

# Intracellular crowding defines the mode and sequence of substrate uptake by *Escherichia coli* and constrains its metabolic activity

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**The influence of the high intracellular concentration of macromolecules on cell physiology is increasingly appreciated, but its impact on system-level cellular functions remains poorly quantified. To assess its potential effect, here we develop a flux balance model of *Escherichia coli* cell metabolism that takes into account a systems-level constraint for the concentration of enzymes catalyzing the various metabolic reactions in the crowded cytoplasm. We demonstrate that the model's predictions for the relative maximum growth rate of wild-type and mutant *E. coli* cells in single substrate-limited media, and the sequence and mode of substrate uptake and utilization from a complex medium are in good agreement with subsequent experimental observations. These results suggest that molecular crowding represents a bound on the achievable functional states of a metabolic network, and they indicate that models incorporating this constraint can systematically identify alterations in cellular metabolism activated in response to environmental change.**

flux balance analysis | metabolic networks | systems biology

An important aim of systems biology is the identification of the organizing principles and fundamental constraints that characterize the function of molecular interaction networks and the limits of an organism's phenotypic diversity (1–3). Flux balance-based modeling approaches, combining the constraints imposed by the metabolic network's structure with, e.g., mass- or energy-conservation principles (3–6), are especially successful in providing experimentally testable predictions on an organism's metabolic flux state and growth rate. A relative shortcoming of these approaches, however, is that they do not take into account the physical and spatial constraints resulting from the cell's unique intracellular milieu (7–9). For example,  $\approx 20$ – $30\%$  of the *Escherichia coli* cytoplasm is occupied by macromolecules, many of them enzymes, whose cytoplasmic concentration cannot be further increased without drastically affecting protein folding, protein–protein association rates, biochemical reaction kinetics, and transport dynamics within a cell (9, 10). This suggests that constraint-based modeling approaches, such as flux balance analysis (FBA) (3, 11), could be improved if we take into account that the enzymes catalyzing each reaction compete for the available cytoplasmic space (12, 13), potentially limiting the attainable flux rates.

Current flux balance-based modeling approaches also have limited ability to predict substrate uptake from the environment. Extensive experimental data indicates that when grown in complex medium bacterial cells use the available substrates either preferentially or simultaneously depending on the growth condition (see, e.g., refs. 14–17). Efforts to model mixed-substrate growth have assumed specific kinetic expressions for substrate uptake and biomass growth rates, and their predictions are formulated in terms of known model parameters (15, 18). Similarly, FBA predictions are based on previous knowledge of the maximum uptake rates in the corresponding medium (the actual variables one aims to predict), and, in contrast to empirical evidence, FBA in itself predicts the

simultaneous utilization of all carbon sources from a mixed-substrate growth medium. One way to overcome this deficiency is the superposition of regulatory mechanisms (in the form of mRNA expression signatures) on the FBA model, assessing which substrates are taken up and which are not (19). Yet regulatory mechanisms appear during the course of evolution because they result in a selective advantage for the cell. This selective advantage results from better use of the available resources within the metabolic constraints of the organism. Therefore, the metabolic constraint can be considered as the primary cause, whereas the regulatory processes represent the specific molecular mechanism developed to cope with this constraint. This fact opens the possibility for a FBA model that, after imposing the relevant constraints, predicts the selective advantage of implementing a regulatory mechanism. Here, we develop a modified FBA model that incorporates a solvent capacity constraint for the attainable enzyme concentrations within the crowded cytoplasm. Using this model, we predict the maximum growth rate of *E. coli* MG1655 wild-type and mutant strains on single carbon sources and for the dynamic patterns of substrate utilization from a mixed-substrate growth medium. We test the model predictions by using growth rate measurements and microarray and substrate concentration temporal profiles, and we obtain a good agreement between model predictions and experimental measurements. Taken together, these results suggest that macromolecular crowding indeed imposes a physiologically relevant constraint on bacterial metabolic activity and that incorporating this constraint allows for improved modeling of cell metabolism from system-level principles.

## Results

**FBA with Molecular Crowding.** In the flux balance model of cellular metabolism a cell's metabolic network is mathematically represented by the stoichiometric matrix,  $S_{mi}$ , providing the stoichiometric coefficient of metabolite  $m$  ( $m = 1, \dots, M$ ) in reaction  $i$  ( $i = 1, \dots, N$ ) (3, 20), where  $M$  and  $N$  are the number of metabolites and reactions, respectively. The cell is assumed to be in a steady state, where the concentration of each intracellular metabolite (other than the metabolites that constitute the biomass) remains

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Abbreviations: FBA, flux balance analysis; FBAwMC, FBA with molecular crowding.

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constant in time. Thus, the stationary reaction rates (fluxes) consuming and producing a metabolite should balance,

$$\sum_{i=1}^N S_{mi}f_i = 0, \quad [1]$$

where  $f_i$  denotes the flux of reaction  $i$ . The study of the solution space defined by Eq. 1 together with maximum capacity constraints for the uptake rates of extracellular substrates constitutes the basis of FBA (3).

We extend this framework to consider the physical and spatial constraints resulting from the very high intracellular concentration of macromolecules (7–9). Given that the enzyme molecules have a finite molar volume  $v_i$ , we can fit only a finite number of them in a given volume  $V$ . Indeed, if  $n_i$  is the number of moles of the  $i$ th enzyme, then

$$\sum_{i=1}^N v_i n_i \leq V. \quad [2]$$

Eq. 2 represents a constraint on the enzyme levels  $n_i$ , potentially affecting their maximum attainable values and relative abundance. Dividing by cell mass  $M$ , we can reformulate this constraint in terms of the enzyme concentrations  $E_i = n_i/M$  (moles per unit mass), resulting in

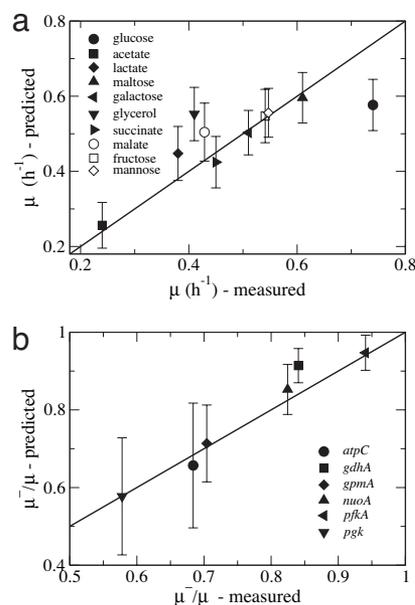
$$\sum_{i=1}^N v_i E_i \leq \frac{1}{C}, \quad [3]$$

where  $C = M/V \approx 0.34$  g/ml is the *E. coli* cytoplasmatic density (21). Eq. 3 imposes a constraint on the maximum attainable enzyme concentrations and, therefore, we refer to it as the enzyme concentration constraint. This constraint is reflected in the metabolic fluxes as well. Indeed, an enzyme concentration  $E_i$  results in a flux  $f_i = b_i E_i$  over reaction  $i$ , where the parameter  $b_i$  is determined by the reaction mechanism, kinetic parameters, and metabolite concentrations. Therefore, the enzyme concentration constraint (Eq. 3) is reflected in the metabolic flux constraint

$$\sum_{i=1}^N a_i f_i \leq 1, \quad [4]$$

where  $a_i = C v_i / b_i$ , affecting the maximum attainable fluxes and the flux distribution among different metabolic reactions. From here on, we refer to this mathematical framework as “flux balance analysis with molecular crowding” (FBAwMC). Furthermore, because the coefficient  $a_i$  quantifies the contribution to the overall crowding by reaction  $i$  we refer to it as the “crowding coefficient of reaction  $i$ ,” or simply “crowding coefficient.” Finally, we note that the enzyme concentration constraint is not the only additional constraint that could potentially restrict the metabolic capabilities of *E. coli* (for example, transporter capacities may be similarly limiting). Yet, our aim here is to test the predictive value of a model that assumes that the enzyme concentration constraint is indeed a main factor limiting the maximal metabolic capabilities of *E. coli*.

**FBAwMC Predicts the Relative Maximum Growth of *E. coli* Growing on Single Carbon Sources.** To examine the validity of macromolecular crowding as a constraint on a cell’s metabolic activity, and to test the predictive capability of the FBAwMC framework, we first examined the phenotypic consequences of extracellular substrate availability during growth in single carbon-limited medium with oxygen being in abundance, focusing on the maximum growth rate. The FBAwMC contains as a free parameter the average crowding



**Fig. 1.** Predicted and measured maximal growth rates comparison. (a) Comparison between the predicted (y axis) and measured (x axis) growth rates  $\mu$  of *E. coli* MG1655 grown in M9 minimal medium with various carbon sources. For a perfect match between experiments and theory the symbols should fall on the black diagonal. The symbols indicate the carbon substrate identified in the key. The predicted growth rates were obtained by using  $\langle a \rangle = 0.0040$  h·g/mmol (see *SI Text* sections S1 and S2). The error bars represent standard deviation over 1,000 sets of specific  $a_i$  parameters. (b) Same plot for single gene deletion *E. coli* mutants growing in glucose, the deleted genes being indicated in the key. The mutant growth rates  $\mu^-$  are given relative to the predicted and measured maximal growth rate  $\mu$  of wild-type *E. coli* cells growing in glucose-limited medium.

coefficient  $\langle a \rangle$ , and the model predictions for the maximum growth rate are proportional to  $\langle a \rangle$ . We first assumed that  $\langle a \rangle$  is a constant independent of the substrates. In this case it is possible to make predictions for the maximum growth rate in different substrates in arbitrary units. To obtain the maximum growth rates in specific units we fit  $\langle a \rangle$  to minimize the mean-square deviation between the predicted and measured growth rates, resulting in  $\langle a \rangle = 0.0040 \pm 0.0005$  h·g/mmol, in which g is grams of dry weight. We have obtained an independent estimate of  $a_i$  for  $\approx 100$  *E. coli* enzymes as well [supporting information (SI) Datasets 1 and 2], resulting in values between  $10^{-6}$  and  $10^{-1}$  and most probable values between  $10^{-5}$  and  $10^{-2}$  (in units of h·g/mmol). The obtained  $\langle a \rangle$  is, therefore, within the expected range.

Using the reconstructed *E. coli* MG1655 metabolic network (22) (SI Dataset 1), we first tested the maximal growth rate of *E. coli* MG1655 cells in various single carbon-limited media and compared the results with the theoretically predicted growth rates (Fig. 1a). In most cases the line of perfect agreement falls within the standard deviation, implying an overall good agreement between the model predictions and the measured maximum growth rates. For glucose and glycerol, the line of perfect agreement is outside the standard deviation, indicating that our assumption of a substrate-independent  $\langle a \rangle$  is not valid for these two substrates. *E. coli* is better adapted to growth on glucose, suggesting a smaller average crowding coefficient than in any of the other carbon sources. Indeed, the average crowding coefficient necessary to obtain a perfect agreement for glucose is smaller:  $\langle a \rangle = 0.0031 \pm 0.0001$  h·g/mmol. In contrast, in some carbon-limited media *E. coli* reaches its predicted maximal growth rate only after a period of adaptive evolution (23, 24), suggesting a higher average crowding coefficient before metabolic adaptation. Indeed, the average crowding coefficient neces-







is the transcriptome profile of galactose-limited cultures, which shows some similarity to that of cells at the stage of switching from exclusive glucose utilization to a mixed-substrate-utilization phase (3.5 h), and an even higher similarity to the transcriptome profiles of cells when all carbon sources are depleted (8 h). Thus, *E. coli* cells display a partial adaptation/stress response at each major metabolic transition, followed by a generic stress response (SI Fig. 27) and implementation of a foraging program (35) at complete exhaustion of all extracellular substrates that seems to be most primed for acetate and galactose catabolism.

## Discussion

A key aim of systems biology is the identification of the organizing principles and fundamental constraints that characterize the function of molecular interaction networks, including those that define cellular metabolism. In the present work we have focused on the identification of principles that define the growth and substrate utilization mode of bacterial cells in complex environments. Our experimental results indicate the occurrence of three major metabolic phases during the growth of *E. coli* on one type of mixed-substrate medium. Glucose, which by itself provides the highest growth rate, is preferentially used by *E. coli*, followed by simultaneous utilization of maltose, L-lactate, and galactose. Glycerol and (secreted) acetate are used at a third and final stage of growth. In addition, global mRNA expression data indicate that the organism-level integration of cellular functions in part involves the appearance of partial stress response by *E. coli* at the boundaries of major metabolic phases, and, as previously shown (35), the activation of a foraging program upon exhaustion of substrates from the growth medium (Fig. 4).

The simulation results show that the FBAwMC model introduced here successfully captures all main features of the examined metabolic activities. First, there is a significant correlation between *in vivo* relative maximal growth rates of *E. coli* in different carbon-limited media and the *in silico* predictions of the FBAwMC (Fig. 1). Second, the FBAwMC model predicts remarkably well the existence of three metabolic phases and hierarchical mode (i.e., single- or mixed-substrate utilization) of substrate utilization in mixed-substrate growth medium (Figs. 2–4). In essence, our modeling approach indicates that when *E. coli* cells grow in conditions of substrate abundance their growth rate is determined by the solvent capacity of the cytoplasm; vice versa, the solvent capacity should be saturated at the maximal growth rate. Therefore, when growing in a mixture of abundant carbon sources *E. coli* cells should preferentially consume the carbon source resulting in the highest growth rate. At solvent capacity saturation, the synthesis of metabolic enzymes for the utilization of a second, less efficient, carbon source can take place only at the expenses of degrading metabolic enzymes involved in the consumption of the more efficient carbon source.

However, this would result in a growth rate reduction and, therefore, cells preferentially using the more efficient carbon source would outgrow those that allow the simultaneous utilization of other carbon sources.

We observe, however, two discrepancies of the FBAwMC model predictions: (i) a higher than predicted amount of secreted acetate in the growth medium, and (ii) a somewhat earlier uptake and consumption of various substrates from the medium compared with that predicted by the model. The first discrepancy is likely rooted on the contribution of other cell components apart from metabolic enzymes. With increasing growth rate higher concentrations of ribosomal proteins, mRNA, and DNA are required in addition to metabolic enzymes (36). This observation indicates that the FBAwMC model may underestimate the impact of macromolecular crowding and the resulting excretion of acetate. The second discrepancy is quite likely a consequence of the first one, as acetate secretion is generally correlated with an increased carbon source uptake rate (27).

Taken together, our results show that *in silico* models incorporating flux balance and other physicochemical constraints can capture increasingly well the metabolic activity of bacterial cells, and that the maximum enzyme concentration is a key constraint shaping the hierarchy of substrate utilization in mixed-substrate growth conditions. Yet, while the metabolic capabilities of a cell are limited by such constraints, in reality any change in metabolic activity is controlled by regulatory mechanisms evolved in the context of these constraints. Therefore, constrained optimization approaches are also expected to help us better understand and/or uncover regulatory mechanisms acting in *E. coli* and other organisms.

## Materials and Methods

**Mathematical Framework.** The FBAwMC modeling framework has been established, as described in *Results* and as detailed in *SI Text*, S1 and S2.

**Growth Experiments, Carbon Substrate, and Microarray Analyses.** The *E. coli* K12 strain MG1655 ( $F^- \lambda^- ilvG rfb50 rph1$ ) was used throughout the work. Isogenic *E. coli* mutants (*pgk*, *atpC*, *gpmA*, *nuoA*, *gdhA*, and *pfkA*) were obtained from F. Blattner (University of Wisconsin, Madison) (37). The experimental details of the growth rate measurements, substrate concentration assays and microarray analyses are detailed in *SI Text*, S3–S12.

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