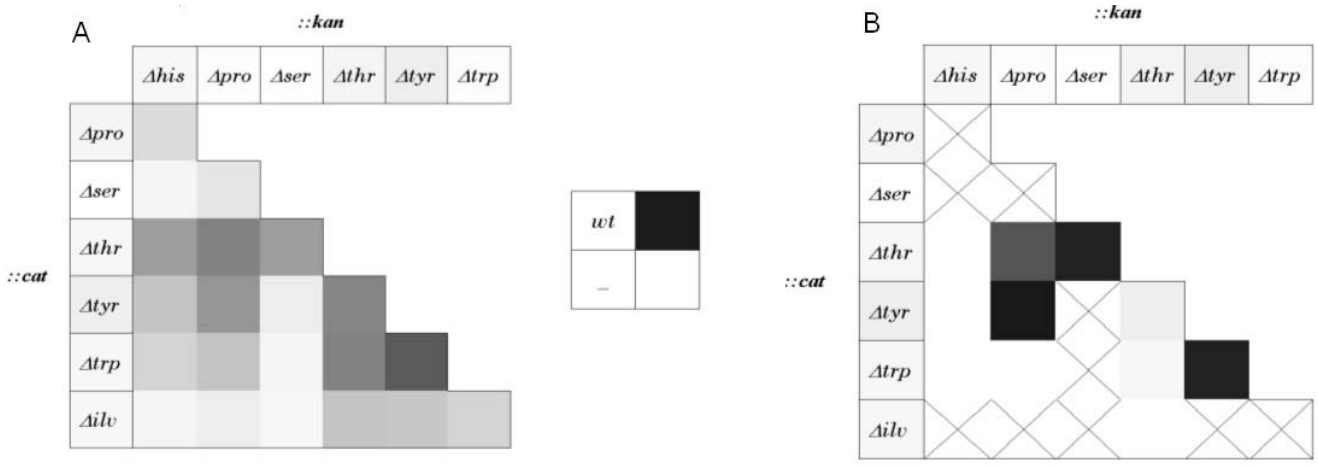

Figure 1: Cross-feeding of 21 pairs of differentially marked *E. coli* amino acid auxotrophs. The final level of culture turbidity is indicated by the intensity of the grey-scale shading. **A** Results of 1st round co-culturing; wild-type (wt) and uninoculated control (-) are indicated in inset. **B** Results of 2nd round co-culturing after passaging. An 'X' indicates a 1st round auxotroph pair that was not passaged as it failed to grow sufficiently in the first round. The tyrosine/tryptophan auxotrophic co-culture was selected for further analysis as it grew the best.

Experimental evolution of Δtrp-Δtyr co-culture

The EcNR1$\Delta tyrA$ :: kan + EcNR1$\Delta trpLEDCBA$ :: cat co-culture, which grew the best in the experiment described previously, was taken for further study. We evolved this cross-feeding co-culture as three independent lineages by daily passaging into fresh minimal medium over the course of approximately 100 days. Glucose was abundant throughout to minimize the potential complication of the appearance of acetate cross-feeders [1]. Exponential growth was maintained throughout. In all three lineages, we found that the doubling time of the co-culture decreased; an example is shown in Figure 2.

By periodically plating out onto solid medium, co-cultures were checked for the presence of both auxotrophs as well as that of any contaminating organisms. In all lines, a ratio of $\Delta tyrA$:$\Delta trpLEDCBA$ cells of $\sim$1:1–1:6 was maintained. In addition, we observed that after just a single passage, both auxotrophs had become polymorphic for colony size and appearance. On minimal medium plates, relative to such plates supplemented with tyrosine and/or tryptophan, colonies took three times as long time to appear and those that did invariably comprised both mutants – as expected of mutualistic growth. Notably, the increase of co-culture growth rate seen in liquid culture is mirrored by the apparent improvement of co-colony growth on solid minimal medium plates (Figure 3).

On the solid minimal medium plates, a colony develops from a pair of single cells, demonstrating that the symbiosis is maintained through cross-feeding or exchange of nutrients. To identify the underlying mechanism, i.e. to confirm that the mutualism is indeed mediated by free tyrosine and free tryptophan,

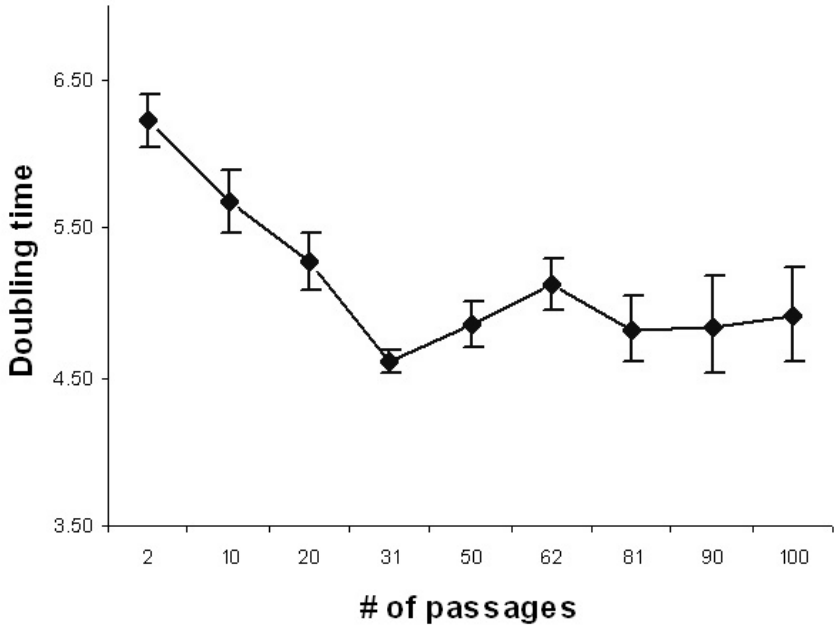

Figure 2: Improvement of growth rate of one of the independent lineages of the tyrosine/tryptophan auxotrophic co-culture. A plateau is reached after approximately 35 passages.

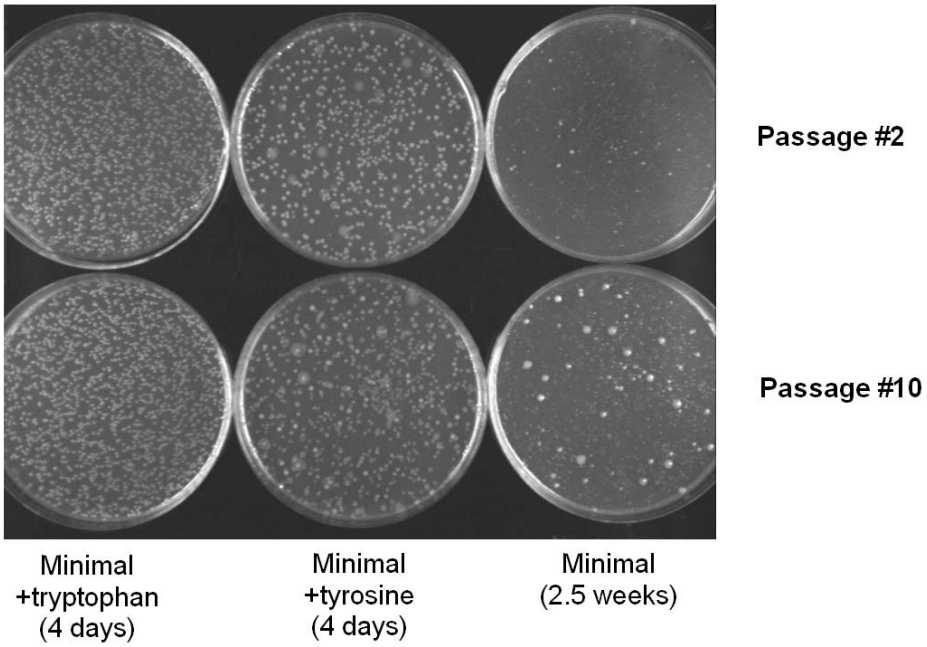

Figure 3: Solid minimal medium growth of one lineage of the tyrosine/tryptophan auxotrophic co-culture at 2 different passage times. The $\Delta tyrA$ strain is rapidly and stably invaded by a mucoid colony morph. On minimal medium without added amino acids, co-colonies form more efficiently after 10 passages than after 2, reflecting mutual adaptation.

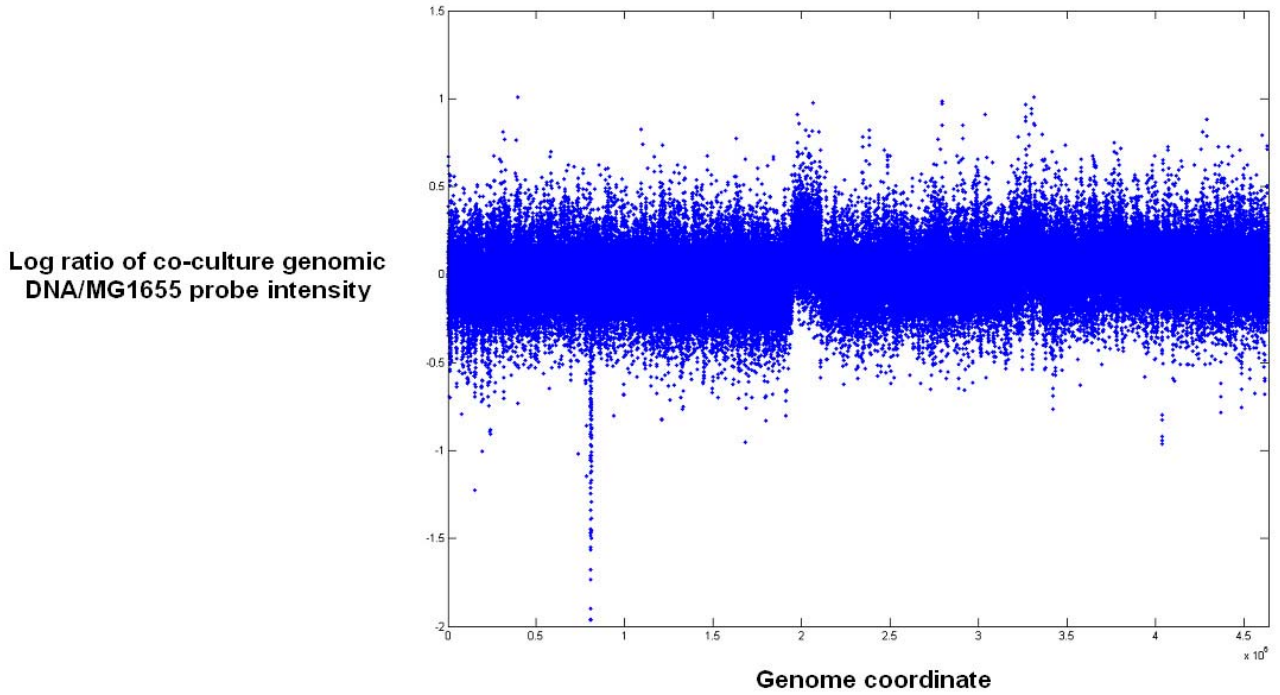

Figure 4: Representative plot of log10 ratio of co-culture/control probe intensities versus genome coordinate for genomic DNA isolated from one of the tyrosine/tryptophan auxotrophic co-cultures after 50 passages (1:1 auxotroph ratio). A downward spike indicates a deletion, e.g. that at 0.8 MB presents the deletion of 4 kb due to the integration of the recombinogenic phage in both auxotrophs. Conversely, an upward spike indicates an amplification, i.e. that centered at 2.1 MB.

we grew separated $\Delta tyrA$ and $\Delta trpLEDCBA$ cultures with a dialysis membrane with a 500 molecular weight cutoff. Preliminary results confirm that the syntrophy is mediated by small molecules ($<$ MW 500) - consistent with free amino acids being responsible for growth. We are also using mass spectrometry to directly measure free tyrosine and tryptophan in spent co-culture media.

Elucidation of genetic basis of coevolutionary adaptation

In an effort to discover the genetic basis for the observed co-culture mutualistic adaptation, we are currently constructing a genomic DNA library of an isolated clone of EcNR1$\Delta trpLEDCBA$ isolated after 40 rounds of passaging for whole-genome sequencing by polony technology. As a prelude to this high resolution genetic analysis, we first sought simply to identify any gross changes in genome structure due to chromosomal amplifications or deletions in either of the two auxotrophs. To this end, we analyzed the hybridization signatures of genomic DNA isolated from several different lines and passage numbers on Affymetrix Antisense Genome arrays, relative to that of MG1655 genomic DNA. We observe a consistent $\sim$163kb amplification centered at 2.1 MB in all cross-feeding genomic DNA samples, strongly suggesting that the amplification was present in the single ancestral co-culture that was used to start all three lineages (Figure 4).

Although the 163 kb region contains many genes, it is of particular interest that tyrP, a gene encoding a tyrosine-specific transporter, is one of them. It seems likely that this amplification is associated with the

$\Delta tyrA$ auxotroph. In support of this hypothesis, real time PCR copy number analysis indicates that the tyrP region is amplified in a EcNR1$\Delta tyrA$ growing on its own in the presence of minimal amounts of tyrosine (1 g/ L) (data not shown). We are carrying out further experiments to test this hypothesis.

An ODE based population dynamics model

We have developed an ordinary differential equation (ODE) system to model the population dynamics of the cross-feeding Δtrp-Δtyr E. coli auxotrophs in a batch liquid culture. Using this model, we are interested in understanding two types of changes:
(1) When Δtrp cells and Δtyr cells are mixed in the minimum medium, how do the cell population and the amino acid concentrations change over time?
(2) When the strains evolve (e.g., improve on amino acid imports and/or synthesis/exports), how does the behavior of the system change?

We start with the following relationship between bacterial growth rate and the concentration of limiting nutrients, first proposed by Monod in 1942 and generally confirmed more recently [2].

$$\mu = \frac{k_{max} c}{K_s + c} \tag{1}$$

where μ is the growth rate at a concentration c of limiting nutrient; k_{max} and K_s are constants. For the cross-feeding system we have constructed and evolved, we assume that the growth rate of each auxotroph is limited by the corresponding amino acid. The concentration of the limiting nutrient for each auxotroph, i.e. tryptophan and tyrosine respectively, is very low and hence we can simplify the Monod equation 1 to a linear relationship between growth rate and limiting nutrient concentration.

$$\mu \approx \frac{k_{max}}{K_s} c \equiv kc \tag{2}$$

Now we define four dynamic variables:
$n_{\Delta trp}$ density of Δtrp cells (gBM/ml)
$n_{\Delta tyr}$ density of Δtyr cells (gBM/ml)
c_{trp} concentration of trp (mmol/ml)
c_{tyr} concentration of tyr (mmol/ml)
Three pairs of parameters are involved in this system:
$k_{\Delta trp}$ growth rate constant of Δtrp cells (gBM/gBM-(mmol/ml trp)-hr)
$k_{\Delta tyr}$ growth rate constant of Δtyr cells (gBM/gBM-(mmol/ml tyr)-hr)
$\alpha_{\Delta trp}$ tyr excretion rate constant of Δtrp cells (mmol/gBM-hr)
$\alpha_{\Delta tyr}$ trp excretion rate constant of Δtyr cells (mmol/gBM-hr)
$\beta_{\Delta trp}$ trp requirement for growth of Δtrp cells (mmol/gBM)
$\beta_{\Delta tyr}$ tyr requirement for growth of Δtyr cells (mmol/gBM)
Note that the k's are related to the import of the required amino acids and the α's are related to the over-production and export of the amino acids.

The ODEs governing the symbiotic system in a liquid batch co-culture are as follows:

$$
\begin{aligned}
\frac{dn_{\Delta trp}}{dt} &= k_{\Delta trp} \cdot c_{trp} \cdot n_{\Delta trp} \\
\frac{dn_{\Delta tyr}}{dt} &= k_{\Delta tyr} \cdot c_{tyr} \cdot n_{\Delta tyr} \\
\frac{dc_{trp}}{dt} &= \alpha_{\Delta tyr} \cdot n_{\Delta tyr} - \beta_{\Delta trp} \cdot \frac{dn_{\Delta trp}}{dt} \\
&= \alpha_{\Delta tyr} \cdot n_{\Delta tyr} - \beta_{\Delta trp} \cdot k_{\Delta trp} \cdot c_{trp} \cdot n_{\Delta trp} \\
\frac{dc_{tyr}}{dt} &= \alpha_{\Delta trp} \cdot n_{\Delta trp} - \beta_{\Delta tyr} \cdot \frac{dn_{\Delta tyr}}{dt} \\
&= \alpha_{\Delta trp} \cdot n_{\Delta trp} - \beta_{\Delta tyr} \cdot k_{\Delta tyr} \cdot c_{tyr} \cdot n_{\Delta tyr}
\end{aligned}
\tag{3}
$$

The initial conditions are:

$$
\begin{aligned}
& n_{\Delta trp} = n_{\Delta trp,0}, \quad n_{\Delta tyr} = n_{\Delta tyr,0} \\
& c_{trp} = 0, \quad c_{tyr} = 0
\end{aligned}
\tag{4}
$$

The above initial value ODE system cannot be solved analytically. However, we can use computer simulation to obtain its solution very effectively. Figure 5 shows the results of an illustrative example with a specific set of parameters.

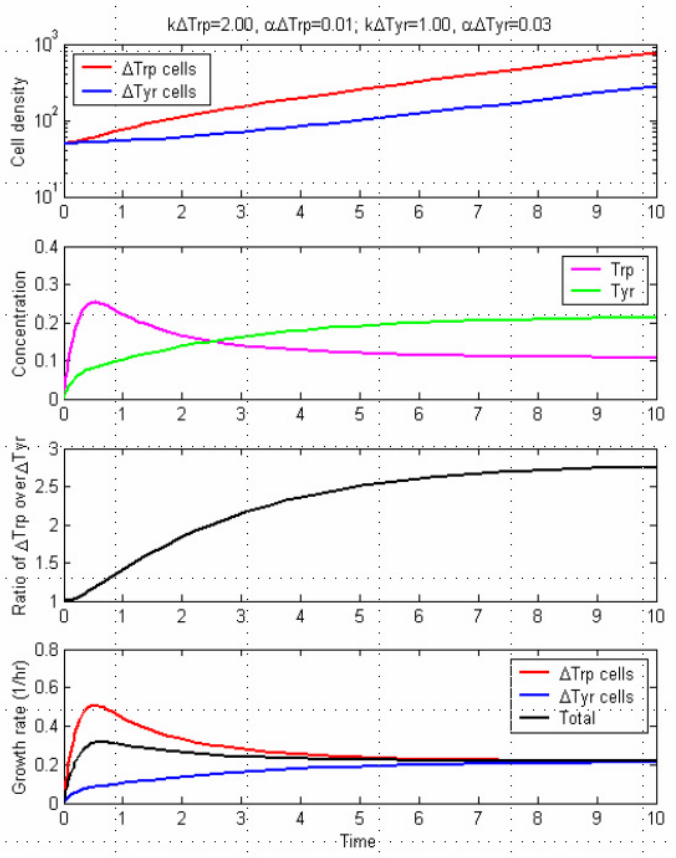

Figure 5: Example simulation results

Three observations can be made from these simulations.

- The ratio of the two cell types and the concentrations of trp and tyr all converge towards constants, no matter what the initial ratio is.
- The growth rates of the two cell types increase from zero at the beginning and reach a constant.
- The rate of approaching the constants only depend on the α's.

Note that the parameters of k's and α's are currently not available, though we can measure them in the lab. The values we use in the simulations may deviate from the true ones, nevertheless the qualitative predictions on the system's behavior should be the same. As a matter of fact, we can derive the "steady-state" solution analytically by making use of two conditions: (i) constant trp and tyr concentrations; and (ii) constant ratio of the two cell types. The final formula are as follows.

$$\mu_{tot} = \mu_{\Delta trp} = \mu_{\Delta tyr} = \sqrt{\frac{\alpha_{\Delta trp}\alpha_{\Delta tyr}}{\beta_{\Delta trp}\beta_{\Delta tyr}}} \tag{5}$$

$$\frac{n_{\Delta trp}}{n_{\Delta tyr}} = \sqrt{\frac{\alpha_{\Delta tyr}}{\alpha_{\Delta trp}}\frac{\beta_{\Delta tyr}}{\beta_{\Delta trp}}}$$

$$c_{trp} = \frac{1}{k_{\Delta trp}}\sqrt{\frac{\alpha_{\Delta trp}\alpha_{\Delta tyr}}{\beta_{\Delta trp}\beta_{\Delta tyr}}} \quad c_{tyr} = \frac{1}{k_{\Delta tyr}}\sqrt{\frac{\alpha_{\Delta trp}\alpha_{\Delta tyr}}{\beta_{\Delta trp}\beta_{\Delta tyr}}}$$

Therefore, the ODE model suggests that:
(i) The two auxotrophs reach the same final growth rate, which is also the growth rate of the overall system, and this growth rate only depends on the secretion parameters (α's). It's worth pointing out that the direct growth parameters (k's) do not affect the final growth rate, which is completely different from the single strain system. This happens because each auxotroph's growth depends not only on the growth parameter k, but also on the concentration of the corresponding amino acid, which is in turn affected by the other auxotroph, and eventually the concentration is adjusted in such a way that the growth parameters cancel out in determining the final growth rate of the system.
(ii) The final ratio of the two auxotrophs only depends on the ratio of their α's. The more one auxotroph secretes, the more there are the other auxotroph.

Now let's consider the evolution of the system by introducing advantageous mutants. More specifically, we introduce a second Δtrp auxotroph with different parameters after the original Δtrp and Δtyr pair reaches "steady state". As shown in Figure 6, the mutant can only invade when it has achieved a higher growth rate constant k. This is consistent with the notion of "the fastest growing one wins", which applies to each subpopulation in the cross-feeding system. In other words, the competition among different cells in the same sub-population selects for those that have the largest growth rate constants (k's). Note that the growth rate of the system only depends on the secretion rate constants (α's). Hence, an advantageous mutant can either improve the growth rate of the whole system or decrease it, depending on whether the mutant has an improved secretion rate (Figure 6 right part) or a reduced one (Figure 6 left part). We would expect random changes of overall growth rate if the two parameters k and α are evolved independently. It is also possible that they are correlated positively or negatively, in which case the growth rate of the system will keep improving or reducing, respectively. Therefore, based on the aforementioned experimental observation that the co-culture has improved its growth rate consistently in all three independent lineages, it is very likely that the two parameters are correlated positively during mutations. One possible scenario is that a bi-directional transporter, which can import one amino acid and export the other, has been over-expressed or improved during the co-evolutionary process.

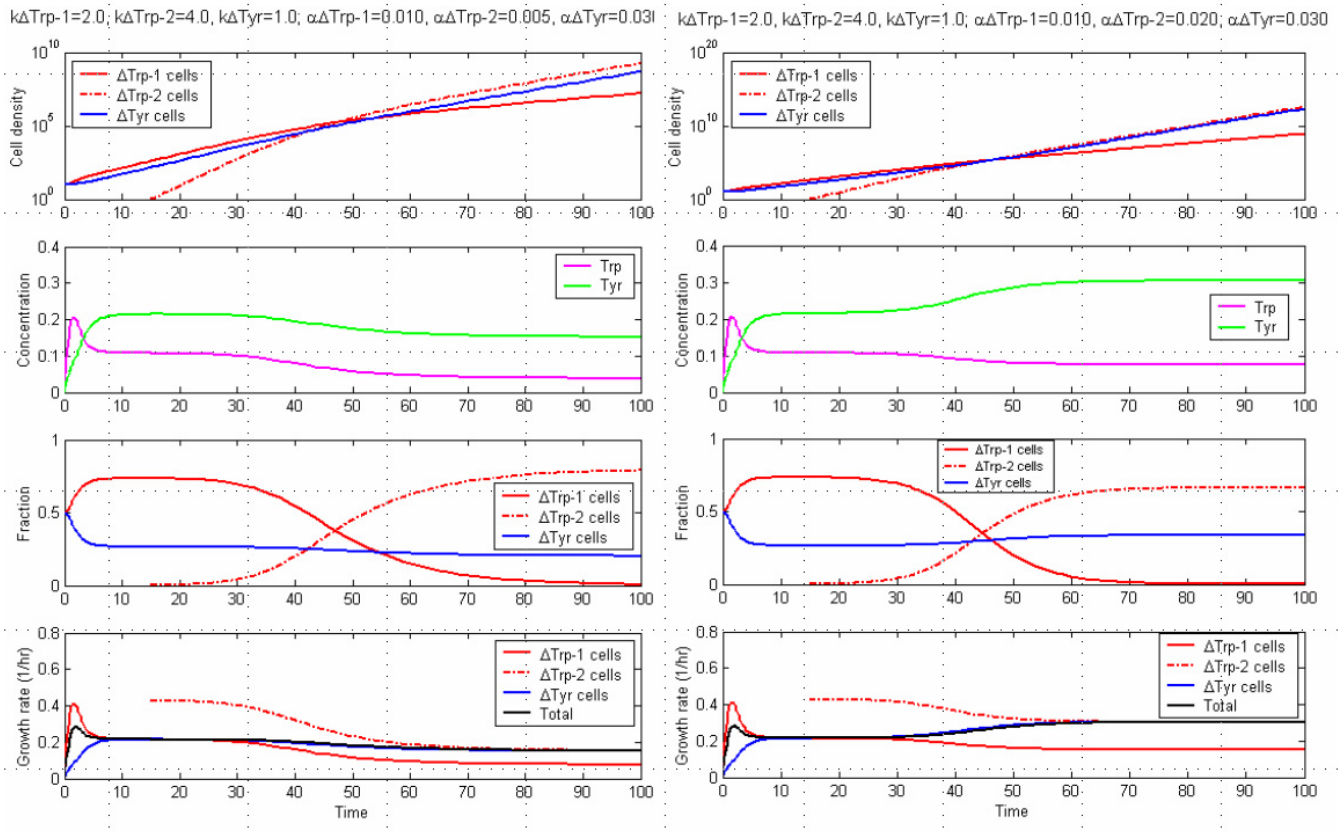

Figure 6: Invasion of advantageous mutants

Conclusions

In this work, we investigate microbial symbiotic systems consisting of two cross-feeding *E. coli* mutants, which can be seen as a simplified model of mutualistic symbiosis. We have constructed 21 pairs of *E. coli* auxotrophs each requiring a specific amino acid for growth. These pairs were co-cultured and were shown to be able to co-grow to varying degrees. The Δtrp-Δtyr pair demonstrated the strongest cross-feeding effect and was selected to undergo experimental evolution by passaging into fresh minimal medium repeatedly a daily basis. The growth rate of the system has improved. Through global Affymetrix chip analysis, we have discovered a $\sim$163kb amplification in all cross-feeding genomic DNA samples. This was further confirmed with real time PCR copy number analysis. We have also developed an ODE based model to investigate the population dynamics of the system, which provides insights on the interactions between different cells in the complex system. Our ongoing work include measuring related quantities to test whether our theoretical predictions agree with the experiments.

References

[1] Maren L. Friesen, Gerda Saxer, Michael Travisano, and Michael Doebeli. Experimental Evidence for Sympatric Ecological Diversification Due to Frequency-Dependent Competition in *Escherichia coli*. *Evolution*, 58:245–260, 2004.

[2] Frederick C. Neidhardt, John L. Ingraham, and Moselio Schaechter. *Physiology of the Bacterial Cell: A Molecular Approach*. Sinauer Associates, Inc., Sunderland, MA 01375, 1990.

[3] Shuji Shigenobu, Hidemi Watanabe, Masahira Hattori, Yoshiyuki Sakaki, and Hajime Ishikawa. Genome Sequence of the endocellular bacterial symbiont of aphids Buchnera sp.APS. *Nature*, 407:81–86, 2000.

[4] John M. Smith and E*ö*rs Szathm*á*ry. *The Major Transitions in Evolution*. Oxford University Press Inc., New York, 1995.

[5] Daiguan Yu, Hilary M. Ellis, E-Chiang Lee, Nancy A. Jenkins, Neal G. Copeland, and Donald L. Court. An Efficient Recombination System for Chromosome Engineering in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA*, 97:5978–5983, 2000.