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NEW INSIGHTS ON THE MONITORING OF A BIOTRANSFORMATION PROCESS USING SYSTEMS BIOLOGY

A. SEVILLA, M. CANOVAS and J.L. IBORRA

Departamento de Bioquímica y Biología Molecular B e Inmunología.
Facultad de Química. Universidad de Murcia.

Abstract: Monitoring is one of the most important tasks previous to control and to optimize a bioprocess. Signalling intermediates are usually not employed to monitor a bioprocess since they are involved in complex networks. However, Systems Biology can help to understand this complexity in order to develop new monitoring agents. In this work, we predicted with the help of a model, which included the signalling pathways related to carnitine metabolism, that the signal factor cAMP can be used to monitor the biotransformation of trimethylammonium compounds into L-carnitine. Experiments in high density cell continuous reactors of *E. coli* using different carbon sources assessed these findings. *Copyright © 2007 IFAC*

Keywords: Systems Biology, monitoring, fermentation, mathematical model, reactor modelling, signal processing.

1. INTRODUCTION

In recent years, primary objectives of the bioprocess industry are the monitoring and the optimization of the fermentation process. Despite the fact that there are many established monitoring techniques, a high demand in the bioprocess industry to develop new methods to monitor a bioprocess is still obvious (Ahlqvist et al., 2006). The most of the attention has traditionally been given to common analytes as oxygen, pH, phosphate and the principal compounds of the metabolic pathways involved in the bioprocess. However, little information was used concerning the signalling structure regulating the enzymatic machinery as a means of optimizing and monitoring a bioprocess. The reason for that is the relatively little information about signalling networks, in contrast with the knowledge available on the metabolic network (Muller et al., 2005), probably due to their complexity. However, mathematical models can be able to fill the gap between discontinuous experimental results and the continuous functions needed to optimize or monitor a bioprocess. Systems Biology can give the mathematical formulations which are necessary to completely understand the signalling networks in order to be finally integrated.

The biotransformation of trimethylammonium compounds into L-(-)-carnitine (R-(-)-3-hydroxy-4-trimethylaminobutyrate) served as a model to illustrate the integration of the signalling networks into the monitoring of a bioprocess. This compound transports long-chain fatty acids through the inner mitochondrial membrane, in consequence, several clinical applications for L(-)-carnitine have been identified and the consequent world wide increase in its demand has led to the development of chemical and biological processes for its production (Kleber, 1997). Strains belonging to the genera *Escherichia*, *Proteus* and *Salmonella* racemice D(+)-carnitine, a waste product and an environmental problem resulting from L(-)-carnitine chemical synthesis, and/or biotransform crotonobetaine (dehydrated D(+)-carnitine) to produce L(-)-carnitine (Cánovas et al., 2002). The carnitine metabolism in *E.coli* has been studied in depth by Elssner et al. (2001). In brief, crotonobetaine (CB) is transformed into L-(-)-carnitine (LC) by the involvement of CoA esters (LCCoA and CBCoA) and two enzymes, an enoyl-CoA hydratase (CaiD) and a CoA-transferase (CaiB) which are induced anaerobically in the presence of D,L-(-)-carnitine mixture and/or crotonobetaine (see Fig. 1). The structural components of the *E. coli* carnitine pathway are encoded by two divergent operons, *caiTABCDE* and *fixABCX* (Fig. 1). The *caiF* gene is located downstream of the *cai* operon but is transcribed in the opposite direction (Fig. 1), playing the role of an activator protein and mediating the induction of carnitine metabolism when an inducer (L-(-) carnitine or crotonobetaine) is present (Eichler et al., 1996). A CRP recognition sequence is found on the transcription start site of *caiF*. Also the intergenic common regulatory region shared by *cai* and *fix* harbours putative motifs of recognition for the binding of CRP.

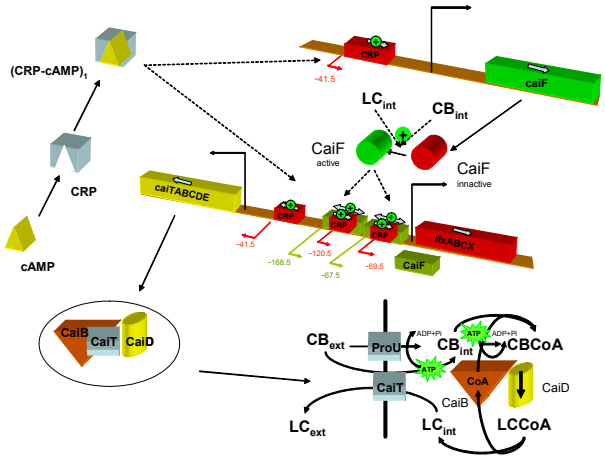

Fig. 1. Genetic regulation of the carnitine metabolism. For simplicity, only the regulation of CaiF and CRP-cAMP transcription factors has been included. Continuous lines indicate reactions, dashed lines show interactions.

Several studies have stated that the addition of glucose leads to total suppression of carnitine metabolism (Eichler et al., 1996; Buchet et al., 1999) where is suggested that the glucose repressive effect is mediated via the interaction of CRP with both the regulatory regions of *caiF* and the *cai* and *fix* operons since the presence of glucose lowers of cAMP concentration (Postma et al., 1993; Wang et al., 2001). However, ATP was demonstrated as essential in this process (Cánovas et al., 2003) and glucose has a higher energetic yield than glycerol, which is normally used in this process as the carbon source. This fact is the principal cause to decide to use glucose as the carbon source instead of glycerol in order to increase the productivity of the bioprocess.

The objective of this work was to establish a Systems Biology approach able to ascertain whether external cAMP can be utilized as a sensor of the biotransformation state when heterogeneous C-sources, such a glycerol and glucose mixture, are involved. The mathematical developed model assessed a linear correlation between the L-carnitine and the external cAMP concentrations which was experimentally proved with continuous high density cell reactors of *E. coli* O44 K74 biotransforming crotonobetaine into L-carnitine.

2. MATERIALS AND METHODS

2.1 Bacterial strain and culture media

The bacterial strain used, *E. coli* O74 K74 (DSM 8828), contained the complete *cai* and *fix* operons and was stored in a minimal medium containing glycerol (20%) at –20ºC.

2.2 Continuous reactor operation.

Continuous experiments were performed in reactors equipped with temperature, pH and pump controllers (Biostat B, Braun Biotech International GMBH, Melsungen, Germany). A 1 L culture vessel with 0.5-0.8 L working volume was used. *Escherichia coli* 044 K74 was grown under different conditions, in order to optimize the induction of the carnitine metabolism enzymes. The culture was inoculated with a 3% (v/v) of the liquid culture stored at -20ºC in 20% (v/v) glycerol. The cells were grown by continuous feeding under anaerobic conditions at 37ºC. Anaerobic conditions were maintained to induce the enzymes involved in the carnitine metabolism, while crotonobetaine was supplied as inducer. Nitrogen was used to maintain anaerobiosis during the experiments. The reactor vessel was also coupled to a cross-flow filtration module (Minitan, Millipore, USA) equipped with four 0.1 µm hydrophilic polyvinylidene difluoride Durapore plates of 60 cm^2 area (Millipore, USA) (Cánovas et al., 2002). The cell broth was recycled into the reactor with a peristaltic pump adjusted to a high flow rate (70 mL/min) to minimize membrane fouling. *E. coli* cells for the inoculum were grown as explained previously and transferred to the fermenter. The initial growing medium contained (g/L): bacteriological peptone, 20; NaCl, 5; glucose (carbon source), 25; crotonobetaine, 4 and fumarate, 2. At time t=0, the above feed medium was switched over to another similar medium but with glycerol (12.5 g/l) instead of glucose. The experiment was carried out in duplicate. Average and standard deviations are depicted in the figures.

2.3. Biotransformation carried out with cAMP and Glucose or Glycerol as C-source.

Using the same device as before but in a batch mode operation and without the cell retention system (the cross-flow filtration module), the biotransformation was carried out with in eight different conditions. The medium contained bacteriological peptone, 20 g/l; NaCl, 5 g/l; carbon source glycerol, 12.6 g/l, or glucose 12.6 g/l; fumarate 2 g/l, with and without inducer, crotonobetaine 4 g/l. Additionally, we accordingly added 5 mM cAMP, since this concentration was shown to be sufficient to suppress diauxic growth (Inada et al., 1996). First, we used glycerol or glucose as the carbon source but without crotonobetaine as the carnitine metabolism inducer. Second, both C-sources and inducer were used. Thirdly, cAMP was added to the growing medium before the inoculation and after the medium was autoclaved since cAMP is heat sensitive. All of these reactors were maintained in anaerobiosis until 36h when L(-)-carnitine and glucose levels were measured to ensure that the concentration of glucose was sufficient to inhibit the carnitine metabolism. Due to the different cellular concentration reached in these experiments and for comparative purposes, the L-carnitine concentration was referred to the dry weight of the cells. The experiments were carried out in duplicate. Average and standard deviations are depicted in the figures.

2.4. Enzyme assays

The crotonobetaine hydration reaction (CHR) is defined as the combination of CaiD and CaiB activities (Fig. 1). The assay was carried out according to Jung et al. (1989) and was started by using crotonobetaine as substrate. Enzyme activity was defined either as the total µmol of substrate consumed per minute (U) or as specific activity, µmol of substrate consumed per minute and mg of protein (U/mg). In all the experiments fumarate was used to inhibit the crotonobetaine-reducing reaction (CRR) activity and then to increase L-(-)-carnitine production.

The enzyme activity assays were optimized for the conditions and media. In each case, reactor bulk liquid samples were withdrawn and centrifuged at 16.000x g at 4 ºC. The supernatant was removed and cells were re-suspended within the extraction buffer. Cells were sonicated for 6 cycles (10 s each), at 10 µm amplitude, with a 1 cm diameter probe and below 20 ºC. The extract was centrifuged for 15 min at 16,000g and 4 ºC to remove cell debris. The protein content was determined by the method of Lowry et al., (1951).

2.5 Determination of metabolite concentrations.

L(-)-carnitine concentration was determined enzymatically with the carnitine acetyl transferase method (Jung et al., 1989). Bacterial growth was followed spectrophotometrically at 600 nm, using a Novaspec II from Pharmacia-LKB, (Uppsala, Sweden), and converted to dry weight accordingly. Extracellular cAMP was determined in the supernatant using an enzymatic immunoassay kit (Sigma, code CA-201).

3. RESULTS

In this work, a model that includes the signalling arquitecture of the CRP modulon was developed. This modulon comprises a high number of operons and regulons. We draw our attention to the glucose and glycerol dissimilation as well as the carnitine metabolism. The model is based on a hierarchical view of the regulatory network (Kremling and Gilles, 2001). In order to prove the findings of the model, the crotonobetaine biotransformation was followed in continuous reactors using as C-source glucose, glycerol or a mixture of them in order to check the relationship between the external cAMP concentration and the product formation.

3.1 Model equations.

The mathematical model is composed of ordinary differential and algebraic equations (DAE system). This model is based on the model of Bettenbrock et al., 2006, in which, the carnitine metabolism was included. Herein we present only the equations referred to L-carnitine metabolism. More information about the previous model and its nomenclature could be found in Bettenbrock et al., (2006) and in Kremling and Gilles (2001).

Ordinary differential equations and rate laws

Results are summarized in Table 1 for the differential equations. In Table 2, the rates which construct the differential equations are presented.

Table 1. Model equations. Ordinary differential equations. The differential equations are constructed with rates (r_i) which are described in Tables 2 and 3.

Ordinary differential equations	
Carnitine Unit	
$\cdot [LC]_{int} = r_{sinLC} + r_{CaiTinLC} + r_{ProULC} - r_{CaiC} - r_{CaiToutLC}$	(1)
$[CB]_{int} = [CB]_{int0} - [LC]_{int}$	(2)
$\cdot [CHR] = r_{CHR} - r_{dCHR}$	(3)
$\cdot [CaiF] = r_{CaiF} - k_d \times [CaiF]$	(4)

Table 2. Model Equations. Kinetic rate laws. Some of the rates needed to understand the whole model are described as algebraic equations and represented in Table 3. The parameters involved in the following equations are shown in Table 4.

Rate laws			
Rate	**Enzyme**	**Kinetics**	
Carnitine Unit			
$\mathbf{r_{sinLC}}$	**CHR**	$k_{sinLC} \times [CB]_{int} \times [CHR]$	(5)
$\mathbf{r_{ProULC}}$	**ProU**	$k_{ProULC} \times [LC]_{ext}$	(6)
$\mathbf{r_{CaiC}}$	**CaiC**	$k_{CaiC} \times [LC]_{int}$	(7)
$\mathbf{r_{CaiTLCout}}$	**CaiT**	$k_{CaiTLCout} \times [LC]_{int} \times [CB]_{ext}$	(8)
$\mathbf{r_{CHR}}$	**CHR**	$k_{CDH} \times [LC]_{int} \times [CB]_{ext}$	(9)
$\mathbf{r_{dCHR}}$		$k_{dCHR1} + k_{dCHR2} \times [CHR] + k_{dCHR3} \times [CHR]^2$	(10)

Algebraic equations.

The genetic expression represented by algebraic equations and relationships is summarized in Table 3. The formalism selected for representing the genetic structure was a hierarchical structure with

functional units developed as in Kremling and Gilles (2001).

Table 3. Model Equations for gene expression. Algebraic equations, mass balance and function and relationships definitions. The parameters involved in the following equations are shown in Table 4.

Algebraic equations			
Operon	**Rate**	**Equation**	
Carnitine Unit			
caiF	$\mathbf{r_{CaiF}}$	$k_{caiF} \times \bar{\psi}_{caiF} \times [D_{caiF}]_0$	(11)
		$\bar{\psi}_{caiF} = \frac{[D_{caiF}^*] + [D_{caiFC}^*]}{[D_{caiF}]_0}$	(12)
caiABCDE	$\mathbf{r_{CHR}}$	$k_{cai} \times \hat{\psi}_{cai} \times [CaiF]_{act} \times [D_{cai}]_0$	(13)
		$K_{eqCaiF} = \frac{[CaiF]_{act}}{[CaiF]_{inact} \times ([LC]_{int} + [CB]_{int})}$	(14)
		$[CaiF]_0 = [CaiF]_{act} + [CaiF]_{inact}$	(15)
		$\hat{\psi}_{cai} = \frac{[D_{cai}^{++}] + [D_{caiC}^{++}]}{[D_{cai}]_0}$	(16)

Parameter identification.

The complete set of parameters is presented in Table 4. To identify the model parameters, the following approach was used: (i) Starting with parameters from literature, the model is analyzed calculating a combination of parameters which has a maximal effect on the states of interest (biomass, glucose and L-(-)carnitine) and (ii) optimal parameter estimation by considering that the difference between the measured data and the computed profiles becomes a least squared error, in order to minimize the square difference between the measured concentration and the computed one.

3.2 Experimental results: continuous reactor using as C-source a mixture of glucose and glycerol.

E. coli was cultivated in a continuous reactor with glucose as carbon source (see Materials and Methods) until the steady state was reached, in this moment, a medium with glycerol was added instead of glucose until its exhaustion. The results are given in Figure 2. The experiment was carried out to measure the long time response of a culture of *E. coli* biotransforming in a mixture of C-sources like an example of a heterogeneous carbon feed. That is also important to predict the response of the biotransformation process when glucose was not deliberately added, or in the case that impure C-sources containing glucose were used.

Table 4**.** Model Parameters. The complete set of parameters used in the model is summarized herein. Markers for units: (a) $\frac{1}{h}$; (b) $\frac{\mu mol}{gDW}$;(c) $\frac{gDW}{h \times \mu mol}$;(d) $\frac{\mu mol}{gDW \times h}$; (e) $\frac{\mu mol}{gDW \times mM \times h}$; (f) $\frac{1}{mM \times h}$; (g) $\frac{gDW}{\mu mol}$. Markers for References: (1) Values determined within the model with experimental results; (2) Values estimated.

Parameters					
Name	**Value (Units)**	**Ref.**	**Name**	**Value (Units)**	**Ref.**
Carnitine Unit					
k_{sinLC}	0.213 (c)	(1)	k_{cai}	$1.39 \cdot 10^{8}$ (a)	(1)
k_{ProULC}	0.147 (e)	(1)	K_{eqCaiF}	1000 (g)	(2)
k_{CaiC}	12.185 (a)	(1)	$[D_{caiF}]_0$	$1.32 \cdot 10^{-5}$	(2)
$k_{CaiTLCout}$	0.381 (f)	(1)	$[D_{cai}]_0$	$1.32 \cdot 10^{-5}$	(2)
$r_{CaiTinLC}$	0.282 (d)	(1)	K_{Ccai}	$5 \cdot 10^{-2}$(b)	(1)
k_{dCHR1}	0.796 (d)	(2)	K_{CcaiF}	$2.44 \cdot 10^{-5}$(b)	(1)
k_{dCHR2}	0.073 (a)	(2)	K_{caiF}	$2.44 \cdot 10^{-5}$	(1)
k_{dCHR3}	0.011 (c)	(2)	K_{cai}	$5.0 \cdot 10^{-2}$	(1)

In the Figure 2 (A and B) is shown the evolution of the concentrations glucose and external cAMP. At initial time (t=0h) the steady state was completely reached and glucose is suppressed in the inlet, like an obvious consequence its concentration started to decrease. At the same time the external cAMP increased deeply. Result in agreement with previous findings (Postma et al., 1993; Wang et al., 2001).

The response of carnitine metabolism (Fig 2 C and D) was as expected: the CHR activity rose up and, consequently, carnitine was synthesized and its concentration increased. When the medium has no glucose, a new steady state concentration of cAMP, LC and CHR activity were reached. These results demonstrated that the effect of glucose is reversible but needed almost complete depletion of glucose to be abolished. Result in agreement with NotleyMcRobb et al., (1997) who established that the residual glucose concentration is above 0.2 mM to

raise markedly the cAMP levels. Therefore, this study reveals that at the moment in which cAMP levels increased as a consequence of the glucose exhaustion (t=8h), LC and CHR activity rose probably due to the synthesis of cAMP and CRP is able to bind it, expressing carnitine metabolism and then the biosynthesis is able to work.

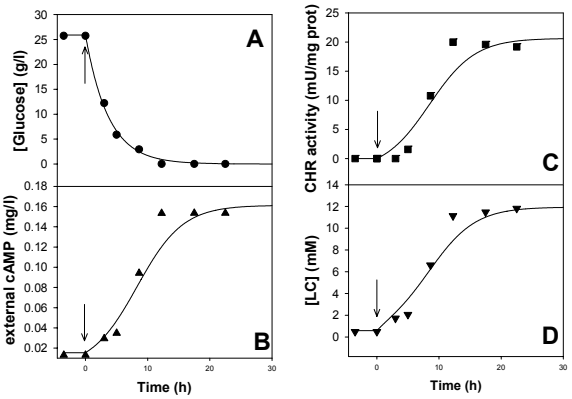

Fig. 2. Evolution of glucose (●), extracellular cAMP (▲), CHR activity (■) and L-carnitine concentration (▼) in the biotransformation of crotonobetaine using *E. coli* 044 K74 grown on a complex medium with glucose in a high density cell recycle membrane reactor system under anaerobic conditions. The feed medium was changed to glycerol (12.6 g/l) as C-source after the steady state was reached (t=0, indicated by an arrow). Model results are represented by continuous lines.

The mathematical model found a relationship between the external cAMP concentration and the bioprocess state (L-carnitine synthesis) almost linear (Fig. 3). The experimental results also confirmed the model results. Moreover, a linear correlation fitted the experimental data (r^2 = 0.9994) what means that the model was able to predict this linear relationship. Additional experiments were carried out (results not shown) in order to prove this relationship: *E. coli* cells were grown in a continuous reactor with glycerol as the carbon source until the steady state was reached, then the response to a glucose pulse was examined. Besides, *E. coli* cells were grown in a continuous reactor with glycerol as the carbon source until the steady state was reached. In this moment, the growing medium was switched over to another containing glucose (the order of feeding was inversed compared to the initial experiment). Results are in agreement with the previous ones and the linear relationship between the L-carnitine concentration and the external cAMP level was always maintained on all the experiments carried out.

3.3 Biotransformation carried out adding exogenous cAMP.

In this experiment, the biotransformation was carried out with glucose or glycerol as C-source. Additionally exogenous cAMP was added (see experimental procedures in Materials and Methods). The experimental results are resumed in Fig. 4 and reveal that if glucose was present, no biotransformation is carried out (the initial levels of L-carnitine could be a residue from the substrate). However, almost a normal productivity was reached with glucose as C-source when cAMP is added to medium. Thus, glucose effect in carnitine metabolism is abolished almost completely if cAMP is present in the reaction medium.

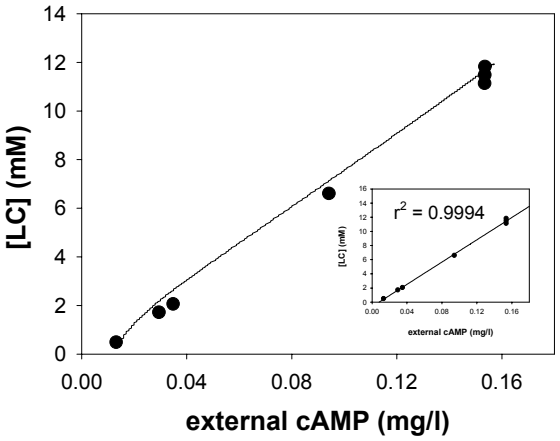

Fig. 3. Representation of the relationship between the external cAMP concentration and the L-carnitine synthesis found by the developed model. Continuous line represents the simulated results. The experimental data regression analysis is represented in the box.

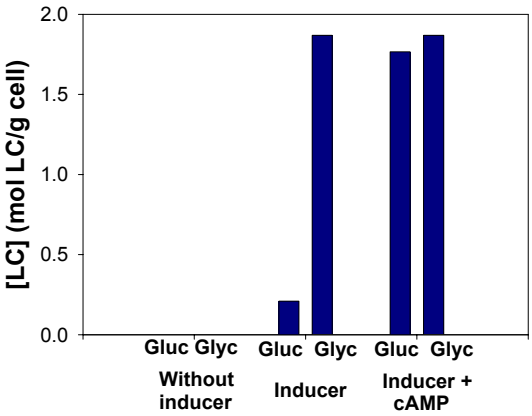

Fig. 4. Results of the biotransformation productivity in the following conditions: without inducer, with inducer (crotonobetaine, 4 g/l); and adding cAMP (5 mM). All of them were carried out with glucose (Gluc) OR glycerol (Glyc) as the carbon source.

4. DISCUSSION

The present work provides new insights on how Systems Biology can help to describe and monitor a bioprocess using a signalling intermediate. Besides, it also shows, for the first time, the linear relationship of the carnitine metabolic network with the alarmone cAMP in *E. coli*. Moreover, the results presented in this work demonstrate that the alarmone cAMP can

be used as an indicator of the state of biotransformation, as shown in Figures 2 and 3, since a linear correlation was found to describe the relationship between the cAMP concentration and the L-carnitine synthesis. This relationship was also found in several scenarios: a mixture of glycerol and glucose as C-sources until glucose or glycerol were exhausted and also with a pulse of glucose in a steady state continuous reactor. The results from the model match adequately the behaviour of the metabolic network involved in the biocatalytic system. Additionally, extracellular cAMP is easily detectable using enzymatic or ELISA methods. Taking together these facts, we could utilize the alarmone cAMP like a marker of the biotransformation if a heterogeneous and/or a not well identified C-source was used.

The aim in the future would be to optimize the developed method in order to use external cAMP as a marker of the biotransformation state. For example, developing an automatizated method able to measure external cAMP on line. Besides, this procedure should be tested on other metabolic pathways that depend on genes regulated by the CRP protein, not only the carnitine metabolism, in order to check its validity as a marker of the catabolite repression exerted by glucose.

In summary, based on a Systems Biology approach, a new reporter of the state of a biotransformation has been stated in this work, especially useful when the composition of a heterogenic C-source used in the bioprocess in study is unknown.

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REFERENCES

Ahlqvist, J., Dainiak, M.B., Kumar, A., Hornsten, E.G., Galaev, I.Y., and Mattiasson, B. (2006). Monitoring the production of inclusion bodies during fermentation and enzyme-linked immunosorbent assay analysis of intact inclusion bodies using cryogel minicolumn plates. *Anal. Biochem.*, 354, 229-237.

Bettenbrock, K., Fischer, S., Kremling, A., Jahreis, K., Sauter, T., and Gilles, E.D. (2006). A quantitative approach to catabolite repression in *Escherichia coli*. *J. Biol. Chem.*, 281, 2578-2584.

Buchet, A., Nasser, W., Eichler, K., and Mandrand-Berthelot, M.A. (1999). Positive co-regulation of the *Escherichia coli* carnitine pathway *cai* and *fix* operons by CRP and the CaiF activator. *Mol. Microbiol.*, 34, 562-575.

Cánovas, M., Bernal, V., Torroglosa, T., Ramírez, J.L., and Iborra, J.L. (2003). Link between primary and secondary metabolism in the biotransformation of trimethylammonium compounds by *Escherichia coli*. *Biotechnol. Bioeng.*, 84, 686-699.

Cánovas, M., Maíquez, J.R., Obón, J.M., and Iborra, J.L. (2002). Modeling of the biotransformation of crotonobetaine into L-(-)- carnitine by *Escherichia coli* strains. *Biotechnol. Bioeng.*, 77, 764-775.

Eichler, K., Buchet, A., Lemke, R., Kleber, H.P., and MandrandBerthelot, M.A. (1996). Identification and characterization of the *caiF* gene encoding a potential transcriptional activator of carnitine metabolism in *Escherichia coli*. *J. Bacteriol.*, 178, 1248-1257.

Elssner, T., Engemann, C., Baumgart, K., and Kleber, H.P. (2001). Involvement of coenzyme A esters and two new enzymes, an enoyl-CoA hydratase and a CoA-transferase, in the hydration of crotonobetaine to L-carnitine by *Escherichia coli*. *Biochemistry*, 40, 11140-11148.

Inada, T., Kimata, K., and Aiba, H.J. (1996). Mechanism responsible for glucose-lactose diauxie in *Escherichia coli*: Challenge to the cAMP model. *Genes Cells*, 1, 293-301.

Jung, H., Jung, K., and Kleber, H.P. (1989). Purification and properties of carnitine dehydratase from *Escherichia coli*: A new enzyme of carnitine metabolization. *Biochim. Biophys. Acta*, 1003, 270-276.

Kleber, H.P. (1997). Bacterial carnitine metabolism. *FEMS Microbiol. Lett.*, 147, 1-9.

Kremling, A. and Gilles, E.D. (2001). The organization of metabolic reaction networks - II. Signal processing in hierarchical structured functional units. *Metabol. Eng.*, 3, 138-150.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, 193, 265-275.

Muller, D., Aguilera-Vázquez, L., Reuss, M., and Mauch, K. (2005). Integration of metabolic and signalling networks. In: *Systems Biology: Definitions and Perspectives* (Westerhoff, H. V. and Alberghina, L. (Ed.)), 235-256. Springer-Verlag, Berlin Heidelberg.

NotleyMcRobb, L., Death, A., and Ferenci, T. (1997). The relationship between external glucose concentration and cAMP levels inside *Escherichia coli*: Implications for models of phosphotransferase-mediated regulation of adenylate cyclase. *Microbiol. UK*, 143, 1909-1918.

Postma, P.W., Lengeler, J.W., and Jacobson, G.R. (1993). Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria. *Microbiol. Rev.*, 57, 543-594.

Wang, J., Gilles, E.D., Lengeler, J.W., and Jahreis, K. (2001). Modeling of inducer exclusion and catabolite repression based on a PTS-dependent sucrose and non-PTS-dependent glycerol transport systems in *Escherichia coli* K-12 and its experimental verification. *J. Biotechnol.*, 92, 133-158.