The benefit of cooperation: Identifying growth-efficient interacting strains of Escherichia coli using metabolic flux balance models

Eleftheria Tzamali and Martin Reczko

Abstract **Cross-feeding, where two or more strains of an organism coexist on a single limiting resource, has been observed to emerge in long-term evolution experiments of E. coli in continuous culture [1]. Here, we describe a computational method to model and systematically identify synergistic strains that have superior growth by exploiting their metabolic by-products with mutual benefit. A mutual flux balance simulation considers all possible single gene deletions growing on various substrates. Several synergistic strains are found to have higher growth than any single-strain cultures given the same limiting substrates.**

As the method is based on a detailed genome scale metabolic flux balance model of the organism [2], the results are not only consistent with several observed cross-feeding E. coli strains, but can also explain the exact mechanism of the synergy. We expect a broad range of applications for this method in metabolic engineering.

I. Introduction

The evolution and maintenance of cross-feeding conditions in microbial population even in homogeneous environments is one example that contradicts the competitive exclusion principle that supports a single competitor in a single limiting resource [1]. Diversity in microbial populations may arise when certain beneficial coexistences of heterogeneous cell populations are possible to evolve. Cell communities, unlike single cells, dynamically shape the environment and the fitness landscape give rise to new metabolic capabilities that were not expected before. The definition of a beneficial coexistence has multiple aspects though.

In this study, the performance of a cell population will be measured with respect to the maximum biomass that the system is capable to produce in a given environment. The maximization of molecules required to make new cells (biomass) is commonly used [4] as the optimization strategy that the bacteria follow to efficiently orchestrate their components and accomplish certain cellular tasks. The rationale behind this assumption is that bacteria strive to maximize their growth under the evolution pressure that has driven the biological systems towards a better survival and source utilization. In order to model the initial evolutionary steps towards microbial diversity maintained by the synergy within the heterogeneous cell populations, 'selfish' cells that strive to maximize only their own biomass are assumed. A posteriori metabolic justification of a microbial mutualism among certain species is nicely supported and analyzed in [5] where a common objective is used instead. This is the first reported construction of a flux-balance model for a two-organism system that analyzes the syntrophic association between the microbes *Desulfovibrio vulgaris* and *Methanococcus maripaludis* which are capable of degrading organic materials in a various environments.

We initiate the study of beneficial heterogeneous cell communities by investigating heterogeneous pairs of cells having superior performance with respect to biomass production and we define a relative and an absolute synergistic benefit. Specifically, we systematically apply single gene deletions to the wild type E. coli cell and we investigate the possibility of having superior performance in cases where each of these strains mutually grows with the wild type cell under a dynamic environment of limited sources. To quantitatively describe superior performance in that coexistence two definitions that might be interesting under probably different perspectives are given. The coexistence of the heterogeneous cell population should first have superior performance in comparison to the performance of the wild type population and second should be superior to the performance of any possible mutant (absolute benefit). Examples of mutants that have shown superior growth when compared to the wild type cells have been observed for certain environmental conditions. Thus, the second condition can be relaxed to include cases (relative benefit) where the biomass produced by the coexistence of the wild type with a certain mutant is above the biomass of the wild type population as well as the biomass of that mutant even if there are other mutants of better performance. The relaxed definition assures that at least when the certain mutant appears, it will not be capable to dominate in the population, since coexistence with the wild type is more beneficial. For bioengineering purposes the absolute benefit might be far more interesting, however, biodiversity might support the relative benefit as well.

The mutual growth of the wild type with each possible knockout is simulated within the dynamic flux balance analysis framework [4] for several environmental conditions. Simulations are based on the main assumption that bacterial populations strive to maximize their growth

Manuscript received July 5, 2008. This work was supported by the Greek General Secretariat of Research and Technology under the PENED 2003 grant "Development of computational methods for genomic data analysis".

E. Tzamali is with the Institute of Molecular Biology and Biotechnology of the Foundation for Research and Technology-Hellas (FORTH) and the department of Computer Science, University of Crete, Heraklion, Crete, 71110 Greece (phone: 302810391721; fax: 302810391101; e-mail: tzamali@ imbb.forth.gr).

M. Reczko is with Synaptic Ltd., P.O. Box 1447 Voutes, Heraklion, Crete, 71110 Greece. (e-mail: mreczko@gmail.com).

under any genetic modification and environmental condition. The genetic modifications concern evolved strains that are assumed to have optimal growth characteristics that can be calculated by flux balance analysis. The two types of cell population grow in the same environment, consume the available resources, produce their own byproducts and proliferate with their own growth rates modifying dynamically the mutual environment. Each cell type population is initialized with the same biomass and eventually produces different amounts of biomass respecting its constraints, its corresponding biomass efficiency and the mutual growing environment. Analysis of the dynamic profiles of the concentrations of the exchange substrates that define the mutual environment, show that the production of certain byproducts such as acetate play significant role in beneficial synergy.

II. Method

Constraint-based models are widely used top-down models that aim to integrate knowledge at different levels in the cascade from genes to proteins and further to metabolic fluxes in a genome-scale metabolic network to describe and understand the overall cellular functions [3, 4]. Flux balance analysis (FBA) models are constraint-based models that estimate the *optimal* flux distribution of the entire biochemical reacting system, providing a quantitative description of the system when the intracellular fluxes are in balance. The flux balance method has proved successfully in analyzing the metabolic capabilities of several organisms, including its ability to predict deletion phenotypes, to determine the relative flux values of the metabolic reactions, to identify alternate optimal growth states.

Flux balance analysis has been further developed to embody in its original framework dynamic phenomena that affect the metabolic capabilities of the system. The so called dynamic flux balance analysis includes the effect of a temporarily varying environment which describes batch and fed-batch cultures [4]. In *silico* predictions that concern growth rates, uptake rates as well as secretion rates of the E. coli metabolic network have proved to be consistent with experimental data under certain conditions [6].

The concentrations of the environmental conditions in which cells will grow are initially specified. In batch cultures that we are interested in, the concentrations of the exchange substrates vary over time as the biomass that is produced consumes the available resources and produces byproducts at certain rates (2). When simulate dynamic phenomena, the whole time regime that represents the time of growth in cell populations is properly divided into constant time intervals, δt. The current exchange concentrations ($excC$) that describe the environmental conditions in which the population grows for the certain time interval, properly scaled by the amount of biomass (bm) that has been produced (1) shape the actual boundaries of the uptake fluxes ($excBounds$) as shown in (3). The metabolic network dynamically constrained by the substrate availability (4) will in turn determine the optimum flux distribution including the uptake flux vector $excFlux$ and the growth rate μ of the next time interval. When the current environmental conditions cannot support further growth in the cell population the iterative algorithm terminates.

$$bm(t) = bm(t-\delta t)\, e^{\mu \delta t} \quad (1)$$

$$\underline{excC}(t+\delta t) = \underline{excC}(t) - \underline{excFlux} \cdot bm\left(1 - e^{\mu \delta t}\right)/\mu \quad (2)$$

$$\underline{excBounds} = \underline{excC}/(bm \cdot \delta t) \quad (3)$$

$$\underline{excFlux} \le \underline{excBounds} \quad (4)$$

In heterogeneous cell populations, each population grows respecting the constraints that its network imposes and shapes the mutual environment according to the biomass it produces as well as its uptake and secretion rates of the exchange reactions. Under that manner, the update equations are reformulated as follows:

$$bm_1(t) = bm_1(t-\delta t)\, e^{\mu_1 \delta t} \quad (5a)$$

$$bm_2(t) = bm_2(t-\delta t)\, e^{\mu_2 \delta t} \quad (5b)$$

$$\underline{excC}(t+\delta t) = \underline{excC}(t) - \underline{excFlux}_1 \cdot \frac{bm_1}{\mu_1}\left(1 - e^{\mu_1 \delta t}\right) - \underline{excFlux}_2 \cdot \frac{bm_2}{\mu_2}\left(1 - e^{\mu_2 \delta t}\right) \quad (6)$$

$$bm = bm_1 + bm_2 \quad (7)$$

$$\underline{excBounds} = \underline{excC}/(bm \cdot \delta t) \quad (8)$$

When none of the different populations can grow further in the shaped medium the total biomass (7) that has been accumulated is compared to the final biomass that the homogeneous population has produced given the same initial environment.

The performance of both the homogeneous (wild type (WT), mutant (KO)) and heterogeneous (wildtype-mutant (WTKO)) cell populations was tested for each environmental condition and out of all genes included in the model [2].

III. Results

In the following we will present the different substrate conditions and gene knockout simulations that lead to superior growth performance of heterogeneous strains.

All our simulations are performed using the genome-scale metabolic model of *E. coli* (*i*JR904) by Reed et al. [2] which includes 904 genes and 931 biochemical reactions. Our simulations are done in aerobic conditions using the 69 diverse carbon sources defined in [7] with the following modifications: Oxygen was assumed to be in excess (50 m mol/lt) to avoid possible *diauxic* shift in growth due to oxygen deprivation. Mutants abstemious to oxygen might prove beneficial when grown with wild type cells in limited oxygen, however we preferred to keep the problem as

unrestricted as possible and understand the alternative underlying mechanisms of synergism that lead to superior performance. Ammonia was assumed to be in excess as well and is initially set to 30 m mol/lt. These are the two modifications we made with respect to the initial metabolite concentrations referred in [7]. All simulation are performed using a modified version of the COBRA toolbox [8].

Table I summarizes the conditions in which certain mutants show superior performance when mutually grown with the wild type cells. The first column presents the carbon source as abbreviated in the supplementary files of [7]. Table I is split in four main parts. The first part (WT) depicts the performance (BM_{WT}) of the homogeneous wild type population. The second part (BestKO) presents the performance (BM) of the best homogeneous mutant population ($gene\_{KO}$). The yield of the best mutant ($Yield_{WT1)}$ which describes the relative difference of the performances with respect to the wild type homogeneous population growth is also shown. For the conditions with a beneficial synergistic strain, we also always find a mutant with superior performance with respect to the wild type. The third part (Best synergistic KO) shows the performance of the mutant ($gene_{S\_KO}$) that when grown with the wild type in the given environment performs better than the homogeneous population of both the specific mutant and the wild type growth. In most of the cases the performance (BM) of this mutant in a homogeneous population is worse than the wild type as the corresponding yield ($Yield_{WT2}$) depicts. The last part presents the performance of synergy. BM_{S_WT} is the final biomass of the wild type population while BM_{S_KO} is the biomass that the mutant ($gene_{S\_KO}$) has produced. The relative ($Yield_{WT3}$) and absolute ($Yield_{abs}$) yield of the synergy, which are calculated with respect to the performance of the homogeneous wild type population (BM_{WT}) and best mutant ($gene\_{KO}$) performance respectively are depicted in the last two columns. Only for the growth on L argenine (arg_L) the best homogeneous mutant is also the best synergistic mutant. For growth on glycine (gly) and glycolate (glyclt) no mutant is found to perform better than the wild type in homogeneous populations. Table I is sorted according to the value of the absolute yield.

The way each of the cases depicted in Table I shapes the mutual environment and leads the system towards a superior performance is particularly interesting. In the following, we present a detailed analysis of the synergistic growths on glycolate, citrate, pyruvate and L arginine.

A. Case study: Aerobic growth on glycolate

Systematic examination of all possible single gene deletions reveals the mutant ‘b2276’ to have superior synergistic performance under growth on glycolate. The ‘b2276’ gene is related to NADH dehydrogenase involved in the respiratory chain of bacteria. The dynamic evolution of biomass for both the homogeneous and heterogeneous populations is shown in Fig. 1. The certain mutation affects the growth rate, thus a slower growth is observed in the homogeneous mutant population than that of the wild type under the same initial conditions. Fewer amount of biomass is also produced by the mutant population. However, the mutual growth of the wild type with the mutant populations shapes the environment in a beneficial way conducting supreme performance of about 8% benefit (Table I).

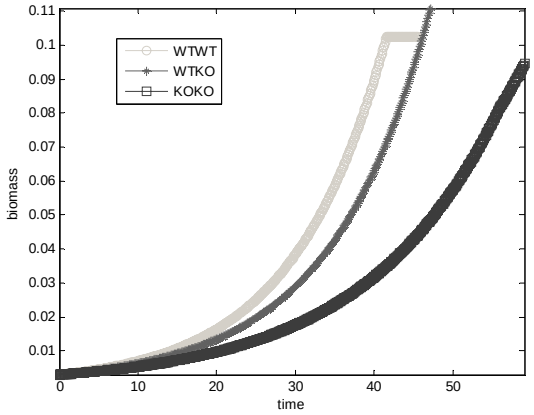

Fig. 1. Predictions of biomass in batch cultures of homogeneous wild type (WTWT), homogeneous mutant (KOKO) and heterogeneous (WTKO) cell populations grown on glycolate. The mutant of best synergistic performance is produced by the knockout of gene ‘b2276’. Time is in hours and the biomass is in gram [dry weight]/lt.

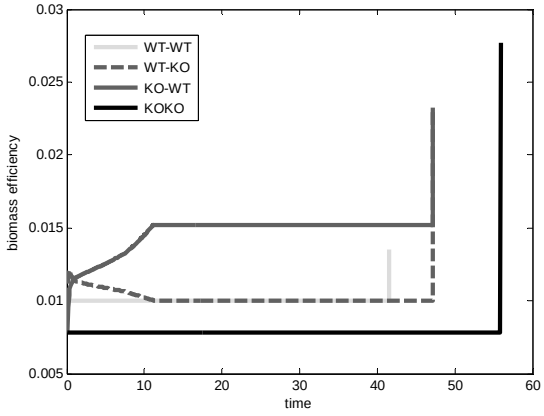

Fig. 2. Predictions of biomass (growth) efficiency with respect to glycolate uptake in batch cultures of homogeneous wild type (WT-WT), homogeneous mutant (KO-KO) and heterogeneous cell populations grown on glycolate as the main carbon source. The figure shows separately how the WT population depicted as WT-KO and how the KO population depicted as KO-WT performs in heterogeneous growth. The mutant of best synergistic performance is produced by the knockout of gene ‘b2276’. Time is in hours and the biomass efficiency is in gram [dry weight]/mmol.

This superior performance of synergism is actually due to the ratio of biomass rate to glycolate uptake rate (Fig. 2) that measures the efficiency of the cell system to convert the main carbon source available to essential biomass and plays key role as shown in the equation (6). It should be noticed though that apart from the main carbon source, essential byproducts may also play critical role in biomass production. However, in aerobic growth on glycolate when glycolate is exhausted no further growth is observed in both the homogeneous wild type and the heterogeneous wild type − mutant populations. The byproducts only constrain the

system fluxes and the growth efficiency with respect to glycolate is determinant for the certain conditions. Why the mutant population becomes that efficient when growing with the wild type? Dynamic profiles of the exchange substrates reveal that the mutant population becomes efficient because of formate, a byproduct which only the wild type population is capable to produce. The increase and decrease of the biomass efficiency that is observed for the first 11.3 h (Fig. 2), in the mutant and the wild type population respectively is due to the acetate production-consumption that only the mutant population produces. Apart from this initial acetate metabolism phase, the reasons that make synergistic growth on glycine also superior are similar.

Furthermore, under homogeneous growth no mutant was found to be superior when compared to the wild type performance under growth on either glycolate or glycine.

B. Case study: Aerobic growth on citrate

Dynamic simulations on citrate reveal the deletion strain of the 'b0728' gene out of all genes in the model to show superior performance when grows with the wild type. This gene is related to the Succinyl coenzyme A synthetase and is involved in the citrate cycle.

The certain gene deletion affects the growth rate and a slower growth is observed (Fig. 3) in the homogeneous

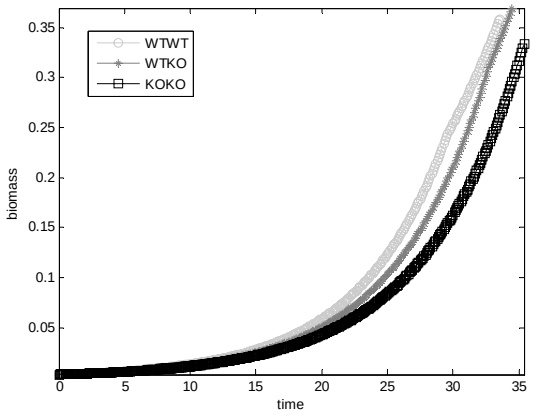

Fig. 3. Predictions of biomass in batch cultures of homogeneous wild type (WTWT), homogeneous mutant (KOKO) and heterogeneous (WTKO) cell populations grown on citrate. The mutant of best synergistic performance is produced by the knockout of gene 'b0728'. Time is in hours and the biomass is in gram [dry weight]/lt.

mutant population growth. The temporal evolution of biomass (Fig. 3) for both the synergistic and the homogeneous wild type growth show a shift in slope at certain time points (at 32.8h and 29.6h respectively). This shift is due to hydrogen deprivation that takes place in both the above cases as illustrated in the concentration profile of hydrogen (Fig. 4). Study of the effect of hydrogen in the growth rate further verifies these observations. Cells can continue to grow even if hydrogen is exhausted but at lower growth rate. Deprivation of H is not observed in the homogeneous mutant population growth under the same initial conditions. This is why no shift in the growth slope is observed in the homogeneous mutant growth. In that manner, synergy becomes beneficial since it delays the exhaustion of hydrogen allowing longer growth at higher rate and therefore more biomass to be eventually produced. Growth ends when citrate is completely exhausted.

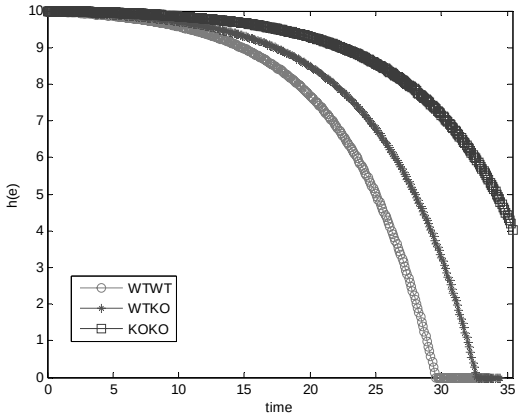

Fig. 4. Predictions of hydrogen concentration in batch cultures of homogeneous wild type (WTWT), homogeneous mutant (KOKO) and heterogeneous (WTKO) cell populations grown on citrate. Only the homogeneous mutant population does not deplete hydrogen during growth. Hydrogen concentration is in m mol/lt and time is in hours.

C. Case study: Aerobic growth on pyruvate

The metabolic pathways that are activated for the optimal consumption of pyruvate lead to the production of acetate. Acetate is an essential common byproduct which is also consumed by the cells. A mutant with the capacity of producing acetate at high concentrations is a potentially efficient partner to mutually grow with. However, acetate has a low growth rate when compared to the growth rate of pyruvate, thus a slight benefit might arise depending significantly on the growth efficiency of the mutant.

Simulations on pyruvate discriminate the mutant that is generated after deleting the gene 'b0721' to have superior synergistic performance as shown in Table I. This mutant produces similar effects under other conditions such as growth on D serine (ser_D), 2 oxoglutarate (akg) and L glutamate (glu_L). The gene 'b0721' encodes the enzyme succinate dehydrogenase which is involved in two metabolic reactions of different pathways; the citrate cycle as well as the oxidative phosphorylation.

The growth profiles of the heterogeneous wild type - mutant populations as well as of the homogeneous wild type population show two exponential phases (Fig. 5). The first phase (rapid growth) coincides with the consumption of pyruvate while the second (slower growth) is mainly related to the consumption of the by product acetate generated during the first phase. The mutant of 'b0721' gene produces acetate but it is not capable to consume it. Thus, no exponential shift is observed in the growth profile of the homogeneous mutant population. Furthermore, the growth rate is significantly affected by the deletion of gene 'b0721' leading the homogeneous mutant population to poor performance as shown in Fig. 5. In synergy, the biomass ratio of the mutant to the wild type population at the end of growth is about 1/7.7. Surprisingly, even though the mutant contribution to the total biomass is minor in synergy and no

synergistic benefit is observed during the first phase the acetate production efficiency of the mutant cells eventually results in a superior performance.

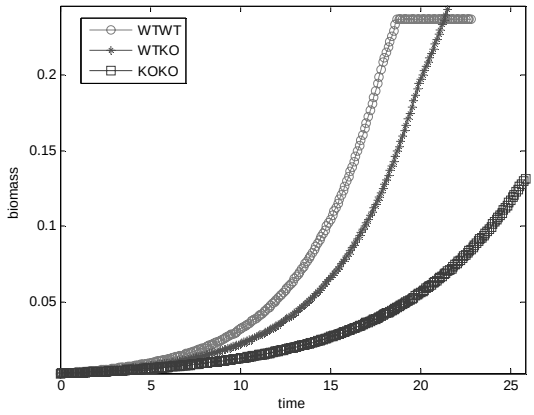

Fig. 5. Predictions of biomass in batch cultures of homogeneous wild type (WTWT), homogeneous mutant (KOKO) and heterogeneous (WTKO) cell populations grown on pyruvate. The mutant of best synergistic performance is produced by the knockout of gene 'b0721'. Time is in hours and the biomass is in gram [dry weight]/lt.

D. Case study: Aerobic growth on L arginine

Dynamic simulations of aerobic growth of E. coli cells on L arginine reveal a mutant that at the end of homogeneous growth has produced more biomass than the wild type (Fig. 6). Furthermore, the synergy between this mutant and the wild type populations is predicted to be even more efficient as shown in Table I. These observations arise two interesting questions. One concerns the reason that the homogeneous mutant population exhibits superior performance when compared to the homogeneous wild type growth. The second concerns again the synergistic benefit.

The temporal concentration profiles reveal that the mutant of the knockout gene 'b1744' redirects the fluxes towards the production of putrescine with no significant effect on the growth rate. Putrescine is the intermediate product of the arginine metabolic pathway. However, simulations predict that the wild type population does not produce putrescine. Applying flux variability analysis (FVA) [9] as a method to identify reactions that are critical for the optimal fluxes on the initial conditions on the wild type cells gives zero flux variability related to the putrescine exchange reaction. This means that the metabolic path towards the production of putrescine is not useful with respect to the growth rate. However, in a dynamic environment in which the main substrate L arginine will eventually get exhausted, the putrescine production plays an important role. It is consumed by the organism when L arginine has been consumed providing a long-term benefit in cell growth. These observations justify the superior performance of the mutant 'b1744' in a source limited environment.

Further experiments show that the presence of putrescine in the environment even at minor amounts (0.0002 m mol/lt) redirects the pathways and constrains the uptake flux of L arginine to 3.12 mmols/gram/h in contrast to 4.6 mmols/gram/h that is observed when no putrescine is present in the environment. In that way the synergistic environment is beneficial since the WT cell population efficiently consumes L arginine with respect to the biomass it produces. A slight increase in growth rate (0.5% relative increase) is observed as well. L arginine lasts longer allowing more biomass to be produced. When it is exhausted the system has already produced more biomass than the homogeneous mutant population. Growth ends when the remaining acetate and putrescine are consumed as well.

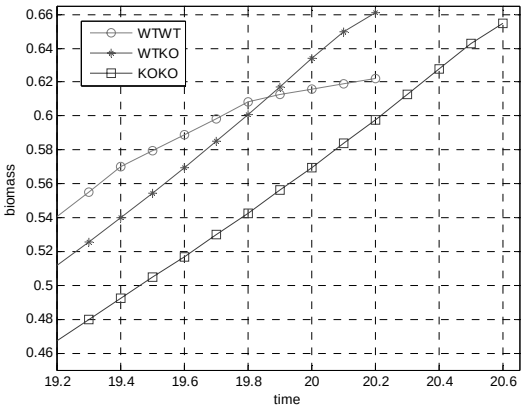

Fig. 6. Predictions of biomass in batch cultures of homogeneous wild type (WTWT), homogeneous mutant (KOKO) and heterogeneous (WTKO) cell populations grown on L arginine. The mutant of best synergistic performance is produced by the knockout of gene 'b1744'. Time is in hours and the biomass is in gram [dry weight]/lt. Slight difference is predicted on the final biomass between the homogeneous mutant population and the heterogeneous cell population. The final time period is shown.

IV. Conclusion

Why is it possible to have homogeneous mutant populations of superior performance with respect to the performance of wild type populations? The dynamic environment shapes the fitness function and alternative pathways that produce byproducts essential for living when the main substrate is exhausted are revealed. Such an example is the growth on L arginine. The metabolic pathways toward the production of the intermediate product putrescine are not optimal with respect to the growth rate under the initial conditions of a medium rich in L arginine (Flux Variability Analysis experiments). However, these pathways show long-term benefits. When this main substrate is exhausted, putrescine plays an essential role leading the system to superior performance. Under the above consideration heterogeneous cell populations can exhibit superior growth as well by exploiting their metabolic by-products with mutual benefit. Synergistic heterogeneous mutants of superior performance can also be found if the mutual environment they grow in is beneficial. When the best mutant and the best synergistic mutant are grown on pyruvate or D serine they exhibit further benefit with respect to the total biomass that is produced.

An encouraging experimental observation supporting the results of our simulation is the emergence of cross feeding

strains in several long-term evolution experiments with E. coli [1] showing that the superior solutions we suggest occur also in nature.

Investigation of coexistences of different mutants is an obvious next step. An efficient, computational method that explores the search space of all possible mutant pairs, triples or any multiples for superior performance comprises part of our planned future work. The method described here and its simple extension to the simulation of more than two interacting strains has many implications for research on the ecology of increasingly complex microbial communities in natural and engineered environments. Furthermore, the identification of heterogeneous bacterial cultures with superior desired properties might further exhibit a broad range of applications in metabolic engineering.

ACKNOWLEDGMENT

We thank George Thireos for support in the experimental verification of our results.

REFERENCES

[1] Treves, D., S. Manning, and J. Adams, "Repeated evolution of an acetate-crossfeeding polymorphism in long-term populations of Escherichia coli," Mol. Biol. Evol., vol. 15, no. 7, pp. 789-797, 1998.

[2] Reed, J.L., et al., "An expanded genome-scale model of Escherichia coli K-12 (iJR904 GSM/GPR)," Genome Biol, vol. 4, no. 9, pp. R54, 2003.

[3] Covert Markus, Schilling Christophe, and P. Bernhard, "Regulation of Gene Expression in Flux Balance Models of Metabolism," J Theor Biol., vol. 13, issue 1, pp. 73-78, 2001.

[4] Varma, A. and B.O. Palsson, "Stoichiometric flux balance models quantitatively predict growth and metabolic by-product secretion in wild-type Escherichia coli W3110," Appl. Environ. Microbiol., vol. 60, no. 10, pp. 3724-31, 1994.

[5] Sergey Stolyar, Steve Van Dien, Kristina Linnea Hillesland, Nicolas Pinel, Thomas J Lie, John A Leigh & David A Stahl1, "Metabolic modeling of a mutualistic microbial community," Molecular Systems Biology, vol. 3, no. 92, 2007.

[6] Jeremy S. Edwards, Rafael U. Ibarra & Bernhard O. Palsson, "In silico predictions of Escherichia coli metabolic capabilities are consistent with experimental data," Nature Biotechnology, vol. 19, pp. 125-130, 2001.

[7] Markus W. Covert, Eric M. Knight, Jennifer L. Reed, Markus J. Herrgard and Bernhard O. Palsson, "Integrating high-throughput and computational data elucidates bacterial networks," Nature, vol. 429, pp. 92-96, 2004.

[8] Scott A Becker, Adam M Feist, Monica L Mo, Gregory Hannum, Bernhard Ø Palsson & Markus J Herrgard, "Quantitative prediction of cellular metabolism with constraint-based models: the COBRA Toolbox," Nature Protocols, vol. 2, pp. 727-738, 2007.

[9] Mahadevan R, Schilling CH," The effects of alternate optimal solutions in constraint-based genome-scale metabolic models," Metab Eng., vol. 5, no. 4, pp. 264-76, 2003.

TABLE I
CONDITIONS OF SUPERIOR PERFORMANCE

	WT	Best KO			Best synergistic KO			Synergy			
condition	**BM_{WT}**	**$gene\_{KO}$**	**BM**	**$Yield_{WT1}$ (%)**	**$gene\_{KO}$**	**BM**	**$Yield_{WT2}$ (%)**	**BMs_{WT}**	**BMs_{KO}**	**$Yield_{WT3}$ (%)**	**$Yield_{abs}$ (%)**
glyclt	0.1025	-	-	-	'b2276'	0.0944	**-7.95**	0.085116	0.025721	**8.07**	**8.07**
gly	0.0660	-	-	-	'b2276'	0.0399	**-39.4**	0.06132	0.006615	**2.89**	**2.89**
cit	0.3585	'b0331'	0.3603	**0.49**	'b0728'	0.3331	**-7.08**	0.23057	0.1389	**3.05**	**2.54**
pyr	0.2365	'b3403'	0.2417	**2.19**	'b0721'	0.1313	**-44.8**	0.217158	0.028121	**3.71**	**1.48**
ser_D	0.2375	'b3403'	0.2433	**2.44**	'b0721'	0.1362	**-42.6**	0.218683	0.027194	**3.53**	**1.06**
arg_L	0.6223	'b1744'	0.6544	**5.16**	'b1744'	0.6544	**5.16**	0.36028	0.30053	**6.18**	**0.97**
4abut	0.5206	'b1849'	0.5211	**0.09**	'b0451'	0.3982	**-23.5**	0.39198	0.13054	**0.36**	**0.27**
melib	1.3837	'b1602'	1.4142	**2.20**	'b2276'	1.0878	**-21.4**	1.1073	0.28443	**0.58**	**-1.58**
tre	1.3883	'b1602'	1.4172	**2.08**	'b1241'	1.3788	**-0.68**	0.76453	0.62803	**0.31**	**-1.74**
sucr	1.3883	'b1602'	1.4172	**2.08**	'b1241'	1.3788	**-0.68**	0.76453	0.62803	**0.31**	**-1.74**
malt	1.3883	'b1602'	1.4172	**2.08**	'b1241'	1.3788	**-0.68**	0.76453	0.62803	**0.31**	**-1.74**
mnl	0.7782	'b1602'	0.7965	**2.34**	'b2288'	0.6283	**-19.2**	0.65929	0.12161	**0.34**	**-1.95**
akg	0.3933	'b4015'	0.4147	**5.44**	'b0721'	0.1343	**-65.8**	0.32267	0.078385	**1.97**	**-3.29**
glu_L	0.4764	'b4015'	0.5089	**6.83**	'b3236'	0.4774	**0.21**	0.26654	0.21778	**1.66**	**-4.83**