J Mol Evol (2006) 63:583–590
DOI: 10.1007/s00239-006-0018-x

JOURNAL OF MOLECULAR EVOLUTION

On the Origins of a Crowded Cytoplasm

Luis Acerenza, Martin Graña*

Systems Biology Laboratory, Faculty of Sciences, University of the Republic, Montevideo, Uruguay

Received: 25 January 2006 / Accepted: 26 June 2006 [*Reviewing Editor:* Dr. Antony Dean]

Abstract. Contemporary cells show a highly crowded macromolecular content, the processes which originated this state being largely unknown. We propose that a driving force leading to the crowded cellular state could be the increase in growth rate produced by an enhanced cytoplasmic protein concentration. Briefly, in a diluted scenario, an increase in protein concentration has two opposing effects on growth rate. The favorable effect is the increase in the activity per unit volume of the component proteins and the disadvantageous effect is the concomitant increase in the protein mass per unit volume which has to be produced. In this work we show that the first effect is quantitatively more important, resulting in an overall increase in growth rate. This result was obtained with a model of *E. coli* and using non-mechanistic physiological arguments. The proposed driving force operates even at low protein concentrations, where the nonspecific interactions of macromolecular crowding are not significant, and could be as ancient as the first protocells. Experimental measurement of this cytoplasmic protein concentration effect in present organisms is hindered by the prevailing nonspecific interactions, product of long-term evolution. However, chemical/biochemical systems, built up to mimic properties of living cells, could be an adequate tool to test this effect.

**Present address:* Unité de Biochimie Structurale, Institut Pasteur, Paris, France
Correspondence to: Luis Acerenza, Laboratorio de Biología de Sistemas, Departmento de Biología Celular y Molecular, Instituto de Biología, Facultad de Ciencias, Universidad de la República, Iguá 4225 Montevideo, 11400, Uruguay; *email:* aceren@fcien.edu.uy

Key words: Bacterial evolution — Cell physiology — Cellular models — Macromolecular crowding — Origins of life — Systems biology

Introduction

In present organisms, cellular macromolecular content is highly concentrated and remains between tightly bounded values (Fulton 1982; Zimmerman and Trach 1991). In *Escherichia coli*, this constancy of cell content holds across many physiological conditions and through experimental evolution (Schaechter et al. 1958; Bremer and Dennis 1996; Lenski et al. 1998). However, to our knowledge, no previous work has been much concerned with the driving forces and evolutionary mechanisms leading to highly crowded cellular states.

The idea that high total macromolecular concentration and the associated little free water available could condition cytoplasm organization and function is by no means new (e.g., Minton 1981). Effects of macromolecular crowding have been reported in phenomena spanning several levels of cellular organization: enzyme kinetics, protein folding, biochemical pathways, signal transduction processes, cell volume regulation, active transport, DNA conformation and function, among others (Minton 1983; Zimmerman and Minton 1993; Garner and Burg 1994; Walter and Brooks 1995; Zimmerman and Murphy 1996; Rohwer et al. 1998; Bray 1998; van den Berg et al. 2000; Kornberg 2000; Al-Habori 2001; Ellis 2001; Goobes et al. 2003; Ellis and Minton

2003). All these effects originate at the physiochemical level of nonspecific macromolecular interactions and have been studied in well-defined experimental systems (Zimmerman and Minton 1993; Hall and Minton 2003).

Regarding the potential advantages of a crowded environment, several considerations and findings have been made previously. Cellular crowding has been invoked to explain stabilizing mechanisms for DNA-DNA and protein-DNA interactions (Goobes et al. 2003; Record et al. 1998). For example, exposing *E. coli* to a wide range of osmolarities did not affect the kinetics and equilibria of protein-DNA interactions, suggesting that this was the result of compensation mechanisms underpinned by macromolecular crowding (Record et al. 1998). In another context, a study by Westerhoff and colleagues showed how crowding reduced the dissociation constants of enzyme complexes, leading to longer-lived functional structures (Rohwer et al. 1998). Furthermore, macromolecular crowding—along with diffusion—was described as a fundamental constraint in a discussion of the evolution of cell signal transduction pathways (Bray 1998). In the context of enzyme complexes sustaining sequential metabolic pathways in the cell, Srere (1987; Srere and Ovadi 1990) linked high macromolecular concentrations to tendencies toward organizing cytoplasm into discrete microenvironments—"metabolons"—where substrate channeling could take place.

The physiochemical considerations described above provide evidence that some of the changes in the properties of proteins, in the evolution from a diluted to a crowded cytoplasm, could confer the organism an evolutionary advantage. However, the changes in other proteins, for example, the change in the catalytic activity of certain enzymes, could contribute negatively to fitness (see, e.g., Cayley et al. 1991).

To explain the origins of a crowded cytoplasm, in this work we shall move from the molecular level to the cellular level of description, that is, from macromolecular interactions to systemic physiological processes. We shall show that the sole increase in the concentrations of proteins, without having to invoke modification of their properties due to nonspecific interactions, suffices to elaborate a plausible physiological explanation about the evolutionary advantage of having a highly concentrated intracellular milieu. First, we shall test the effect of increasing overall protein concentration in a model of *E. coli* that describes its cell cycle dynamics and long-term experimental evolution of cell volume and growth rate. Next we show that the results obtained with this particular model can be generalized using model free arguments from control analysis, macromolecular crowding, conservation constraints, and scaling, among others. Finally, we sketch a plausible mechanism for the evolution toward a highly crowded cytoplasm and discuss a way in which the existence of the newly proposed driving force, acting at the physiological level, could be tested experimentally.

Crowding and Growth Rate

A Bacterial Model

In order to test the effect that increasing overall cellular protein concentration has on growth rate, we used a mathematical model of *E. coli* previously developed to explain the parallel increase in cell volume and fitness in laboratory evolution experiments (Graña and Acerenza 2001).

In Richard Lenski's (2004, 2005) laboratory, populations of *Escherichia coli* have evolved, under controlled conditions, during thousands of generations. In these experiments, the bacteria showed a parallel increase in mean cell volume and relative fitness (Lenski and Travisano 1994). From a purely scaling perspective the result was rather unexpected, since it implied that a larger organism—with a lower surface-to-volume ratio and a smaller input of nutrient per unit volume—would grow faster. We found that explaining this parallel increase required considering processes operating at two levels: the organism and the population (Graña and Acerenza 2001). First, the physiology relevant to the evolutionary question was implemented in an organismic model. Our aim was to describe how cell volume, v, and relative fitness (i.e., growth rate of the evolved populations relative to the ancestor, μ/μ_0) change through evolution. Therefore, the relevant physiology to answer the evolutionary question was the relationship between cell volume and growth rate, v vs μ (Donachie and Robinson 1987), and the response of the growth rate to changes in external nutrient concentration, μ vs X, represented by the so-called Monod (1949) equation. These physiological responses were implemented in a modular model consisting of only three variables: cell volume, v, number of molecules of nutrient internal to the cell, n, and number of molecules of signal for initiation of DNA replication, s (see Appendix). The temporal behavior of these variables during the cell cycle was described by a system of three differential equations. Finally, we considered the processes operating at the population level. It has been experimentally shown that many catabolic functions, e.g., the capability to degrade substrates not present in the medium, decay during evolution (Cooper and Lenski 2000). This genetic decay was incorporated in the model via a simplified population genetics formalism. The resulting model, combining the physiology and population genetics,

succeeds in reproducing, quantitatively, the parallel increase in cell volume and fitness, and several other properties of the physiology and evolution of *E. coli*, determined under laboratory conditions (Graña and Acerenza 2001).

The essence of the explanation given by our model is as follows. The decay of unused functions during evolution decreases the consumption of nutrient for physiological adaptation purposes, leaving more nutrient available for growth. Since the organism maintains its composition approximately constant during the cell cycle and through evolution, the surplus of nutrient results in an increase in volume. The rate at which the signal is produced also increases with the amount of nutrient available for growth and the doubling time is reached in a shorter time, resulting in an enhanced growth rate.

In the present paper, the organismic model described above is used to test the effects of changing total protein concentration on growth rate. In the model, the total protein concentration remains approximately constant during the time scale of several cell cycles, in which short-term physiological responses take place. Therefore, it is considered a parameter. On an evolutionary time scale, total protein concentration could change, which may be represented by a slow change in time of the parameter. If this parameter change is advantageous, i.e., it increases the relative fitness of the population, the new value of total protein concentration and its phenotypic consequences would be fixed by selection.

It is important to note that, in the model, all the rates of the processes are proportional to the concentrations of the proteins that catalyze them. Therefore, the formulation of this model represents the situation that would occur in a putative diluted ancestral scenario, where the changes in protein concentrations maintain their proportions and specific properties unchanged. This additive effect on their functions results in macromolecular activities proportional to protein concentrations (this is described in more detail in the next section).

We shall now use the organismic model to simulate changes that could have occurred during the course of evolution. In Fig. 1, we represent the growth rate as a function of the factor by which protein concentrations and activities have been changed. The result obtained is an increasing function. Moreover, the growth rate divided by the surface-to-volume ratio is approximately constant (Fig. 2), indicating a close correlation between them. In conclusion, the analysis of the model suggests that an increase in the concentrations of proteins under additive conditions, i.e., maintaining their proportions and specific properties unchanged, produces an increase in the fitness of the organism.

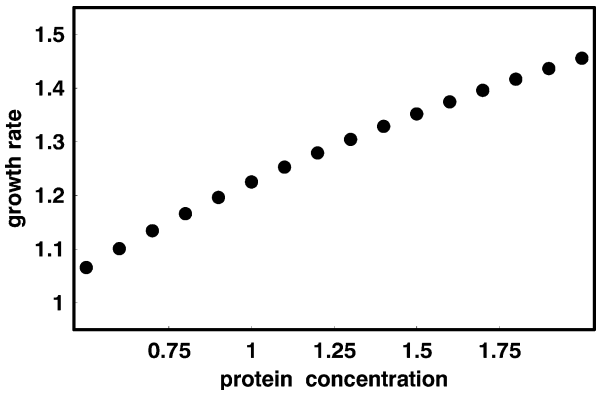

Fig. 1. Growth rate versus relative total protein concentration. The model used to generate this plot is briefly described in the Appendix (see also Graña and Acerenza 2001).

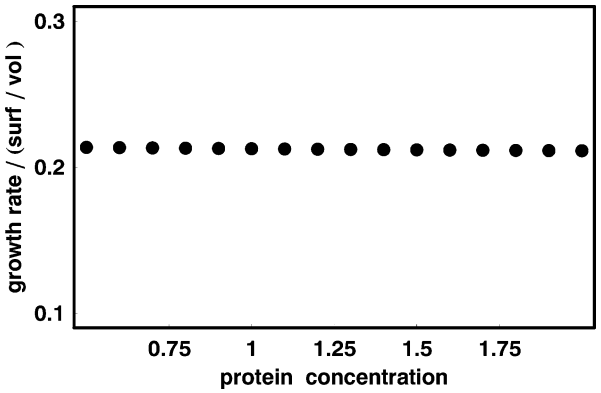

Fig. 2. (Growth rate)/(surface/volume) versus relative total protein concentration. The model used to generate this plot is briefly described in the Appendix (see also Graña and Acerenza 2001).

In the model, fitness augments and cell volume diminishes indefinitely with higher total protein concentrations. In reality, a limit exists for these tendencies, set by physical and physiological constraints of cellular processes. For example, a higher limit exists to the number of macromolecules that can be accommodated in a certain volume, and protein concentration may not be indefinitely increased. Similarly, there must be a lower limit to the volume of the cell capable of containing the minimal set of macromolecular species able to sustain life (Koch 1996).

In the next section, using model independent physiological considerations, we obtain results on the relationship between growth rate and total protein concentration similar to the ones that we have determined in this section, with the particular model of *E. coli*.

General Physiological Considerations

Let us consider a diluted intracellular milieu, where concentrations of molecules and macromolecules are

very low. This is an *additive* milieu, because in a highly diluted medium protein association-dissociation processes are displaced to the dissociated forms, causing the macromolecules to function independently, i.e., they only affect each other through metabolite effects (Kacser et al. 1990). In this scenario, when the protein concentrations are increased their proportions and specific properties remain unchanged, resulting in a proportionality relationship between concentration and activity. The alterations occurring in the system are therefore only due to the increase in the number of molecules per unit volume. Under these conditions, the increase in the concentration of all proteins by a certain factor produces two simultaneous effects—an increase in protein activity per unit volume and an increase in protein mass per unit volume—both by the same factor. Next we shall analyze the consequences of these two effects on the growth rate of a unicellular organism.

In an in vitro biochemical system, the protein concentrations are externally adjusted, i.e., they are parameters. Owing to the additivity property described above, if the experimenter increased them by a factor k, the processes would be performed, and their products would be produced, k times faster. In addition, in vitro systems operate at constant volume. Therefore, in the time courses of the metabolite concentrations, the same values would be reached in a period k times shorter (Acerenza 1990; Acerenza and Kacser 1990). In contrast to what normally happens in in vitro systems, in living systems proteins and volume are internally adjusted, i.e., they are variables. As mentioned above, the increase in protein activity per unit volume k times comes together with an increase in the protein mass per unit volume by the same factor. Mass increase takes time, contributing to decrease the growth rate. But since all the processes occur k times faster, the production of the signal that starts DNA replication would be faster and the time required to reach its threshold value smaller. As a consequence, the cell would divide in a shorter time, resulting in cells with smaller volumes. Smaller volumes give higher surface-to-volume ratios, and more nutrient per unit time is incorporated to the cell per unit volume. Therefore, these higher surface-to-volume ratios produce faster increases in protein mass per unit volume.

In summary, when total protein concentration is increased, there is a simultaneous increase in protein activity per unit volume and in protein mass per unit volume. The activity effect tends to increase growth rate and the mass effect to decrease it. We have argued that the activity effect is quantitatively more important than the mass effect, the increase in total protein concentration resulting in a net increase in growth rate. Note that, in these arguments, a key property leading to a faster growth rate is the increase in the mean surface-to-volume ratio, which is in agreement with what we found for the specific model of *E. coli* described under A Bacterial Model, above (see Fig. 2).

The general physiological reasoning that we have developed shows that even in an ideal additive scenario, where macromolecular nonspecific interactions are still not very significant, it would be expected that unicellular organisms showing a higher total protein concentration grow faster, being more fit. It is noticeable that this conclusion arises within a systems perspective, without postulating detailed mechanisms.

Final Remarks

We have argued that the highly concentrated cytoplasm found in contemporary unicellular organisms could be explained by an increase in fitness, produced by the effect that increasing protein concentrations, under additive conditions, has on cellular physiology. Superimposed on this effect are the contributions of crowding originated in nonspecific macromolecular interactions, which may increase or decrease fitness.

Another major component of fitness is the way in which the total protein concentration is distributed among different proteins performing the whole set of cellular functions. For a fixed total protein concentration, increasing the concentration of an enzyme, for example, would cause a fall in the concentration of others, resulting in perturbations of metabolic variables. This type of perturbation, called protein burden, has been measured experimentally (Koch 1983; Snoep et al. 1995). Under constant environmental conditions, one could hypothesize that in cellular organisms there is a distribution (or set of distributions) of enzyme concentrations maximizing fitness, which would be most difficult to determine with our present level of knowledge. However, in simple metabolic systems, the distributions of enzyme concentrations that maximize or minimize a variable, e.g., that maximize the flux of a metabolic pathway, can be calculated using standard optimization procedures (see, e.g., Heinrich et al. 1991; Heinrich and Schuster 1996; Kacser and Beeby 1984; Ortega and Acerenza 1997). In the present work, we did not treat the effects of enzyme distribution on fitness. On the contrary, we studied the effect of changing total protein concentration, while leaving the proportion of protein allocated to each enzyme or process unchanged.

Under our physiological arguments, a plausible mechanism of evolution of total cellular protein concentration could be as follows. At the early stages of prebiotic evolution, it is more likely that rather diluted environmental conditions prevailed (Lazcano

and Miller 1999). If the first cells originated at this stage, the protein concentration increase was driven solely by its effect on cellular physiology, in the absence of nonspecific interactions. At some point, protein concentrations sufficiently high for macromolecular nonspecific interactions to enter into operation in the cytoplasm were reached. In contrast, if the first cells appeared only after the prebiotic conditions attained a rather concentrated state, then their protein concentration increase would be driven by the effect on cell physiology and the effect of nonspecific interactions acting simultaneously. Nonspecific interactions could have positive or negative effects on fitness. Advantageous interactions reinforce the positive effect on physiology and are retained. In contrast, disadvantageous interactions may prevent additional increases in protein concentration by neutralizing the advantageous effect on physiology. These would be erased by mutation/selection, and only protein variants not promoting such negative interactions would be kept. As a consequence, further increases in protein concentration driven by the effects at the cellular level, or by newly evolved favorable nonspecific molecular interactions, could be obtained.

This process would continue until a highly crowded cytoplasm was obtained. At this stage, further small increases in total macromolecular concentration are expected to have major simultaneous effects on the thermodynamic and kinetic properties of many intracellular reactions. Therefore, a slowdown in the rate at which evolution to higher concentrations takes place would occur. In addition, concomitant to total macromolecular concentration increase during evolution, physiological mechanisms to maintain and restore this concentration, similar to those exhibited by present organisms, would have to have evolved to prevent harmful effects of external perturbations (Minton 2001).

The experimental test of the effect of increasing total protein concentration on growth rate would require measuring these physiological effects in the absence of nonspecific interactions. This poses a problem because, in the highly crowded cytoplasm of present living cells, the thermodynamic and kinetic effects of nonspecific interactions dominate. Note, for example, that with an increase in the external osmolarity, the cytoplasmic protein concentration of *E. coli* K-12 could be increased by more than 50%, resulting in a linear decrease in the growth rate (Cayley et al. 1991). This behavior can be explained by the disadvantageous effect of the newly added nonspecific interactions. The physiological effect of increasing the total protein concentration proposed in the present work, could well find a test bed in experimental chemical/biochemical systems similar to those that have been built to mimic essential properties of life, such as self-reproduction and homeostasis (Zepik et al. 2001; Luisi et al. 2004). It appears that more elaborated systems than the ones that have been constructed up to date would be required, but adequate synthetic systems would probably be obtained during the accomplishment of the challenging project of synthesizing life (Szostak et al. 2001).

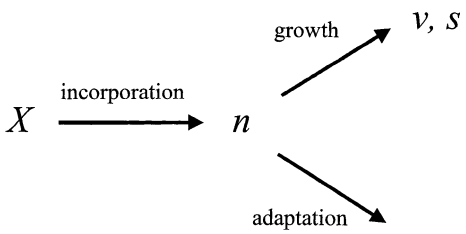

Fig. 3. Bacterial modular model.

Appendix: Bacterial Model

In the model (Graña and Acerenza 2001), cellular processes are partitioned into three modules, each one associated with an essential function of the cell, namely, nutrient incorporation, growth, and physiological adaptation. The nutrient (X) enters the cell by the nutrient incorporation module and is consumed by the growth and adaptation modules (Fig. 3). The growth module includes all the cellular processes that participate in growth and maintenance; the adaptation module, the processes responsible for physiological adaptation.

The model has three variables: cell volume, v, number of molecules of nutrient internal to the cell, n, and number of molecules of signal for initiation of DNA replication, s. Their time courses during the cell cycle are described by a system of differential equations:

$$\frac{dn}{dt} = k_i \frac{X}{K_X + X} \left((\pi d^2/2) + 4\, v/d \right) - (k_g + k_a)\, n$$

$$\frac{dv}{dt} = \frac{k_g}{C_T}\ n - k_d\, v$$

$$\frac{ds}{dt} = k_{gs}\, n - k_{ds}\, s$$

In these equations k_i, k_g, k_a, k_X, d, k_d, k_{gs}, k_{ds}, and C_T are parameters. The system of differential equations is integrated until a time t_u, at which the number of signal molecules, s, reaches a threshold value s_u. At this point, replication of DNA starts and production of signal ceases. After this moment, the number of molecules of signal remains at the threshold value until cell division is accomplished. The cell divides at

time t^* after initiation of replication. Therefore, the doubling time t_d and the growth rate μ are given by $t_d = t_u + t^*$ and $\mu = 60/t_d$.

t^* corresponds to the time for a round of chromosome replication and the time between the completion of chromosome replication and the subsequent cell division. It has been shown experimentally that, for the range of growth rates considered in this work (i.e., growth rates of between 1.0 and 1.5 doublings/h and duplication times of between 60 and 40 min), there is little variation in t^* for several strains of *E. coli* (Helmstetter 1996, p 1633). To take this fact into account, we have assumed in the model that t^* is not determined by the system of three differential equations, depending directly on the parameter values by the equation $t^* = 60/\mu^*$ where $\mu^* = (k^* X/(K^* + X))\ (2000/C_T)$. In this relationship, the hyperbolic dependence on X was included to comply with the Monod (1949) equation and the dependence on C_T to take into account the effect that a change in protein mass per unit volume has on μ^*. Note that μ^* was defined such that when k^*, representing growth activity, and C_T are simultaneously increased by the same factor, t^* does not change. The reason for this choice will be explained below.

The parameter values used were $k_i = 40$, $K_X = 5$, $X = 5$, $d = 1$, $k_g = 1.5$, $k_a = 4.5$, $k_d = 10^{-6}$, $k_{gs} = 2.5 \times 10^{-5}$, $k_{ds} = 10^{-6}$, $C_T = 2000$, $k^* = 3$, $K^* = 1$, and $s_u = 0.02$. With these values, the model just described succeeds in reproducing, quantitatively, the parallel increase in cell volume and relative fitness in *E. coli*'s evolution during 10,000 generations. This was the aim that originally motivated the development of the model.

But, with the same parameter values, the model can also explain the following experimental facts related to *E. coli*'s physiology and evolution of cell volume and fitness.

1. The fact that certain division mutants of *E. coli*, for example, *ftsZ* mutants, form filaments attaining sizes several hundreds of times the size of the wild type is explained by the property of the model that the volume grows, in principle, indefinitely if cell division is not imposed (Lutkenhaus and Mukherjee 1996).
2. The fact that incorporating in *E. coli* an inducible plasmid carrying the *dnaA* gene and overproducing its product (the signal protein DnaA) results in smaller and faster-growing cells corresponds in the model to increasing the catalytic capacity to produce the signal, i.e., to increasing k_{gs}, and obtaining a decrease in cell volume and an increase in growth rate (Løbner-Olesen et al. 1989).
3. The experimental evidence that the mechanism of antagonistic pleiotropy (consisting of mutations that were detrimental in the previous environment and are beneficial in the new one) is more important than the neutral process of mutation accumulation for the decay of unused catabolic functions is in agreement with a feature of the model consisting in that the genetic decay of the adaptation module in the laboratory evolution medium increases the growth rate and, therefore, is beneficial in this new environment (Cooper and Lenski 2000).
4. The experimental curves representing the physiological relationship between cell volume and growth rate, at different stages of evolution, have the same shape as those obtained with the model (Mongold and Lenski 1996).
5. Yield increase in processes converting glucose into cell mass during experimental evolution of *E. coli* is explained by the fact that, in the model, as a consequence of the genetic decay of the adaptation module, nutrient previously consumed for adaptation is used for growth (Lenski and Mongold 2000).

Moreover, we are not aware of any experimental results relating to the coarse patterns exhibited in the physiology and evolution of cell volume and relative fitness of *E. coli* that could be tested in the model and that the model could not explain.

The parameter C_T represents the total concentration of molecules and macromolecules inside the cell. In this work, we focus on the functional consequences of changing C_T. So, for our purposes, we will assume that a change in C_T could be considered as a change in total protein concentration. When the total protein concentration is changed by a certain factor, in an additive scenario, not only does the parameter C_T change by that factor, but also the activities of all the processes in the cell, mediated by proteins, will change by the same factor. Therefore, in the model, the change in total protein concentration will produce a simultaneous change in C_T, k_i, k_g, k_a, k_{gs}, and k^* by the same factor, and its effect on the growth rate will depend on the effect of a simultaneous change in these six parameters. We have seen that $\mu = 60/t_d$ with $t_d = t_u + t^*$. There are, in principle, two ways to affect μ by parameter changes, through t_u and through t^*. Since $\mu^* = (k^* X/(K^* + X))\ (2000/C_T)$ was defined so that when C_T and the five activities are changed, t^* does not change, the only effect of changing total protein concentration on growth rate is through t_u. The reason for defining μ^* independent of total protein concentration is as follows. We are interested in calculating, in this model, how μ depends on total protein concentration. But to our knowledge, the experimental relationship between μ^* and total protein concentration has not been determined. A parsimonious way to handle this lack of knowledge is to assume that μ^* is independent of total protein concentration, so that the effect of total

protein concentration on μ is only exerted through t_u. In this way, the dependence of μ on total protein concentration is only determined by the system of three differential equations, given above, which was built based on well-known experimental facts.

In Fig. 1, we plot the growth rate as a function of the factor by which C_T, k_i, k_g, k_a, k_{gs}, and k^* were changed. Increasing simultaneously these six parameters produces an increase in growth rate. Therefore, we can conclude that increasing the total protein concentration would produce an increase in growth rate, the derived bacterial population being more fit. The arithmetic mean of the surface-to-volume ratio (for the cell cycle) was determined for the same sets of values of the six parameters. Finally, in Fig. 2, we plot the growth rate divided by the surface-to-volume ratio as a function of the factor by which the six parameters were changed, obtaining an approximately constant relationship. This suggests that the increased mean surface-to-volume ratios appearing under higher total protein concentration conditions could be contributing significantly to the growth rate increase.

Acknowledgments. The authors are grateful to Allen Minton for useful comments relating to the physical properties of macromolecular crowding. L.A. also acknowledges funding from Comisión Sectorial de Investigación Científica de la Universidad de la República (CSIC, Montevideo) and Programa de Desarrollo de las Ciencias Básicas (PEDECIBA, Montevideo).

References

Acerenza L (1990) Temporal aspects of the control of metabolic processes. In: Cornish-Bowden A, Cárdenas ML (eds) Control of metabolic processes. Plenum Press, New York, pp 297–302

Acerenza L, Kacser H (1990) Enzyme kinetics and metabolic control. A method to test and quantify the effect of enzymic properties on metabolic variables. Biochem J 269:697–707

Al-Habori M (2001) Macromolecular crowding and its role as intracellular signalling of cell volume regulation. Int J Biochem Cell Biol 33:844–864

Bray D (1998) Signaling complexes: biophysical constraints on intracellular communication. Annu Rev Biophys Biomol Struct 27:59–75

Bremer H, Dennis PP (1996) Modulation of chemical composition and other parameters of the cell by growth rate. In: Neidhart FC, et al. (eds) *Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington, DC, pp 1553–1569

Cayley S, Lewis BA, Guttman HJ, Record MT Jr (1991) Characterization of the cytoplasm of *Escherichia coli* K-12 as a function of external osmolarity. Implications for protein-DNA interactions *in vivo*. J Mol Biol 222:281–300

Cooper VS, Lenski RE (2000) The population genetics of ecological specialization in evolving *Escherichia coli* populations. Nature 407:736–739

Donachie WD, Robinson AC (1987) Cell division: parameter values and the process. In: Neidhart FC, et al (eds) *Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington, DC, pp 1578–1593

Ellis RJ (2001) Macromolecular crowding: obvious but underappreciated. Trends Biochem Sci 26:597–604

Ellis RJ, Minton AP (2003) Cell biology: join the crowd. Nature 425:27–28

Fulton AB (1982) Why is the cytoplasm so crowded? Cell 30:345–347

Garner MM, Burg BB (1994) Macromolecular crowding and confinement in cells exposed to hypertonicity. Am J Physiol 266:C877–C892

Goobes R, Kahana N, Cohen O, Minsky A (2003) Metabolic buffering exerted by macromolecular crowding on DNA-DNA interactions: origin and physiological significance. Biochemistry 42:2431–2340

Graña M, Acerenza L (2001) A model combining cell physiology and population genetics to explain *Escherichia coli* laboratory evolution. BMC Evol Biol 1:12

Hall D, Minton AP (2003) Macromolecular crowding: qualitative and semiquantitative successes, quantitative challenges. Biochim Biophys Acta 1649:127–139

Heinrich R, Schuster S (1996) The regulation of cellular systems. Chapman and Hall, New York

Heinrich R, Schuster S, Holzhütter H-G (1991) Mathematical analysis of enzymic reaction systems using optimization principles. Eur J Biochem 201:1–21

Helmstetter C (1996) Timing of synthetic activities in the cell cycle. In: Neidhart FC, et al (eds) *Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington, DC, pp 1627–1639

Kacser H, Beeby R (1984) Evolution of catalytic proteins or On the origin of enzyme species by means of natural selection. J Mol Evol 20:38–51

Kacser H, Sauro HM, Acerenza L (1990) Enzyme-enzyme interactions and control analysis. The case of non-additivity: monomer-oligomer associations. Eur J Biochem 187:481–491

Koch AL (1983) The protein burden of *lac* operon products. J Mol Evol 19:455–462

Koch AL (1996) What size should a bacterium be? A question of scale. Annu Rev Microbiol 50:317–348

Kornberg A (2000) Ten commandments: lessons from the enzymology of DNA replication. J Bacteriol 182:3613–3618

Lang F, Busch GL, Ritter M, Volkl H, Waldegger S, Gulbins E, Haussinger D (1998) Functional significance of cell volume regulatory mechanisms. Physiol Rev 78:247–306

Lazcano A, Miller S (1999) On the origin of metabolic pathways. J Mol Evol 49:424–431

Lenski RE (2004) Phenotypic and genomic evolution during 20,000-generation experiment with the bacterium *Escherichia coli*. Plant Breed Rev 24:225–265

Lenski RE (2005) The *E. coli* long-term experimental evolution project site. Available at: http://myxo.css.msu.edu/ecoli/

Lenski RE, Mongold JA (2000) Cell size, shape and fitness in evolving populations of bacteria. In: Brown JH, West GB (eds) Scaling in biology. Oxford University Press, Oxford, pp 221–235

Lenski RE, Mongold JA, Sniegowski PD, Travisano M, Vasi F, Gerrish PJ, Schmidt TM (1998) Evolution of competitive fitness in experimental populations of *E. coli*: what makes one genotype a better competitor than another? Antonie van Leeuwenhoek 73:35–47

Lenski RE, Travisano M (1994) Dynamics of adaptation and diversification: a 10,000-generation experiment with bacterial populations. Proc Natl Acad Sci USA 91:6808–6814

Løbner-Olesen A, Skarstad K, Hansen FG, von Meyenburg K, Boye E (1989) The DnaA protein determines the initation mass of *Escherichia coli* K-12. Cell 57:881–889

Luisi PL, Rasi PSS, Mavelli F (2004) A possible route to prebiotic vesicle reproduction. Artif Life 10:297–308

Lutkenhaus J, Mukherjee A (1996) Cell division. In: Neidhart FC, et al (eds) *Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington, DC, pp 1615–1626

Minton AP (1981) Excluded volume as a determinant of macro-molecular structure and reactivity. Biopolymers 20:2093–2120

Minton AP (1983) The effect of volume occupancy upon the thermodynamic activity of proteins: some biochemical consequences: Mol Cell Biochem 55:119–140

Minton AP (2001) The influence of macromolecular crowding and macromolecular confinement on biochemical reactions in physiological media. J Biol Chem 276:10577–10580

Mongold JA, Lenski RE (1996) Experimental rejection of a non-adaptive explanation for increased cell size in *Escherichia coli*. J Bacteriol 178:5333–5334

Monod J (1949) The growth of bacterial cultures. Annu Rev Microbiol 3:371–394

Ortega F, Acerenza L (1998) Optimal metabolic control design. J Theor Biol 191:439–449

Record MT Jr, Courtenay ES, Cayley S, Guttman HJ (1998) Biophysical compensation mechanisms buffering *E. coli* protein-nucleic acid interactions against changing environments. Trends Biochem Sci 23:190–194

Rohwer JM, Postma PW, Kholodenko BN, Westerhoff HV (1998) Implications of macromolecular crowding for signal transduction and metabolite channeling. Proc Natl Acad Sci USA 95:10547–10552

Schaechter M, Maaløe O, Kjeldgaard NO (1958) Dependency on medium and temperature of cell size and chemical composition during balanced grown of *Salmonella typhimurium*. J Gen Microbiol 19:592–606

Snoep JL, Yomano LP, Westerhoff HV, Ingram LO (1995) Protein burden in *Zymomonas mobilis*: negative flux and growth control due to overproduction of glycolytic enzymes. Microbiology 141:2329–2337

Srere PA (1987) Complexes of sequential metabolic enzymes. Annu Rev Biochem 56:89–124

Srere PA, Ovadi J (1990) Enzyme-enzyme interactions and their metabolic role. FEBS Lett 268:360–364

Szostak JW, Bartel DP, Luisi PL (2001) Synthesizing life. Nature 409:387–390

van den Berg B, Wain R, Dobson CM, Ellis RJ (2000) Macromolecular crowding perturbs protein refolding kinetics: implications for folding inside the cell. EMBO J 19:3870–3875

Walter H, Brooks DE (1995) Phase separation in cytoplasm, due to macromolecular crowding, is the basis for microcompartmentation. FEBS Lett 361:135–139

Zepik HH, Blöchliger E, Luisi PL (2001) A chemical model of homeostasis. Angew Chem Int Ed 40:199–202

Zimmerman SB, Minton AP (1993) Macromolecular crowding: biochemical, biophysical, and physiological consequences. Annu Rev Biophys Biomol Struct 22:27–65

Zimmerman SB, Murphy LD (1996) Macromolecular crowding and the mandatory condensation of DNA in bacteria. FEBS Lett 390:245–248

Zimmerman SB, Trach SO (1991) Estimation of macromolecule concentrations and excluded volume effects for the cytoplasm of *Escherichia coli*. J Mol Biol 222:599–620