Modelling and Analysis of Central Metabolism Operating Regulatory Interactions in Salt Stress Conditions in a L-Carnitine Overproducing E. coli Strain

Guido Santos1, José A. Hormiga1, Paula Arense2, Manuel Cánovas2, Néstor V. Torres1*

1 Departamento de Bioquímica y Biología Molecular, Universidad de La Laguna, San Cristóbal de La Laguna, Santa Cruz de Tenerife, Spain, 2 Departamento de Bioquímica y Biología Molecular B, Universidad de Murcia, Murcia, Spain

Abstract

Based on experimental data from E. coli cultures, we have devised a mathematical model in the GMA-power law formalism that describes the central and L-carnitine metabolism in and between two steady states, non-osmotic and hyperosmotic (0.3 M NaCl). A key feature of this model is the introduction of type of kinetic order, the osmotic stress kinetic orders (gOSM), derived from the power law general formalism, which represent the effect of osmotic stress in each metabolic process of the model. By considering the values of the gOSM, linked to each metabolic process we found that osmotic stress has a positive and determinant influence on the increase in flux in energetics metabolism (glycolysis); L-carnitine biosynthesis production; the transformation/excretion of Acetyl-CoA into acetate and ethanol; the input flux of peptone into the cell; the anabolic use of pyruvate and biomass decomposition. In contrast, we found that although the osmotic stress has an inhibitory effect on the transformation flux from the glycolytic end products (pyruvate) to Acetyl-CoA, this inhibition is counteracted by other effects (the increase in pyruvate concentration) to the extent that the whole flux increases. In the same vein, the down regulation exerted by osmotic stress on fumarate uptake and its oxidation and the production and export of lactate and pyruvate are reversed by other factors up to the point that the first increased and the second remained unchanged. The model analysis shows that in osmotic conditions the energy and fermentation pathways undergo substantial rearrangement. This is illustrated by the observation that the increase in the fermentation fluxes is not connected with fluxes towards the tricaboxylic acid intermediates and the synthesis of biomass. The osmotic stress associated with these fluxes reflects these changes. All these observations support that the responses to salt stress observed in E. coli might be conserved in halophiles. Flux evolution during osmotic adaptations showed a hyperbolic (increasing or decreasing) pattern except in the case of peptone and fumarate uptake by the cell, which initially decreased. Finally, the model also throws light on the role of L-carnitine as osmoprotectant.

Introduction

Stress responses and their underlying mechanisms are of foremost interest not only because they are critical for determining cell physiology and metabolism in such conditions but also because they reveal some conserved features that are largely independent of the organism [1].

Extensive investigation into E. coli stress responses [2–5] has shown that major components of the general and specific response regulate key cellular processes. However, most of the studies on the E. coli response to osmotic stress environmental changes refer to the transcriptional level. Despite some analyses [6–10], the mechanisms involved in the adaptation of the central and energy metabolism of bacteria to osmotic conditions remain largely unstudied. Important in this regard is the fact that, contrary to most cases where signaling is involved, the osmoregulation signals which eventually translates into changes in metabolism and physiology, are of a physicochemical rather than of chemical nature [11]. This paper deals with the osmoregulation of E. coli at the metabolic level; that is, the changes in metabolic processes which, prompted by signals associated with the new osmolality conditions, occur during adaptation to increasingly saline environments.

Important in this regard are some aspects that greatly determine the scope of our study. First, attention should be drawn to the distinction between the osmoregulatory mechanisms acting in the long term (when the system has reached a steady state in constant osmolality conditions), and short term mechanisms, of a transient nature that occur in changing osmolality conditions. Interestingly, little information is available concerning the long-term effects of salt stress exposure on the central metabolism of E. coli. In addition, in the case of hyperosmotic stress in E. coli, one well observed homeostatic mechanism is the synthesis of some osmoprotectants, non-toxic molecules that help compensate the osmotic pressure by accumulating in the intracellular medium. These osmoprotectants include K+ amino acids such as glutamate
and proline, and trehalose and zwitterionic organic solutes like L-carnitine, [12,13] a trimethylammonium compound well described as osmoprotectant in this microorganism [14,15]. Further, experimental evidence on the important role played by salt stress on the energetic state and the main metabolic pathways of the cells during metabolic biortransformations of trimethylammonium compounds in E. coli has been shown [6].

Starting with the results of an anaerobic culture of a L-carnitine E. coli overproducing strain subjected to an osmotic stress [10], we have constructed a mathematical model which reproduces both the non-osmotic and hyperosmotic steady states, as well as the observed dynamic behavior of the transition between them. Our aim was to quantify the signals and observed changes in fluxes; in particular, we wished to contribute to the elucidation and quantification of the response generated in E. coli by hyperosmotic stress as regards the primary metabolism (central carbon) and its relationship with the synthesis of an osmoprotectant, L-carnitine, produced by a secondary metabolism.

Materials and Methods

Experimental Methods

The experimental set up and data used to construct and verify the model were taken from [10]. In the experimental set up used by these authors, an L-carnitine overproducer E. coli (O44K74AS) strain adapted to high salt concentration was used. After removal of the storage medium, the bioreactor was operated in the presence of this strain and a standard complex medium at a fixed dilution rate. Once the initial steady state under standard salt conditions (control, 0.085 M NaCl) was reached, a gradual osmotic up-shift was carried out by feeding the cultivi with a medium containing 0.3 or 0.5 M NaCl until a new, hyperosmotic medium steady state was reached. This new steady state was reached 70 h after switching the medium. This time period of 70 hours can be considered a long-term period [10,16] since at least 12 generations were produced to keep the cell population steady within the reactor at the new salt concentration [10]. The osmotic up-shift was carried out when the culture had reached the steady state.

Mathematical modeling

The approach used to model this biochemical system involves mathematical models in ordinary differential equations in the power-law formalism. In this formalism [17] the processes that conforms the biochemical networks are modelled using power-law expansions in the variables of the system and are then included in non-linear ordinary differential equations with the following structure, called Generalized Mass Action (GMA):

$$\frac{dX_i}{dt} = \sum_j \gamma_j \prod_{k=1}^p X_k^{\phi_{jk}} \quad i = 1, \ldots, n_d \quad (1)$$

In Equation 1, $X_i$ represents the model variables (concentrations of compounds of the investigated network: metabolites, salt medium, bacterial concentration, etc.), $n_d$ is the number of model variables, and $\gamma_j$ (rate constants) and $\phi_{jk}$ (kinetic orders) are different kinds of parameters defining the dynamics of the system. Unlike in conventional kinetic models, where the kinetic orders are always integer numbers, power-law models admit non-integer values.

In order to deal with the description and quantification of the effect of the osmotic stress in the metabolic processes we use a new type of kinetic order, the osmotic stress kinetic orders ($g_{OSt}$), derived from the power law general formalism. In the definition of this system parameter, we follow a similar approach to that presented by Fonseca et al. [18] for the study and quantification of the temperature dependence of the system. While these authors introduce a parameter that represents the change in enzymatic activity brought about by an increase in temperature, our kinetic orders ($g_{OSt}$) represent the change caused by the osmotic stress.

In our case, the rate constants $\gamma_j$ can be expressed as $\gamma_j' = k_j E_j$, where $k_j$ depends on the properties of the molecular medium, such as the pH, temperature or salt concentration (in our case only the salt, $k_j = k_0 f_j(salt)$ and $E_j$ is the enzyme concentration. The enzyme concentration, $E_j$, under stress conditions also changes, $E_j = E_0 h_j(salt)$. Accordingly, $\gamma_j' = k_0 f_j(salt) E_0 h_j(salt)$. In the phenomenological approach here proposed the effect of osmotic stress in the rate constant and the enzyme activity is aggregated into a single characteristic power-law term:

$$Salt^{k_{OSt}} = f_j(salt) h_j(salt)$$

Therefore Equation 1 in the osmotic stress conditions under consideration becomes:

$$\frac{dX_i}{dt} = \sum_j \gamma_j' \prod_{k=1}^p X_k^{\phi_{jk}} \cdot Salt^{p_{OSt}} \quad i = 1, \ldots, n_d \quad (2)$$

The most notable property of these power-law equations is that these two such equations with the same structure can be used to describe totally different dynamics (from inhibitory processes to the description of cooperativity) simply by modifying the numerical values of the kinetic orders involved [19]. Moreover, this type of representation has been shown to be suitable and effective for the modelling of dynamic signalling structures. It has been shown that virtually any linear or non-linear system can be represented accurately by a GMA of higher dimension through algebraic equivalence transformations of variables in a process called recasting [20].

Delay definition. A time-delay was applied to the model to represent the different time scale between some distinct qualitative processes within the cell (cell death, gene expression, etc.). This delay was modeled using the linear chain trick [21], whereby the delay was described using time-dependent fictional variables (in our case, $Delay_{name1}$ and $Delay_{name2}$, where $name$ is the name of the variable to which the delay is applied). The features of distributed time-delay (average value and standard deviation) depend on the number of fictitious variables used and the value assigned to the rate constant $k_{delay}$. We noticed that more sophisticated strategies are available to introduce time delay in ordinary differential equation models (see for example, [22]). However, our initial analysis indicated that the simple strategy used to model time-delay was sufficient for our case study.

Parameter estimation. The numerical values for the parameters $\gamma_i$ and $g_k$ are determined using an evolutionary algorithm called simple genetic algorithm (SGA) adapted to determine the values of parameters in power law models [23,24]. This algorithm starts with a population of individuals, which are the set of parameters that generates a solution of the model. The population is mutated and recombined; the convergence stems from minimization of the value of the objective function ($F_{obj}$):

$$F_{obj} = \sum_{i=1}^{n_i} |\gamma_i| + \sum_{j=1}^{n_{exp}} \sum_{t=1}^{n_{exp}} \left( \frac{1}{\sigma_i(t)} \left( \frac{y_j(t) - \bar{y}_j^{exp}(t)}{\bar{y}_j^{exp}(t)} \right)^2 \right)$$

$$+ \sum_{i=1}^{n_i} \sum_{t=1}^{n_{exp}} \left( \frac{1}{\sigma_i(t)} \left( \frac{\bar{y}_j(t) - \bar{y}_j^{exp}(t)}{\bar{y}_j^{exp}(t)} \right)^2 \right)$$

$$+ \sum_{i=1}^{n_i} \sum_{t=1}^{n_{exp}} \left( \frac{1}{\sigma_i(t)} \left( \frac{\bar{y}_j(t) - \bar{y}_j^{exp}(t)}{\bar{y}_j^{exp}(t)} \right)^2 \right)$$

Plagiarism: None
In Equation 3, \( n_{\text{var}} \) and \( n_{\text{tp}} \) are the model variables and time points, respectively; \( n_{\text{i}} \) is the number of rate constants of the model \( x_j(t) \) and \( x_{\text{exp}}^j(t) \) are the value of variable \( j \) at time \( i \) based on the simulation of the model and the experimental data of the variable \( j \) at time \( i \), respectively; \( \sigma_j(t_i) \) is the standard deviation of the experimental value \( x_j(t_i) \). Furthermore, an additional term in Equation 3 was introduced in order to obtain a more restrictive objective function. This term is the sum of the \( c \)'s, in absolute values, which allow the value of these parameters in the solutions to be reduced. \( c \) is associated with the enzyme concentration; the enzyme concentration \( (P_{nc}^k) \) is minimized in the data fitting process.

The initial point integrating for the variables should be at the same time an experimental and analytical steady state value. The last condition is fulfilled by a set of system parameters satisfying equations 3.

\[
\frac{dX_i(t=1)}{dt} = 0 \quad i = 1, \ldots, n_d
\]  

(4)

In the search for a solution, SGA was run through 10,000 iterations using the 0.3 M salt concentration time series data. Initially, a first set of \( \gamma \) and \( g_k \) parameters was found within the range searched \( [\gamma = [0, 20]; \quad g_k = [-3, 3] \] \), showing a low value of \( F_{\text{obj}} \). Then, the previously obtained parameter solution was used in the subsequent searches within a 95% of the first solution, the \( F_{\text{obj}} \) between 25% and 60% of the initial \( F_{\text{obj}} \) in all cases.

Stability analysis. All eligible solutions should show initial and final stable steady states. The stability of the steady states was checked by analyzing the behavior of the model against small perturbations.

Dynamic sensitivity analysis. Sensitivity analysis is a general analysis tool useful for evaluating robustness and characterizing system dynamics. Since our model studies the system dynamics, it is possible to identify those parameters with a major influence on the transient dynamics. For this purpose we used a modified version of previously defined dynamic sensitivity [24], the System Dynamic Sensitivity, \( S_{pk}^{Xi} \), as indicated in equation 5:

\[
S_{pk}^{Xi}(X_i, p_k) = \sgn\left( \frac{A_{k2} - A_k}{p_{k2} - p_k} \right) \frac{D_k}{p_k}
\]

(5)

In the above expression, \( Xi \) is the considered variable, while \( p_k \) is the parameter under scrutiny. \( A_k \) is the area below the solution curve, after a change of \( p_k \) to \( p_{k2} \). \( A_k \) is the area under the solution curve using the original value \( p_k \). \( D_k \) is the area between the two solution curves, using \( p_k \) and \( p_{k2} \), respectively. We have considered changes in two kinds of parameter, the kinetic orders and the rate constants. The \( S_{pk}^{Xi} \) value corresponds to the variation of the area under the variable time course after perturbation in parameter space. For each model variable the absolute values of the area \( A_k \), \( A_{k2} \) and \( D_k \) were calculated using the trapezoidal method. Positive sensitivity means that the area under the new curve is greater than the area under the original curve i.e. that the parameter \( p_k \) has a positive influence on variable \( Xi \). Negative sensitivity means the opposite. Zero sensitivity means that small changes in the parameter have no influence on the variable. All sensitivities were computed for standardized variables.

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**Figure 1. Schematic representation of the central metabolism of a L-carnitine overproducing *E. coli* strain applicable both to normal and osmotic stress conditions.** Solid lines represent rate processes and dotted arrows represent regulatory interactions. The empty arrow represents the output flux of biomass. The “clock” symbols represent a time-delay in a process whose value is a parameter estimated during the model calibration. The OS boxes represent the influence of the osmotic stress on the flux rate. See text for detail.

doi:10.1371/journal.pone.0034533.g001
Kinetic processes

Figure 1 shows the simplified version of the central metabolism of E. coli applicable both to normal and osmotic stress conditions where the intracellular variables are connected to the external measured ones.

Since our objective here is to analyze the effect that changes in the salt concentration of the medium provoke in the dynamics of the system, we focused our attention on the main central metabolism fermentation processes. We also pay attention to the mass flux looking at the metabolite accumulation and biomass production and the flux exchanges among the cell cultures, the medium and the bioreactor. Accordingly, four variables aggregate all the intracellular compounds. As is shown in Figure 1, three pools (Pool1, Pool2 and Pool3) connect the carbon source (glycerol, Gly) with the fermentation products lactate and pyruvate (Lac) reduction into succinate (Suc). Pool4, on its own, aggregates the acetyl CoA, acetyl-P, acetate, acetaldehyde and ethanol, while law term SaltgOSj (indicated as OS, green boxes in Figure 1). In rearrangement.

In this model the influence of osmotic kinetics processes. The central metabolism and the glycerol and peptone uptake by cells are also depicted in Figure 1. V1 is the glycolytic flux (glycerol to phosphoenolpyruvate, indicate by Pool1); V10 the transformation from Pool1 (mainly phosphoenolpyruvate) to Pool2 (pyruvate) through pyruvate kinase, and, V11 is the transformation from Pool2, namely pyruvate, into Pool3 (acetyl CoA) through pyruvate dehydrogenase. Fluxes V12, V14 and V16 are catabolic fluxes mediated by enzymes; V12 is associated with the phosphoenolpyruvate carboxykinase activity and also represents the thehalose degradation processes that yield pyruvate from any other precursor, particularly the TCA intermediates and the amino acid degradation; V14 represents the synthesis of pyruvate from other metabolites. V16 represents different processes leading to the synthesis of Pool3 from precursors other than glycerol (lipids and/or proteins and L-carnitine). V13, V15 and V17 are anabolic processes. V13 is associated with the intracellular production of trehalose, the net flux between Pool1 and Krebs cycle intermediates, the PEP-carboxylase activity and the transformation of these intermediates into Biomass [10]; V15 represents the transformation of the pyruvate component of Pool2 into some precursors (amino acids and osmoprotectants). V17 is related with the flux through citrate synthase, including glutamate biosynthesis. V18 (empty arrow) represents the output of the whole system (open and continuous) has overall input and output fluxes. V19 to V30 represent the influxes and the outflows of the corresponding biomass production. Fumarate (Fum) is antipoted with intracellular succinate and it is not used as carbon source [26]. Once fumarate is within the cell, it is reduced and transformed (V14) into internal succinate (Pool1), which eventually leaves the cell, V6. The positive regulatory influence of fumarate on V6 collects its role as substrate in the production of succinate [10].

Biomass, which is not directly represented in Figure 1, where the yellow area represents the variable biomass as the sum of all the variables within it, is an auxiliary variable, defined as the summation of the four intracellular Pool, variables. Thus defined, Biomass serves to represent the mass of microorganism in the reactor. Biomass is defined as the whole population in the reactor, and it is measured as the total cell dry weight (mg/ml) of the reactor bulk liquid. During the different cell divisions biomass adapts to the new steady state after the salt up-shift [10,25]. Therefore, the individual cell content was adapted to the new conditions and can be represented by the summation of the Pool4, and the total amount of cells can be represented by the total cell dry weight (mg/ml) of the reactor bulk liquid. Since the biomass changes during the transition, fluxes were normalized to the biomass unit, so that they are referred to milligrams of cell mass.

The reactor medium and the extracellular metabolic concentrations are represented by Lac+Py, the combined concentrations of lactate and pyruvate; Ac+Et is the summation of pyruvate, acetate and ethanol; Gly is the glycerol concentration, and Pepto represents the concentration of peptone.

Quantification of direct and indirect influence of osmotic stress on metabolism. In this model the influence of osmotic stress conditions on the flux values is represented by of the power law term SaltgOSj (indicated as OS, green boxes in Figure 1). In this expression Salt is the normalized salt concentration (NaCl) of the medium with respect to the control conditions (0.085 M). Salt quantifies the osmotic stress, being 1 in control conditions, 3.53 in 0.3 M NaCl conditions and 5.88 in 0.5 M.

The exponential terms gOSj are the osmotic kinetic orders. These are phenomenological parameters, that quantify the combined effect on the fluxes (Vj) of the mediating mechanisms between the medium salt concentration (the osmotic stress) and the fluxes. These mechanisms are of a physicochemical and transcriptional (changes in enzyme concentrations) nature. The term gOSj can have positive or negative values, reflecting an increase or decrease of the flux due to the osmotic stress. This modelling strategy permits us to separately quantify the influence of osmotic stress (through the gOSj) and of the metabolite concentration (gOj) on the fluxes

Transport and central metabolic processes. The central metabolism and the glycerol and peptone uptake by cells are also depicted in Figure 1. V1 is the glycolytic flux (glycerol to phosphoenolpyruvate, indicate by Pool1); V10 the transformation from Pool1 (mainly phosphoenolpyruvate) to Pool2 (pyruvate) through pyruvate kinase, and, V11 is the transformation from Pool2, namely pyruvate, into Pool3 (acetyl CoA) through pyruvate dehydrogenase. Fluxes V12, V14 and V16 are catabolic fluxes mediated by enzymes; V12 is associated with the phosphoenolpyruvate carboxykinase activity and also represents the thehalose degradation processes that yield pyruvate from any other precursor, particularly the TCA intermediates and the amino acid degradation; V14 represents the synthesis of pyruvate from other metabolites. V16 represents different processes leading to the synthesis of Pool3 from precursors other than glycerol (lipids and/or proteins and L-carnitine). V13, V15 and V17 are anabolic processes. V13 is associated with the intracellular production of trehalose, the net flux between Pool1 and Krebs cycle intermediates, the PEP-carboxylase activity and the transformation of these intermediates into Biomass [10]; V15 represents the transformation of the pyruvate component of Pool2 into some precursors (amino acids and osmoprotectants). V17 is related with the flux through citrate synthase, including glutamate biosynthesis. V18 (empty arrow) represents the output of the whole system (open and continuous) has overall input and output fluxes. V19 to V30 represent the influxes and the outflows of the corresponding biomass production. Fumarate (Fum) is antipoted with intracellular succinate and it is not used as carbon source [26]. Once fumarate is within the cell, it is reduced and transformed (V14) into internal succinate (Pool1), which eventually leaves the cell, V6. The positive regulatory influence of fumarate on V6 collects its role as substrate in the production of succinate [10].

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The exponential terms gOSj are the osmotic kinetic orders. These are phenomenological parameters, that quantify the
Model Equations

In accordance with the model scheme shown in Figure 1, the system model equations are as follows.

**Mass Balance.** The mass balance equations of the processes take the following form:

\[
\begin{align*}
\frac{d\text{Gly}}{dt} &= V_{21} - V_{22} - V_1 \\
\frac{d\text{Pepto}}{dt} &= V_{23} - V_{24} - V_2 \\
\frac{d\text{Cro}}{dt} &= V_{25} - V_{26} - V_3 \\
\frac{d\text{Fum}}{dt} &= V_{19} - V_{20} - V_4 \\
\frac{d\text{Car}}{dt} &= V_5 - V_{28} \\
\frac{d\text{Suc}}{dt} &= V_6 - V_{27} \\
\frac{d\text{Pool}_i}{dt} &= V_I + V_4 - V_6 - V_{10} + V_{12} - V_{13} - V_{B\text{Pool}_1} \\
\frac{d\text{Pool}_2}{dt} &= V_{10} - V_7 - V_8 + V_{14} - V_{15} - V_{B\text{Pool}_2} \\
\frac{d\text{Pool}_3}{dt} &= V_{11} - V_9 + V_{16} - V_{17} - V_{B\text{Pool}_3} \\
\frac{d\text{Pool}_4}{dt} &= V_2 + V_3 - V_5 - V_9 + V_{13} - V_{12} + V_{15} - V_{14} + V_{17} - V_{16} - V_{B\text{Pool}_4} \\
\frac{d\text{Lac} + \text{Py}}{dt} &= V_7 - V_{29} \\
\frac{d\text{Ac} + \text{Et}}{dt} &= V_8 - V_{30} \\
\frac{d\text{Salt}}{dt} &= K_{d}(\text{Salt}_{\text{medium}} - \text{Salt})
\end{align*}
\]

As said above, biomass is the sum of Pool, \(i = 1, 2, 3, 4\). In Figure 1, \(V_{18}\) represents the output of biomass, but since biomass is a variable composed of four terms, \(V_{18}\) is the sum of four biomass contributions coming from each one of the internal variables of Pool, namely \(V_{B\text{Pool}_1}, V_{B\text{Pool}_2}, V_{B\text{Pool}_3}\) and \(V_{B\text{Pool}_4}\). Accordingly, these fluxes are represented in the corresponding Pool differential equations (see Equations 6).

**Salt_{medium}** is the normalized concentration of salt with respect to the control condition in the input medium to the reactor and Salt as defined above.

**Delay equations.** The delay equations that describe the longer temporal hierarchies of the enzyme expression processes are modelled using a three-equation linear chain trick. Delay is applied to the variable Biomass, which is the sum of the variables of the model Pool1, Pool2, Pool3 and Pool4, so this delay is applied to each of these variables as a proportional part of the biomass that they conform (Equations 6).

\[
\begin{align*}
\frac{d\text{DelayPool}_1}{dt} &= K_{\text{delay}}(\text{Pool}_1 - \text{DelayPool}_1) \\
\frac{d\text{DelayPool}_2}{dt} &= K_{\text{delay}}(\text{Pool}_2 - \text{DelayPool}_2) \\
\frac{d\text{DelayPool}_3}{dt} &= K_{\text{delay}}(\text{Pool}_3 - \text{DelayPool}_3) \\
\frac{d\text{DelayPool}_4}{dt} &= K_{\text{delay}}(\text{Pool}_4 - \text{DelayPool}_4)
\end{align*}
\]

\[
\text{Biomass} = \text{DelayPool}_1 + \text{DelayPool}_2 + \text{DelayPool}_3 + \text{DelayPool}_4
\]

**Power law rate equations.** The rate equations in power law form corresponding to the different fluxes are:

\[
\begin{align*}
V_1 &= \gamma_1 \cdot \text{Gly} \cdot \text{Biomass} \cdot \text{Salt}^{60} \\
V_2 &= \gamma_2 \cdot \text{Pepto} \cdot \text{Biomass} \cdot \text{Salt}^{60} \\
V_3 &= \gamma_3 \cdot \text{Cro} \cdot \text{Biomass} \cdot \text{Salt}^{60} \\
V_4 &= \gamma_4 \cdot \text{Fum} \cdot \text{Biomass} \cdot \text{Pool}^{64} \cdot \text{Salt}^{60} \\
V_5 &= \gamma_5 \cdot \text{Pool}_4 \cdot \text{Pool}_5 \cdot \text{Pool}^{59} \cdot \text{Cro}^{53} \cdot \text{Salt}^{60} \\
V_6 &= \gamma_6 \cdot \text{Pool}_1 \cdot \text{Fum}^{64} \cdot \text{Salt}^{60} \\
V_7 &= \gamma_7 \cdot \text{Pool}_2 \cdot \text{Salt}^{60} \\
V_8 &= \gamma_8 \cdot \text{Pool}_3 \cdot \text{Salt}^{60} \\
V_9 &= \gamma_9 \cdot \text{Pool}_4^{610} \cdot \text{Salt}^{60} \\
V_{10} &= \gamma_{10} \cdot \text{Pool}^{607} \cdot \text{Salt}^{60} \\
V_{11} &= \gamma_{11} \cdot \text{Pool}_1^{118} \cdot \text{Salt}^{60} \\
V_{12} &= \gamma_{12} \cdot \text{Pool}_2^{1210} \cdot \text{Salt}^{60} \\
V_{13} &= \gamma_{13} \cdot \text{Pool}_1^{137} \cdot \text{Salt}^{60} \\
V_{14} &= \gamma_{14} \cdot \text{Pool}_2^{1410} \cdot \text{Salt}^{60} \\
V_{15} &= \gamma_{15} \cdot \text{Pool}_2^{158} \cdot \text{Salt}^{60} \\
V_{16} &= \gamma_{16} \cdot \text{Pool}_4^{1610} \cdot \text{Salt}^{60} \\
V_{17} &= \gamma_{17} \cdot \text{Pool}_1^{179} \cdot \text{Salt}^{60} \\
V_{18} &= \gamma_{18} \cdot \text{Biomass} \cdot \text{Salt}^{60}
\end{align*}
\]

**System parameters and constants.** In this model approach the rate constants (\(\gamma\)) are associated with the net enzyme activity of each process and are considered to remain constant. It should be noted here that since the osmotic kinetic orders measures the effect on the fluxes of the mechanisms that mediate between the osmotic stress and the fluxes, they include in their values the effects on the fluxes associated with changes that occur in enzymes (concentration, effectors, coenzymes etc.). Kinetic orders (\(g_{jk}\)
measure the effect of each variable on the flux $V_j$. Gly_{medium}, Peptone_{medium}, Cro_{medium} and Fum_{medium} are the concentration of glycerol, peptone, crotonobetaine and fumarate in the input medium to the reactor, respectively, which are kept constant.

**Identifiability of solutions.** Many set of parameters showing similar values of the objective function were found. In order to select the most representative ones, a selection criterion was applied. The selection criterion used ensures that the fitting of each variable is independent from the remaining variables. Since the objective function can give solutions with a poor fitting in one of the variables if another variable has a very good fit, the criterion mentioned above eliminate these kinds of solutions. According with this criterion only was admitted as solutions for the osmotic steady state (0.3 M) those in which the values of the dependent variables were within the range $\pm 1.5$ SD of the experimental values of osmotic steady state, except in the case of Lac+Py and Car ($\pm 3$ SD) and Biomass ($\pm 4.5$ SD). The value of the ranges was estimated from the standard deviation of the data.

**Model validation.** The model was validated using a new set of data which were not used in the parameter fitting procedure. This data were obtained at 0.5 M salt concentration. Solutions obtained by fitting with the 0.3 M data were tested against the experimental data obtained at 0.5 M NaCl. In these conditions the only solutions admitted were those in which the dependent variables were within the range $\pm 3$ SD of the experimental values. In the case of the variables Lac+Py and Car and Biomass the range was $\pm 6$ SD and $\pm 9$ SD, respectively.

**Model fitting and verification**

Figure 2 shows the simultaneous integration of the six selected solutions obtained with the procedures described above; this representation shows the differences between them. Panel A shows the model data fitting in 0.3 M of NaCl conditions; panel B shows a comparison of the model predictions with the experimental time series data in 0.5 M of NaCl conditions. In all cases the first vertical line (23 hours) indicates the moment when salt switching begins at the initial 0.085 M NaCl control steady state, and the second one (70 hours) the moment when the system reaches the final steady state (0.5 or 0.5 M NaCl, respectively). The mean values of the parameters of the six solutions as well as the whole set of parameter solutions for each of the solutions are shown in Tables S1 and S2 of Supporting Information files, respectively. The model fits the experimental data well ($F_{obj} = 195.82 \pm 20.84$) and is able to predict the general behavior in different, more saline conditions (0.5 M NaCl).

**Sensitivity and stability analysis**

All solutions were in a stable steady state before and after the salt concentration switch. Variable sensitivities for parameter changes and initial conditions showed distinct pattern distribution for the different solutions considered. However, all sensitivity values were in the range $[1.0825, 20.7377]$, 99% of all sensitivities being between $-0.1$ and $0.1$ (data not included). This indicates that the selected solutions are robust for changes in parameters and initial conditions.

**Results and Discussion**

Figure 2. Model fitting and verification. In all panels continuous lines represent the six best model solutions are represented (they are represented simultaneously in order to see the differences between them). The first vertical line indicates the moment (23 hours) when the medium switch begins by NaCl addition, while the second one indicates when the final steady state is reached (70 hours). Red lines represent the salt concentration in the extracellular medium. A. Model fitting in conditions of 0.3 M of final NaCl concentration. B. Model verification in conditions of 0.5 M of final NaCl.

doi:10.1371/journal.pone.0034533.g002
Concentrations change in variables

Figure 3 shows the relative changes in variables concentrations between the final (0.3 M NaCl) and the initial steady state. It can be seen that the concentration of all the variables increased in osmotic conditions. In particular Pool2 increased more than 6 times with respect to its value in nonosmotic conditions and Pool1 and Fum doubled in concentration.

Kinetic order distribution for metabolic changes and osmotic stress

In previous studies [6,10] it was shown that, as a result of long-term exposure to NaCl, the metabolism of E. coli adapts to stress conditions, the adaptation being mediated by changes in enzyme activities and cofactor levels. Integration of the experimental data into a comprehensive model provides new evidences regarding the actual flux distribution.

Figures 4A and 4B show the magnitude of the flux changes from different perspectives. It can be seen that the fluxes showing the increases in the 0.3 M steady state were V10 and V11. To a lesser degree, V1, V2, V5, V6, V8, V9, V12, V13, V14 and V15, also decreased. Some fluxes (V5, V16 and V17) decreased when in osmotic conditions while V7 remained practically unchanged.

Figure 4C show the estimated gOS kinetic orders. As stated above the value of these parameters measure the effect of osmotic stress on the corresponding fluxes. As can be seen, all of them are within the [-2, 2] range value. Those showing higher positive values, indicating a major positive influence of the osmotic conditions on the corresponding fluxes, are gOSV2 (V2), gOSV6 (V6), gOSV8 (V8), gOSV10 (V10) and gOSV14 (V14). Also positive but of minor magnitude are gOSV1 (V1), gOSV3 (V3), gOSV5 (V5), gOSV12 (V12) and gOSV15 (V15). On the other hand, the gOS showing the greatest negative values are gOSV7 (V7) and gOSV11 (V11). Negative too, but less so, were: gOSV6 (V6), gOSV16 (V16) and gOSV17 (V17). Parameters gOSV4 (V4) and gOSV13 (V13) have zero values.

As stated above, the influence of osmotic stress conditions on the flux values is represented by the term SaltgOSn. Examination of this term in the context of each flux rate equation (see Equations 7) allows us to analyse the magnitude and significance of the osmotic associated interactions on each of the fluxes. The main results of this section are summarized in Figure 4. One observed change after the salt switch was the increase in the flux V1. This flux is dependent on Gly and Salt and represents the flux from external glycerol (Glymedium) to internal Pool1. The corresponding kinetic order gOS1 is 0.3, meaning that the osmotic stress has a moderate positive effect in the input of glycerol. Since variable Gly remains constant (see Figure 2), we conclude that the osmotic stress is responsible for this flux increase. This result shows that there is a mechanism which relates osmotic stress with the entrance and transformation of glycerol into Pool1.

Another result arises from the change in the fluxes V10 and V11. V10, which increase its value in osmotic conditions, represents the transformation from Pool1 to Pool2. Its value is dependent on Pool1 and Salt through the kinetic orders g107 (1.6) and gOS10 (1.1), respectively. Since both kinetic orders are positive and the values of Pool1 and Salt are higher in osmotic conditions (Figure 3) the positive effect of the other variables. A different situation is observed in the case of V11, the flux representing the transformation from Pool2 to Pool3. V11 depends on Pool2 and Salt through the kinetic orders g118 (1.7) and gOS11 (-1.2), respectively. In this case, despite the negative value of gOS11, V11 increased, the positive effect of the combined Pool2 increased and its greater kinetic order g118 being enough to compensate the negative influence of SaltgOS11 term. It may be suggested that the increase in V10 is to meet the energy requirements, while the increase in V11 serves to compensate Pool2 accumulation. This would permit increased energy synthesis in order to resist the selective pressure imposed and would explain the increased rates of generation of fermentation products.

V8, the combined transformation/excretion of Pool3 into Ac+Et, reflects a different situation. This flux, which is dependent on Pool3 and Salt through the kinetic orders 1 and gOS8 (1.6), increases in osmotic conditions. In the stressed steady state, Pool3,

Figure 3. Relative variable concentration changes. Mean values of the variables concentration changes for the selected solutions between the final, osmotic stress (0.3 M NaCl) and the initial steady state. Values were calculated as the ratio between the final and the initial steady states. All values are normalized with respect to the initial steady state values. doi:10.1371/journal.pone.0034533.g003
that this flux increases by 30%, its related osmotic stress kinetic order being positive (g_{OS2} = 0.23). Since Cro remains unchanged, the single reason for the increase in crotonobetaine input is the salt stress response mechanism.

In our model the production of glutamate is represented by flux V_{17}, which is dependent on Pool4 and Salt through the kinetic orders g_{175} (0.3) and g_{OS17} (-0.1), respectively (see Table S1; Supplementary Materials). After the salt switch, a decrease in this flux occurs, indicating that glutamate is not significantly synthesized in response to the salt rise. This suggests that there is, instead, an accumulation of other compatible solutes from the extracellular medium, such as the trimethylammonium monomers involved in the medium (Cro and Car).

L-carnitine metabolism is inhibited in osmotic stress conditions [10]. In our model, the catalysis of L-carnitine is represented by V_{16}, while its synthesis and excretion are represented by V_{15}. V_{16} is dependent on the variables Pool4 and Salt through the kinetic orders g_{1610} (null value) and g_{OS16} (-0.6), respectively. On its own V_{15} is dependent on the variables Pool3, Pool4, Cro and Salt through the kinetic orders 1, g_{50} (0.5), g_{53} (1.15) and g_{OS17} (0.6), respectively. Our results show that the magnitudes of these fluxes are reduced in osmotic conditions (see Figure 4B); In the case of V_{16}, the determinant (negative) factor is the osmotic stress response mechanism since the g_{OS10} value is null. But in the case of V_{15}, the observed decrease in the flux seems to be due to the combined effects of the decrease in Pool4 and Pool3 in spite of the counteracting influence of the osmotic stress. This indicates that the carnitine metabolism is down regulated by an osmotic stress response mechanism, leading to the accumulation of the osmoprotectant L-carnitine, while its excretion is not affected by the osmotic stress.

Another effect revealed by our model refers to the metabolism of peptone, which is present in the reactor medium. Peptone is made up of peptides and amino acids, among them proline (46 mg/g peptone, MC24 bacteriological peptone from LAB M laboratory). It is known that proline uptake from the extracellular medium plays a role as osmoprotectant by eliciting the (rapid) excretion of K\(^+\) and contributing to the depletion of trehalose [11]. Some of our model observations clearly correlate with these observations. First, it can be seen that flux V_{12}, which in our model represents peptone uptake, increases (27%) when in osmotic conditions, suggesting increased proline uptake. At the same time V_{12}, which also represents trehalose catabolism, increases more than two-fold. The values of the related osmotic kinetic orders, g_{OS2} and g_{OS12}, related with V_{2} and V_{12}, respectively, have positive values (1.1 and 0.5, respectively). These results suggest that the input of peptone (V_{2}) and trehalose catabolism (V_{12}) are affected by the osmotic stress.

Concluding remarks

In analyzing the influence of osmotic stress on flux redistribution in the osmotically stressed steady state, not only the regulatory effects inherently associated with the osmotic stress response mechanisms should be considered, but also the simultaneous influence of the changes that occur in other variables in the system. The model approach used to represent the central metabolism and L-carnitine biosynthesis subjected to long term adaptation to...
osmotic stress conditions allows the influence of the osmotic stress condition on fluxes to be quantified.

The model analysis shows that, after salt up-shift, the energy and fermentation pathways in the central metabolism undergo substantial rearrangement as they move towards an enhanced energy production. This is the case of the increase observed in the fermentative (V1, V2, V10, V11), anaerobiotic (V12 and V13) and succinate (V4 and V6; see [10]) fluxes. This is further illustrated by the observation that the increase in the fermentation flux, V1, from Pool0 to Pool1, is not connected with fluxes towards Pool4, representing the tricarboxylic acid intermediates and the synthesis of biomass. The values of the osmotic stress associated with these fluxes reflect these changes.

Pyruvate kinase (V10) and Pyruvate dehydrogenase (V11), which render pyruvate and acetyl-CoA, respectively, control energy yields during growth [10]. In fact, the increase in Pyruvate kinase activity is in good agreement with the observed increased glyceral consumption (V1; gOS1), suggesting that glycolytic rates increased as a consequence of stress towards fermentation (gOS2; gOS3; gOS5; gOS10, and gOS14). On the other hand, under anaerobiosis, acetyl-CoA is transformed to acetate (forming ATP) by phosphotransacetylase-acetate kinase (Pta-Ack) pathway (V4 in our model, which increases, see [27]).

This would permit for increased energy synthesis to withstand the osmotic stress and would explain the increased rates of generation of fermentation products.

Furthermore, under anaerobic conditions, the TCA cycle (Pool1 and Pool3) provides biosynthetic precursors. In osmotic stress conditions, the activities of the controlling anaplerosis and gluconeogenesis enzymes isocitrate lyase, phosphoenol pyruvate carboxylase and phosphoenol pyruvate carboxykinase are altered [10]. This demonstrates the effect of long-term salt stress as regards cellular needs for anaerobiotic intermediaries and energy (Pool1 and Pool4, reversibly connected with Pool0). These pathways allow the oxalacetate pool needed for biosynthesis to be replenished (Pool0 node). Taken together, the model results presented demonstrate the involvement of the biosynthetic pathways in the adaptation to osmotic conditions, since they are required for the production of cellular structural components. Greater fluxes in the central energy-producing and anaerobic pathways have also been found in C. glutamicum when exposed to increased osmolarity [28]. Interestingly, the model outcome confirms the importance of acetate metabolism during long-term exposure to salt and during stress adaptation.

Further, in the transition phase, osmotic adaptations of E. coli metabolism translates into monotonic increases and decreases, with the exception of peptone and fumarate uptake into the cell which initially showed decreased concentrations.

Regarding the role of the osmoregulator L-carnitine, we conclude that its catabolism is negatively influenced by the osmotic stress, while its synthesis and excretion is unaffected by any osmotic stress response mechanism.

Supporting Information

Table S1 Mean parameters and SD values of the selected solutions. (DOCX)

Table S2 System parameters value for six solutions. (DOCX)

Acknowledgments

The authors gratefully acknowledge Dr. Carlos González-Alcon and Hugo Velasco-Beñarrán for helpful observations. The authors want to acknowledge the excellent work done by one of the referees in evaluating the previous versions of the manuscript. Their suggestions and comments have greatly contributed to the improvement of this work.

Author Contributions

Conceived and designed the experiments: MC. Performed the experiments: PA. Analyzed the data: GS JAH NVT. Contributed reagents/materials/analysis tools: GS JAH MC NVT. Wrote the paper: NVT GS. Model development: NVT GS. Parameter stimation, stability analysis and dynamic sensitivity analysis: NVT GS JAH. Model analysis: NVT GS MC PA.

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