UNIVERSIDAD DE MURCIA

DEPARTAMENTO DE BIOQUÍMICA Y
BIOLOGÍA MOLECULAR (B) E INMUNOLOGÍA

Metabolic Engineering and Systems Biology strategies for L(-)-carnitine production in *Escherichia coli*

Estrategias de Ingeniería Metabólica y Biología de Sistemas aplicadas a la producción de L(-)-carnitina por *Escherichia coli*

PAULA ARENSE PARRA

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D. Manuel Cánovas Díaz, Catedrático de Universidad del Área de Bioquímica y Biología Molecular en el Departamento de Bioquímica y Biología Molecular (B) e Inmunología, AUTORIZA:

La presentación de la Tesis Doctoral titulada "ESTRATEGIAS DE INGENIERÍA METABÓLICA Y BIOLOGÍA DE SISTEMAS APLICADAS A LA PRODUCCIÓN DE L(-)-CARNITINA POR *Escherichia coli*", realizada por Dña. Paula Arense Parra, bajo mi inmediata dirección y supervisión, y que presenta para la obtención del grado de Doctor por la Universidad de Murcia.

En Murcia, a 21 de marzo de 2014.

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Que la Tesis Doctoral titulada "ESTRATEGIAS DE INGENIERÍA METABÓLICA Y BIOLOGÍA DE SISTEMAS APLICADAS A LA PRODUCCIÓN DE L(-)-CARNITINA POR *Escherichia coli*", realizada por Dña. Paula Arense Parra, bajo la inmediata dirección y supervisión de D. Manuel Cánovas Díaz y D. José Luis Iborra Pastor, y que el Departamento ha dado su conformidad para que sea presentada ante la Comisión de Doctorado.

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Parte de los resultados que se presentan en esta memoria han dado lugar a los siguientes artículos, capítulo de libro y comunicaciones a congresos:

ARTÍCULOS

- Bernal, V., Masdemont, B., **Arense, P**., Cánovas, M. and Iborra, J.L. 2007. *Redirecting metabolic fluxes through cofactor engineering: Role of CoA-esters pool durihg L(-)-carnitine production by Escherichia coli*. Journal of Biotechnology. 132, (110-117).

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- V. Bernal, B. Masdemont, **P. Arense**, A. Sevilla, C. Bernal, R. Teruel, M. Cánovas y J.L. Iborra. *Conexión del pool de coenzima-A con el metabolismo secundario en Escherichia coli: optimización de la producción de L(-)-carnitina mediante ingeniería de cofactor*. BioSpain Biotec 2006. Madrid (España). Books of Abstract.

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A Jesús Mª

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RESUMEN

Esta Tesis Doctoral recoge el trabajo de investigación que se ha realizado en dos líneas desarrolladas de forma paralela sobre *Escherichia coli*. Por un lado, la optimización de un proceso de biotransformación para mejorar la síntesis de L(-)-carnitina mediante técnicas de ingeniería metabólica. Y por otro, la determinación de los principales efectos que provoca, sobre las células indicadas, la exposición prolongada a altas concentraciones de sal, así como su respuesta de adaptación a dicho estrés osmótico, principalmente cuando las fuentes de carbono pueden contener altas concentraciones de sal y tanto el sustrato como el producto son osmoprotectores. Para ello, se han aplicado técnicas utilizadas por la biología de sistemas y la ingeniería metabólica.

En los dos primeros capítulos se desarrolla la modificación genética de cepas de *Escherichia coli* con la intención de optimizar el proceso de biotransformación de crotonobetaína (D-carnitina deshidratada) en L(-)-carnitina, y así, mejorar la producción de esta última. L(-)-carnitina es un compuesto que, en el caso de mamíferos, permite el transporte de ácidos grasos de cadena larga a través de la membrana interna mitocondrial, para su posterior oxidación, desempeñando un papel esencial en el metabolismo energético. En mamíferos, la forma de obtener L(-)-carnitina es, en mayor medida, mediante los alimentos que se ingieren en la dieta, aunque una pequeña parte es sintetizada por el organismo. La deficiencia de este compuesto se asocia a diversas patologías como cardiomiopatía, fallo cardiaco, encefalopatía, hepatomegalia, alteración en el crecimiento, el desarrollo infantil y desórdenes neuromusculares; por tanto, se hace indispensable su administración como fármaco para suplir tal carencia [1-3]. Debido a su aplicación terapéutica, se ha generado una creciente actividad investigadora en el campo de la producción de L(-)-carnitina. Inicialmente, la síntesis química se estableció como la principal fuente de este compuesto. Sin embargo, uno de los principales inconvenientes que presenta este tipo de síntesis es el coste económico que supone, por un lado, la separación de la mezcla racémica de D.L-carnitina producida, y por otro, las extremas condiciones del proceso (presión, temperatura, etc) necesarias para llevar a cabo la síntesis,

que inhibe la reacción catalizada por CaiA, favoreciendo que todo el sustrato sea transformado en L(-)-carnitina.

Otro de los aspectos estudiados, y que está relacionado con la actividad de CaiC, es la necesidad de disponer de coenzima A libre. Esta necesidad se puso de manifiesto en diferentes ensayos, donde la adición al medio de reacción de compuestos que favorecían el incremento o la disminución de los niveles de este coenzima, provocaron una variación de la producción de L(-)-carnitina. Este efecto, por tanto, señala una herramienta más para conseguir la optimización del proceso de biotransformación, que es conocida como ingeniería de cofactor.

A diferencia de las proteínas del metabolismo de carnitina mencionadas anteriormente, la proteína CaiC tenía asignada su actividad CoA-ligasa por homología de secuencias [13], sin embargo, hasta ahora no se había verificado dicha actividad. En una serie de ensayos realizados se ha demostrado dicha actividad. Además, también se ha podido demostrar que la presencia de ATP es indispensable para la función de la enzima, y que ésta presenta una selectiva especificidad para compuestos muy relacionados con L(-)-carnitina.

Posteriormente, el trabajo se encaminó a conseguir superar ciertas limitaciones que impedían obtener la máxima producción del proceso. El trabajo con plásmidos presentó buenos resultados pero la capacidad de biotransformación conseguida podría perderse debido a la segregación de los mismos. Además, pese a que los medios de reacción fueron suplementados con fumarato para impedir la reacción catalizada por CaiA, la eliminación del gen permitiría que el sustrato en su totalidad fuera transformado en L(-)-carnitina. A esto hay que añadir el requisito esencial de condiciones anaerobias, para que la expresión de los genes del metabolismo de carnitina tenga lugar de forma apropiada en *E. coli*. Por tanto, en un intento por seguir mejorando la producción de L(-)-carnitina y siguiendo una metodología, en la que se han implementado diversas estrategias de ingeniería metabólica, se obtuvo una cepa de *E. coli* estable y con una alta capacidad para su

microorganismos pueden adaptarse y dar una respuesta pasajera, si el estrés no perdura en el tiempo, o por el contrario, realizar un proceso de adaptación para mantener su supervivencia mientras el estrés está presente [24]. En la mayoría de los trabajos revisados, los estudios de estrés se centraban en la respuesta celular a corto plazo, y raramente bajo condiciones anaerobias. En este trabajo, se cultivó la cepa *E. coli* O44K74 en un reactor contínuo, con la concentración basal de sal presente en el medio de cultivo (se tomó como control, 0,085 M), y bajo condiciones anaerobias. Una vez que el crecimiento celular permanecía en estado estacionario, se le suministró el mismo medio pero suplementado con tres concentraciones diferentes de sal (moderada, alta y muy alta de 0,3, 0,5 y 0,8 M, respectivamente) y se observó su evolución hasta que alcanzó un nuevo estado estacionario. A continuación se llevó a cabo el análisis de la respuesta celular generada por dicho estrés osmótico, a cada concentración de sal, lo que condujo a determinados mecanismos que se utilizan en la respuesta adaptativa a nivel del metabolismo central y energético. La medida de las actividades enzimáticas de las principales rutas metabólicas, así como la determinación de los metabolitos fermentativos producidos, resaltaron el importante papel ejercido por el metabolismo central en la adaptación y en la supervivencia celular tras una larga exposición a estrés salino. Así, los principales nodos enzimáticos que regularon el flujo de carbono fueron los formados por lo pares isocitrato deshidrogenasa/isocitrato liasa, fosfoenolpiruvato carboxicinasa/fosfoenolpiruvato carboxilasa y fosfotranferasa/ acetil-CoA sintetasa. Las relaciones de estos nodos alterados por las concentraciones de sal evidenciaron la necesidad de disponer de precursores biosintéticos y de energía en forma de ATP. Además, se comprobó el efecto de la adaptación en células aisladas que habían estado expuestas a muy altas concentraciones de sal. Dichas células presentaron unas capacidades metabólicas alteradas que se reflejaron en unas mayores tasas de producción y consumo de metabolitos respecto a la cepa silvestre.

Tras los primeros resultados, se procedió a profundizar en el estudio del comportamiento celular ante este tipo de estrés, analizando la evolución de la

debe destacar que se observaron dos conjuntos de respuestas consecuencia de la concentración de sal presente en el medio. Principalmente las respuestas producidas por la exposición a altas y muy altas concentraciones de sal (0.5 y 0.8 M NaCl) mostraron un mismo patrón dirigido a mantener unos niveles energéticos umbrales en la célula. Así, se observó el incremento de los flujos metabólicos hacia rutas que permitían la generación de ATP, basadas en el consumo de glicerol y la formación de succinato y formiato. Sin embargo, la intensidad de respuesta fue dependiente de la cantidad de sal, siendo mayor en la concentración más alta. Por otro lado, otra respuesta celular generalizada fue la reducción de procesos no esenciales para la supervivencia celular que llevaban asociados un gasto energético. Así los resultados obtenidos avalaron la disminución del nivel de expresión de genes relacionados con la motilidad y la locomoción tales como *cheZ*, *fliS* y *tap*. En este sentido, cabe destacar el consumo masivo de aminoácidos que se observó en el medio del reactor una vez que se comienza a incrementar la concentración de sal en el mismo, independientemente de la concentración utilizada. Este comportamiento indica que la disponibilidad de los mismos permitiría reducir su síntesis de *novo*, lo que se traduciría en un ahorro energético. Por otra parte, se observó una respuesta característica en las células expuestas a alta o a muy alta concentración de sal, que estuvo caracterizada no sólo por cambios en los patrones de fermentación metabólica sino también por una alteración significativa del estado redox celular. Además, el cambio producido en las principales rutas de fermentación también se observó en los cultivos realizados en reactores discontínuos con la cepa adaptada a alta salinidad. Este hecho indica que la respuesta adaptativa se conserva, convirtiéndose en una estrategia esencial para la supervivencia celular.

En resumen, este trabajo recoge diferentes estrategias para abordar la optimización de un proceso de producción de un bioproducto por microorganismos, mediante herramientas proporcionadas por la ingeniería metabólica. Además, se ha conseguido mejorar el proceso de biotransformación de crotonobetaína en L(-)-carnitina con *E. coli*, obteniéndose la máxima producción de este compuesto por

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INTRODUCTION

This work is the result of several years of research concerning the biotransformation of a trimethylammonium compound (crotonobetaine or D(+)-carnitine) into L(-)-carnitine by *Escherichia coli*.

L(-)-carnitine ((R)-3-hydroxy-4-N-trimethylaminobutyrate) was discovered by entomologists at the University of Illinois, who found that the growth of mealworm *Tenebrio molitor* required a growth factor (carnitine). They purified this growth factor and called it vitamin B_T [1]. However, research on carnitine increased once it was observed that mammalian animals were capable of biosynthesizing carnitine only at low levels.

L(-)-carnitine is an ubiquitously occurring substance and has been shown to play several roles in metabolism, especially in the transport of long-chain fatty acids through the inner mitochondrial membrane [2, 3]. Although the role of L(-)-carnitine is well established in eukaryotes, it is not so clear in bacteria [4-6]. In addition to glycine betaine, one of the most widely distributed osmoprotectants, L(-)-carnitine was shown to serve as osmoprotectant in *Escherichia coli* [7] and other microorganisms [8-12]. The function of L(-)-carnitine in the eukaryotic cell metabolism led to a series of clinical applications, e.g. in the treatment of patients with carnitine deficiency syndromes, in the prophylaxis and therapy of various heart diseases, and in replacement therapy for haemodialysis patients. Additionally, it is known that L(-)-carnitine attenuates oxidative stress [13, 14] and has immunomodulatory properties [15, 16]. L(-)-carnitine has been used as an additive in energetic drinks, or added to fermentation media to increase the growth rate of yeast and bacteria.

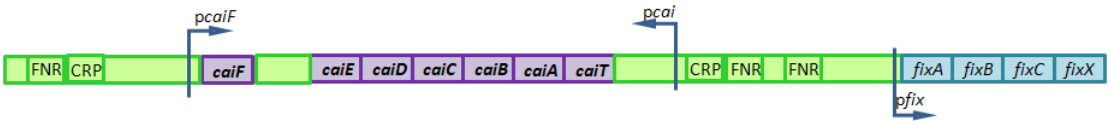
The carnitine metabolism is present in numerous microorganisms [6]; for instance, L(-)-carnitine can be metabolized by various members of the Enterobacteriaceae, e.g. *Escherichia*, *Salmonella* and *Proteus* [17]. L(-)-carnitine metabolism in *E. coli* is usually related to anaerobic conditions, and in fact the enzymes involved in it are only induced when cells are grown in the absence of

oxygen [18, 19]. However, *Escherichia coli* is the target microorganism on this work focuses.

The carnitine metabolism has been established in *E. coli* by means of several genetic works [4, 20]. The main genes are organized in the two divergent *caiTBCDE* and *fixABCX* operons (Fig. 1). The structural *cai* operon is responsible for the expression of the betaine transporter, CaiT, and the carnitine metabolism enzymes (crotonobetainyl-CoA reductase, CaiA; coenzyme A transferase, CaiB; coenzyme A ligase, CaiC and enoyl-CoA hydratase, CaiD). The enzyme activities involved in L(-)-carntine production have been revealed in different works. Firstly, two enzymes, carnitine dehydratase (CDH) and crotonobetaine reductase (CR) [7, 21] were reported. A racemase (CRac) was also postulated for the biotransformation of D(+)- into L(-)-carnitine [4, 18]. Subsequently, CDH activity was demonstrated to depend on two proteins, CaiD and CaiB, and CR activity was carried out by CaiA and CaiB. Moreover, CaiD was postulated to be involved in the racemization process [5, 22, 23].

The *fix* operon encodes a serial of proteins whose function is related to specific electron transfer for carnitine transformation [4]. The regulation of both *cai* and *fix* operons is similar in that they are repressed by the same effectors, in particular, oxygen, glucose and nitrate. On the other hand, a specific regulatory protein, CaiF, is necessary to induce the expression of these operons. This protein is encoded by the *caiF* gene, which lies downstream of the *ca*i operon and in the opposite orientation [24]. Moreover, both *cai* and *fix* operons and the *caiF* gene are subject to global control by the same regulatory proteins including the cyclic AMP (AMPc) receptor protein (CRP), which mediates the activation of catabolic operons, and the transcriptional regulator FNR that is responsible for anaerobic induction, the DNA-binding protein presence of FNR and CRP are required for the expression of both *cai* and *fix* operon. However H-NS acts by partially repressing that expression [20, 25, 26].

A)



B)

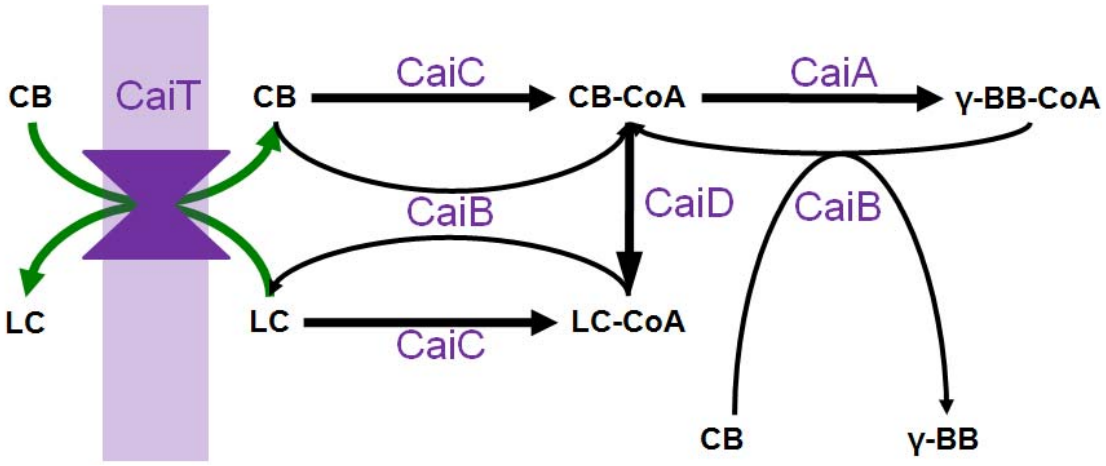


Figure 1. L(-)-carnitine metabolism. A) Operons related to carnitine metabolism and B) enzyme activities involved in the biotransformation from crotonobetaine to L(-)-carnitine by *E. coli [27]*.

The growing demand for the biologically active enantiomer, L(-)-carnitine, for the above mentioned purposes and others has caused a world wide search for different ways of synthesizing this betaine in an optically pure form, since the chemically synthesized racemate cannot be administered. It has been noted that D(+)-carnitine is not only physiologically inactive but also bound and transported by the L(-)-carnitine transport system acts by diminishing L(-)-carnitine in cells and inhibiting the L(-)-carnitine specific reactions. The chemical synthesis of L(-)-carnitine is limited by the optical resolution of racemic carnitine or its derivates.

A waste by-product, D(+)-carnitine, is formed during this process, and effective separation strategies are required [28]. The biotechnological processes used for L(-)-carnitine production have advantages over chemical processes. It has been reported that biotechnological processes produce about 50% less total organic waste and 25% less waste water than chemical processes, while waste incineration is reduced by 90% [29]. Moreover, the biotechnological process saves energy. Biocatalysis offers the possibility to obtain a selectivity in the desired products that is not possible using purely chemical steps. Biocatalysis may be carried out using whole cells, cellular extracts or discrete enzymes. The use of whole cells is not always possible, for example, when the product is toxic to the organism, or where a lack of diffusion limits the yield attainable. However, due particularly to the high costs of cofactors, whole cell processes are generally preferred, as they allow efficient cofactor recycling [30]. Hence, the possibility of L(-)-carnitine production by microorganisms has been widely studied in an attempt to obtain an improved and manipulative process.

Many works have been directed towards improving of the biotransformation process since L(-)-carnitine is considered an important nutraceutical and pharmaceutical compound just as mentioned above. In fact, previous works in our laboratory focused on various aspects in order to enhance production and the yield of the biotransformation process [22, 31-33]. The ways used for this purpose were based, first, on increasing the biomass within the reactor by using high-cell density reactors and, second, improving the biocatalytic environment by optimizing the conditions of the cultures [22, 32, 34]. Moreover, several processes were developed with growing and resting cells, both free and immobilized, with positive results [23, 31, 32, 35-37]. On the other hand, interest in genetically manipulating the microorganism has increased, since this would make it possible to obtain better strains able to increase the production of the compound of interest, and therefore improve the cellular processes involved.

Any improvement of a production process using microorganisms entails the need to know and understand all the functions, which lead to final compound. The manipulation of *E. coli* for this purpose is the starting point of this work, which describes two different and parallel approaches in order to insight and optimize the L(-)-carnitine production process: metabolic engineering and adaptation processes to osmotic stress.

Metabolic engineering allows engineered strains to be obtained to unravel what target genes are most efficient and necessary to enhance L(-)-carnitine production, and to achieve biotransformation in conditions where the process is only limited by the regulatory mechanisms of the carnitine metabolism itself. During recent years, great advances have been made using recombinant DNA technology and successful advances have occurred by means of genetic engineering. Metabolic engineering has contributed significantly to the enhanced production of various value-added compounds [30, 38-40]. This is generally referred to as the targeted and purposeful alteration of the metabolic pathways found in an organism in order to better understand and utilize cellular pathways for chemical transformation, energy transduction, and supramolecular assembly [41]. Traditionally, industrial microorganisms were developed via multiple rounds of random mutagenesis and selection, which might cause unwanted alterations in the cells. More recently, metabolic engineering has emerged as an alternative, whereby genes within metabolic pathways are purposefully amplified or deleted based on the consideration of the metabolic network as an entity [42-44]. In this work, Chapter 1 and Chapter 2 describe the improvement and enhancement of L(-)-carnitine production using metabolic engineering tools on the carnitine metabolism of *E. coli*. In Chapter 1, two tools are used to perform the transformation of the *E. coli* LMG194 strain. First, the *caiC* gene was cloned and integrated in the parental strain using a plasmid of high copy numbers. Subsequently, that modifications led to a new strain with a high capacity to biotransform crotonobetaine into L(-)-carnitine. The effect of the overexpression of caiC was higher than that produced by other genes of the carnitine

metabolism. Moreover, since the *caiC* gene had not been characterized previously, and its function had simply been assigned by sequence homology, it was characterized, finding that the *caiC* gene acted as a crotonobetainyl-CoA ligase:transferase. Furthermore, it was described that the biotransformation process occurred at the CoA level [5]. Therefore, cofactor engineering was also applied as a tool. The caiC enzyme is involved in coenzyme A transfer (Fig.1) and substrate activation during the bioprocess [45]. Moreover, the level of coenzyme A acts by limiting the activity of CaiC. Several studies were carried out to modify the L(-)-carnitine production and to gain insight into the control steps acting in the metabolism of acetyl-CoA.

Given the interest in metabolic engineering to increase production of useful metabolites, improved methods for developing strains were introduced. Chapter 2 describes how genetic modification adopts a new approach to improve the biotransformation process, applying a procedure to disrupt chromosomal genes in *E. coli*. This technique has been widely used [46] and it has allowed wild type cells to be modified and adopt new properties. On the other hand, another technique used is promoter swapping which enables researchers to replace chromosomal promoter sequences with precisely engineered promoters to control chromosomal gene expression [47, 48].

Typically, the deletion and strong overexpression of genes has been used to modify the metabolic pathways in order to achieve a desired product. However, in the case on carnitine production, the recombinant strains engineered with plasmids limited the yield and productivity of the process [22]. In order to overcome the limitations found in the biotransformation, the whole set of genes responsible for the carnitine metabolism were overexpressed by chromosomal mutation. Thus, gene expression was tuned, and the native promoter of the *cai* operon was replaced by an strong artificial promoter. The strains were engineered using the Datsenko and Wanner method [49], which led to more stable modified strains with better properties. Nevertheless, the *caiA* gene was removed since the CaiA enzyme

decreased the biotransformation yield as gene codes an enzyme catalyzing a side reaction, whereby the substrate is consumed, limiting its transformation to L(-)-carnitine. In addition, control points of the central metabolism were taken into account and all the positive modifications were performed in a single strain. Finally, this engineered strain showed quantitative differences in the biotransformation process. Furthermore, this engineered strain allowed the production of L(-)-carnitine in aerobic conditions. This possibility is considered advantageous as the resulting biomass is increased and, therefore, the process yield. Previously, no stable strain with this capability had been obtained.

Thus, metabolic engineering was seen as a feasible solution and was carried out as described. However, the importance of the environment in which the production process is carried out and the possible effects that might affect the physiology of cells and their behaviour must be stressed. As is known, systems biology allows rapid evaluation of the global physiology of a cell with respect to the response generated because of different alterations in the environment. Systems biology studies the cellular response from several points of view, such as metabolic flux distribution, alterations in the concentrations of the main metabolites and transcriptional changes, among others. The results obtained with these analyses might contribute to improving the strain and the production process. Moreover, *in silico* simulation results might provide additional information on the cellular status at various hierarchical levels from genome to fluxome, and other information about fermentation and downstream processes.

The adaptive behaviour of microorganisms plays a crucial role in the development and maintenance of selected production organisms in biotechnology. For example, from environmental microbiology we can learn how microorganisms adapt to changes in the environment. By applying this knowledge, we can improve and stabilize production strains and discover novel activities based on the genetic diversity of a given microorganism [50]. Furthermore, the ability to adapt rapidly to different environmental conditions provides a way to enhance the properties of the

microorganisms used in biotechnological applications [51]. The genomic and metabolic evolution of extremophiles is also an example of such a natural adaptation process in bacteria [52]. For this reason, the *E. coli* metabolism and adaptation process to long-term osmotic stress in a severe environment was studied. Moreover, it is necessary to know the precise metabolic response to stress conditions, since any biotechnological process to generate products at industrial level could give negative results since the environment within the reactor evolves during the production process. Furthermore, in this biotransformation process substrates and products are compatible solutes.

The exposure of *E. coli* to high osmolarity provokes a response called osmoadaptation, in which three phases overlap: dehydratation (loss of some cell water), the adjustment of cytoplasmatic solvent composition and rehydratation [53]. Among the first adaptative responses to osmotic upshifts is an increase in the osmotic pressure inside the cells [54], resulting from the uptake of certain solutes from the medium or through *de novo* synthesis [55, 56]. These osmoregulatory solutes include K^+, amino acids (e.g., glutamate, proline), polyalcohols (e.g., trehalose, glycerol) and other zwitterionic organic solutes (e.g., betaines such as glycinebetaine, crotonobetaine and D,L-carnitine) [24]. Besides, in an effort to overcome this situation due to extreme conditions, both central and secondary metabolisms establish a link in order to optimize their resources. Previous works have shown this link and have emphasized the involvement of critical elements in the biotransformation, such as the level of ATP, the acetyl-CoA/CoA ratio or the reducing power [27, 57]. On the other hand, other studies have focused on understanding the regulatory network involved in osmoadaptation [58, 59], in which changes in gene expression levels as well as in the profile of proteins were observed. Most of the research on the osmoadaptation process has been carried out using short term salt stress with a moderate salt concentration in the medium, so that, the response studied is provoked by a temporal alteration. However, long term stress could exert strong pressure on the adaptative response and force it to modify bacteria

grown in these conditions both physiologically and genetically, leading to a new strain with a capacity to tolerate this kind of stress.

In order to throw light on the osmoregulation process and with the intention of achieving further knowledge, a continuous reactor system (Fig. 2) was used to study the effect of long term exposure to salt stress. In Chapter 3, the evolution of the osmoadaptation is followed by measuring cell growth, L(-)-carnitine production, fermentation products, the energetic and redox levels, some selected enzyme activities related to the central metabolism and the expression of certain genes related to osmotic stress.

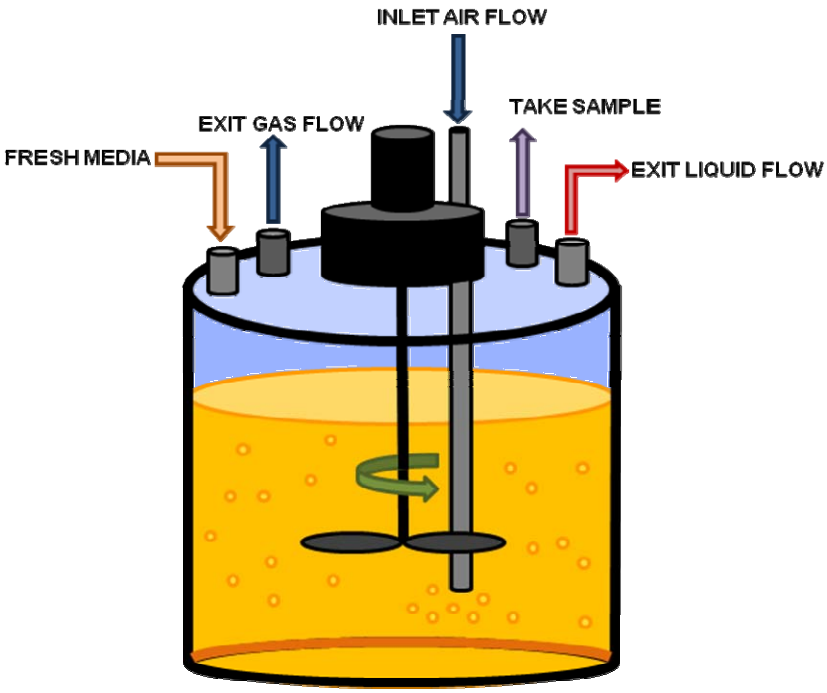


Figure 2. Continuous reactor system to perform experimental procedures. The controller of the system was that of the Braun Biostat B. For further understanding see Chapter 3.

The main alteration observed in the energetic level was emphasized by changing the enzyme activities of the acetate metabolism. On the other hand, the Icdh/Icl node acts by regulating the TCA cycle and the glyoxylate shunt and was found to be balanced as a result of increased Icl activity in the stress condition. In addition, other enzyme activities underwent similar responses, indicating the need of the metabolic intermediates to maintain the biosynthetic pathways. Since salt stress strongly affected the metabolism of *E. coli*, the process of adaptation was studied using the most drastic conditions and isolating an adapted strain. Subsequently, this strain was compared with the wild type strain in different conditions, showing altered metabolic capabilities. Nevertheless, further research is necessary to complete the study about osmoadapation and a high platform would be necessary to implement the obtained results. Along with intracellular metabolite concentrations, the fluxes involved represent the minimal information needed for describing metabolism and cell physiology [60]. This knowledge would allow targets to be identified, which could be modified in order to optimize any metabolic process. In Chapter 4, therefore using different tools and experiments, the overall response pattern to high and very high salt concentration was analyzed. The evolution of the external and internal metabolites, as well as the overall gene expression in the adaptation process to salt stress, indicated that the effect produced on the metabolism depended on the salt concentration in the medium. Furthermore, these experimental results were used in an *in silico* model previously developed by our group which was able to predict the behavior of the *E. coli* strain under the environmental perturbation assayed. In addition, this model showed the distribution of the metabolic fluxes reached after the osmoadaptation process, unveiling new information related to this process. Following the principle of Systems Biology, the results obtained concerning the adaptation process could be integrated in an attempt to unravel the characteristics of cell behavior in response to such adverse conditions.

In Summary, this work attempts:

1. To clone and overexpress the *caiC* gene in *E. coli* in order to optimize L(-)-carnitine production using molecular biology tools. Furthermore, to analyze the enzyme activity and function of caiC, and study the role of CoA-derived compounds in the biotransformation of trimetylammonium compounds into L(-)-carnitine.

2. To develop better engineered strains by applying improved techniques to increase the yield of L(-)-carnitine production and to overcome constraints due to the use of plasmids, the conditions of cell growth and the formation of a useless by-products.

3. To determine the effect of long term exposure to salt stress on the central metabolism and the changes generated by the adaptation process in batch and continuous reactors.

4. To ascertain the global behavior and the main process involved in the response to high salt stress, following the cell biochemical evolution of cells at metabolomic, fluxomic and transcriptomic levels and to establish possible targets in order to improve the production process.

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CHAPTER 1

Genetic and cofactor engineering to improve L(-)-carnitine production in Escherichia coli. Effect of caiC gene.

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Bernal V, Masdemont B, Arense P, Canovas M, Iborra JL: **Redirecting metabolic fluxes through cofactor engineering: Role of CoA-esters pool during L(-)-carnitine production by *Escherichia coli***. *Journal of Biotechnology* 2007, **132**(2):110-117.

Bernal V, Arense P, Blatz V, Mandrand-Berthelot MA, Canovas M, Iborra JL: **Role of betaine : CoA ligase (CaiC) in the activation of betaines and the transfer of coenzyme A in *Escherichia coli***. *Journal of Applied Microbiology* 2008, **105**(1):42-50.

ABSTRACT

L(-)-carnitine is considered an essential therapeutic compound for application in several diseases. Therefore, the development of production systems has increased in the pharmaceutical industry. However, the main way to produce this compound was focused on the chemical synthesis leading to racemic mixtures. In this work, the optimization of an enantioselective and biotechnological process was carried out using growing *Escherichia coli* cells. The cloning and overexpression of the *caiC* gene has allowed the improvement of L(-)-carnitine production from crotonobetaine (dehydrated D(+)-carnitine), increasing L(-)-carnitine production 8-fold in the overexpressing strain compared to the wild type. Moreover, cofactor engineering stated as an efficient strategy to enhance L(-)-carnitine production since carnitine metabolism occurs at the level of coenzyme A. The modification of the acetyl-CoA/CoA ratio by supplementing a CoA precursor or growing the cells on substrates which modified the level of free-Coenzyme A (acetate or pyruvate) altered the L(-)-carnitine production. Furthermore, an enzymatic method based on a cell free extract was used to verify the activity of CaiC for the first time. In fact, CaiC was confirmed as a betaine CoA:ligase establishing the need of ATP and free CoA to carry out its activity. In addition, the CoA-transferase activity was also shown in "*in vitro*" assays. However, the reaction catalyzed by CaiC – synthesis of betaiyl-CoAs – was the main activity "*in vivo*", this being the control step in the biotransfomation of crotonobetaine to L(-)-carnitine.

INTRODUCTION

Microorganisms have been widely used for the production of a great variety of chemical and biochemical compounds. Moreover, the application of DNA recombinant techniques has contributed to improve the metabolic capacities of microorganism in order to enhance the production and yield of biocatalityc processes. L(-)-carnitine [R(-)-3-hydroxy-4-trimethylaminobutyrate] is considered a important compound because of therapeutic benefices on several diseases [1]. Therefore, increasing demand for this compound, particularly in medicine, has encouraged research to develop processes for its production. In this sense, biotechnological methods have been developed to avoid the constraints of chemical synthesis [2-5]. Whole cell biotransformation by Enterobacteria has shown high potential for the recycling of waste products of the L(-)-carnitine chemical production process, such as D(+)-carnitine or crotonobetaine [4, 6]. Genetic studies have led to the description of the structural *cai* operon which is responsible for the expression of the betaine transporter, CaiT, and the carnitine metabolism enzymes (crotonobetainil-CoA reductase, CaiA; coenzyme A transferase, CaiB; coenzyme A ligase, CaiC; enoyl-CoA hydratase, CaiD) [7]. All the enzymes involved in the biotransformation are induced in anaerobiosis in the presence of D,L-carnitine and/or crotonobetaine. Although L(-)-carnitine is a secondary metabolite of *Escherichia coli*, biotransformation occurs at the coenzyme-A (CoA) level [8, 9]. Interestingly, these trimethylammonium compounds are activated by an uncharacterized betainyl-CoA ligase (CaiC), while CoA moiety is transferred between substrates and products by a transferase (CaiB) (Fig. 1), which makes both enzymes crucial in the biotransformation [8]. However, the limitations imposed on the biotransformation by the cellular CoA-thioester pool and the enzymes involved in the metabolic link remain unclear. Furthermore, before bioprocesses based on secondary metabolism can be optimized, the role of the central pathways also needs to be determined.

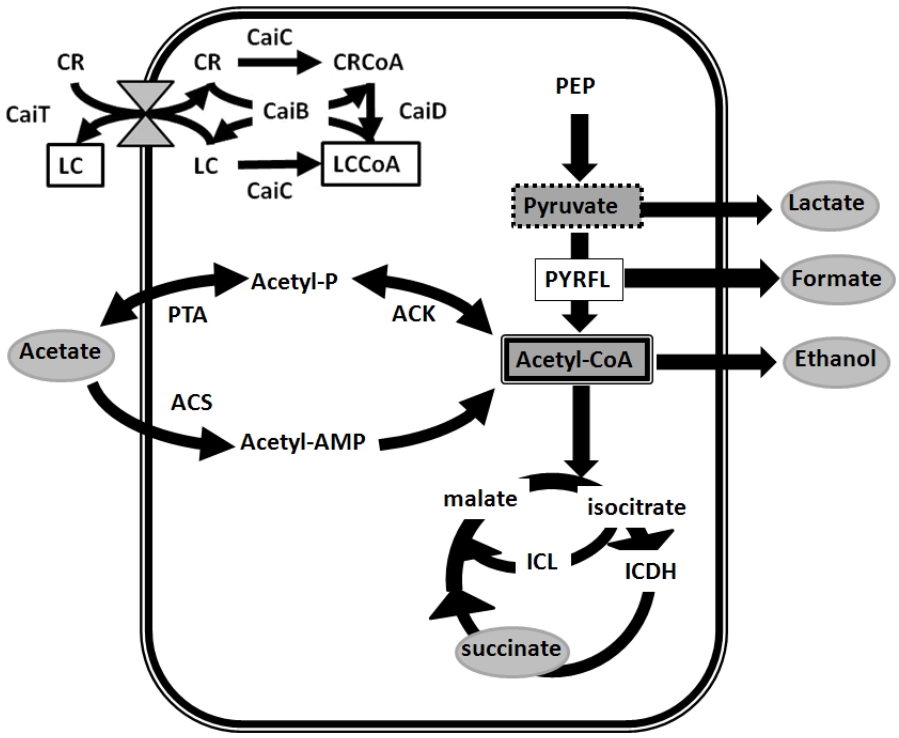

Figure 1. Schematic diagram of central and carnitine metabolims. Abbreviations: LC, L(-)-carnitine; LCCoA, L(-)-carninyl-CoA; CR, crotonobetaine; CRCoA, crotonobetainyl-CoA; CaiT, L-carnitine/γ-butyrobetaine/crotonobetaineprotein transporter; CaiB, CoA transferase; CaiC, L-carnitine/γ-butyrobetaine/crotonobetaine CoA ligase; CaiD, enoyl-CoA hydratase or carnitine racemase activity; PEP, phosphoenolpyruvare; PYRFL, pyruvate:formate lyase; ICDH, isocitrate dehydrogenase; ICL, isocitrate lyase; PTA, phosphotransacetylase; ACK, acetate kinase; ACS, acetyl-CoA synthetase. Adapted from [14].

In this context, cofactor level is one of the control parameters which the cell uses to regulate fluxes through various metabolic pathways, since it affects not only enzyme activity but also gene expression. Cofactor level reflects the physiological state of the cell, and adapts to deal with the various metabolic situations that cells can face [10]. The perturbation of coenzyme pools, also known as cofactor engineering (CE), has shown great potential for metabolic engineering [11, 12]. Coenzyme A and

acetyl-CoA, which regulate the central and intermediary metabolism and link glycolysis, the Krebs cycle, the glyoxylate shunt and acetate metabolism, are recognized as central cofactors [13] (Fig. 1). In the secondary metabolism, CoA-derivatives are involved in the synthesis of PHAs, terpenoids, polyketides and flavonoids.

In this work, the *caiC* gene was cloned and overexpressed, and function of the *caiC* gene product in the biotransformation pathway and the enzyme activity have been analysed. Moreover, the effect of the trimethylammonium compound activating enzyme CaiC and the metabolism of acetyl-CoA are studied to throw light on their involvement in the biotransformation process in *E. coli*. The control imposed by the pool of CoA-thioesters on the interrelation between the central and carnitine metabolisms is discussed.

MATERIAL AND METHODS

1. Strains and plasmids.

Escherichia coli O44K74 overexpresses the genes of carnitine metabolism [2]. *Escherichia coli* LMG194 [F^- *DlacX74 galE galK thi, rpsL DphoA (PvuII) Dara714 leu::Tn10*] is defective in L-arabinose metabolism and was used as expression host [15]. Wild type *E. coli* BW25113 *[rrnB3 DlacZ4787 hsdR514D(araBAD)567 D(rhaBAD)568 rph-1*] and its *caiB* and *caiC* knock-out derivatives [16] were kindly supplied by Prof H. Mori (Keio University, Japan). Unless otherwise stated, all these strains contain the complete structural *cai and fix* operons, and express carnitine racemase (CRac) and carnitine dehydratase (CDH) activities. The strains were stored on culture medium containing glycerol (20%) at -20ºC

2. Batch cultures.

Cells were grown using L-Broth and pH was adjusted to 7.5 with KOH. Ampicillin (10 μg ml^{-1}) and arabinose (0.1 – 0.2%) were added after autoclaving and just before inoculating the media. To study the expression of cloned CaiC, cells were grown in the presence of 0.15% L-arabinose and in the absence of trimethylammonium compounds and aerobic conditions, to avoid the expression of the chromosome encoded carnitine metabolism genes. For the biotransformation experiments, L-broth was supplemented with 50 mmol l^{-1} crotonobetaine or D(+)-carnitine as substrates. Anaerobic conditions were maintained to induce the enzymes involved in carnitine metabolism, while D,L-carnitine or crotonobetaine were supplied as inducers of the *cai* operon. Different concentrations of L-arabinose were used as the inducer of the genes cloned into the pBAD24 vector. Anaerobic batch biotransformation experiments were performed under nitrogen atmosphere in Biostat B (Braun Biotech Intl. GmbH, Melsungen, Germany) reactors. A 0.5 l working volume was used.

3. Assays.

Cell growth was followed as absorbance at 600 nm with a spectrophotometer (Novaspec II; Pharmacia-LKB, Uppsala, Sweden) and correlated with cell dry weight. L(−)-carnitine concentration was determined by an enzymatic test [8], while D,L-carnitine, crotonobetaine and γ-butyrobetaine were determined by HPLC [4].

4. Detection of Betaine:CoA ligase activity and Betaine:CoA transferase activity.

To assay the betaine:CoA ligase activity of CaiC, a modification of the method [17] was followed. The reaction mixture (1 ml) contained 50 mmol l^{-1} sodium phosphate buffer (pH 7.5), 3 mmol l^{-1} ATP, 10 mmol l^{-1} $MgCl_2$, 0.5 mmol l^{-1} CoA and an appropriate amount of cell free extract (approx 0.5–1 mg prot ml^{-1}). After 5 min of incubation at 37ºC, the reaction was started by the addition of 5 mmol l^{-1} substrate [L(-)-carnitine or γ-butyrobetaine, unless otherwise stated in the text]. The reaction was stopped with 15% (w/v) trichloroacetic acid (TCA). After centrifugation (10 min at 19000 g), the supernatant was neutralized with KOH. The reaction was monitored by HPLC and the identity of the products was confirmed by HPLC-ESI-MS.

To assay this activity, betainyl-CoAs were synthesized following the procedure explained above. Upon completion of the reaction (which was confirmed through HPLC), a second betaine (different) was added to the reaction media. The transfer of CoA from the first betaine species to the second was assessed by HPLC and HPLC-ESI-MS.

6. HPLC: detection of CoA derivatives.

CoA derivatives were analysed in an HPLC system (Shimadzu, Kyoto, Japan) equipped with a μ-BondapakTM C18 (Millipore lbérica, Madrid, Spain) column (4.5 mm x 25 cm) with UV detection at 254 nm. Two mobile phases were used: A (200 mmol l^{-1} sodium phosphate pH 5 buffer) and B (200 mmol l^{-1} sodium phosphate pH 5 buffer, 20% acetonitrile) at a flow rate of 1.0 ml min^{-1}. The gradient profile

was: B, 3% at 0 min, 18% at 7.5 min, 28% at 10 min, 30% at 15 min, 40% at 25 min, 42% at 26 min, 90% at 35 min, 3% at 36 min and 3% at 45 min [18].

6. HPLC: detection of ATP, ADP and AMP.

A modification of the method used by Manfredi was followed [19]. The previous HPLC system and column were used. Aliquots from CaiC enzyme activity assays were analysed and compared with standards of ATP, ADP or AMP. The mobile phases were: A (25 mmol l^{-1} NaH_2PO_4, 100 mg l^{-1} tetrabutylammonium, pH 5) and B (10% acetonitrile, 200 mmol l^{-1} NaH_2PO_4, 100 mg l^{-1} tetrabutylammonium, pH 4). Flow rate was adjusted to 1 ml min^{-1}. The gradient profile was modified to the following content of buffer B: 0% isocratic for 10 min, 50% at 20 min, 100% at 25 min and 0% at 30 min. Samples were UV monitored at 254 nm.

7. Mass spectrometry.

The ESI-MS spectra were recorded using an AGILENT VL 1100 HPLC-MS system (Agilent Technologies, Santa Clara, CA, USA). Samples were solid phase extracted using Sep-Pack Cartridges Plus C18 (Waters, Milford, USA). Aqueous samples were applied to the cartridges and the CoA derivatives were eluted with 40% acetonitrile. For the initial characterization of the products, the partially purified samples were directly injected to the MS detector and the spectra were recorded in the positive and negative modes. Mass scans were performed from 300 to 1100 m/z. For acquisition of the spectra, Octopole RF amplitude was set at 150 Vpp, Capillary Exit at -154.6 V and Capillary Exit Offset at -90.4 V. Dry gas flow rate was 9 l min^{-1} and the pressure of the nebulizer was set at 60 psi. Dry temperature was set at 350ºC. For the analysis of enzyme activity, HPLC-ESI-MS was performed following a modification of the method by Pfleger [20]. Two mobile phases were used (A, 100 mmol l^{-1} ammonium acetate buffer, pH 6; B, 70% phase A, 30% acetonitrile). For the elution, phase B profile was as follows: 8% at 0 min, 50% at 5 min, 100% at 13 min, keep at 100% until 19 min and back to 8% at 24 min. The column was allowed to equilibrate for at least 10 min. Flux was set at: 0.25 ml min^{-1};

drying gas, 12 l min^{-1}; nebulizer pressure, 60 psig; drying gas temperature, 300ºC; capillary voltage, 2500 V. Spectra were acquired in positive mode.

RESULTS

1. Cloning and expression of CaiC. Effect on the carnitine metabolism.

Specific primers were designed to PCR-amplify the complete *caiC* gene from *Escherichia coli* O44K74, a wild-type overproducing strain (Table 1). The *caiC* gene was subsequently cloned into the expression vector pBAD24 [15], downstream of the arabinose inducible pBAD promoter. Plasmid construction was performed using standard molecular biology techniques [21]. Specific oligonucleotide primers were designed to amplify *caiC* by PCR and cleavage sites for *XbaI* and *PstI* were introduced for directed cloning into the arabinose inducible pBAD24 expression vector [15].

Table 1: Specific primers designed to amplify *caiC* gene. Restriction sites are represented in bold and italic.

Primer	Sequence (5' a 3')
CaiC forward	GGT GGT ***TCT AGA*** AAT GGA TAG AGG TGC AAT GGA T
CaiC reverse	GGT GGT ***CTG CAG*** TTA TTT CAG ATT CTT TCT AAT TAT TTT

The correct construction of the plasmid pBAD*caiC* was confirmed by electrophoresis and sequencing (Fig. 2). Subsequently, the plasmid pBAD*caiC* was introduced in *E. coli* LMG194 as the expression host, obtaining an engineered strain capable of overexpressing the *caiC* gene.

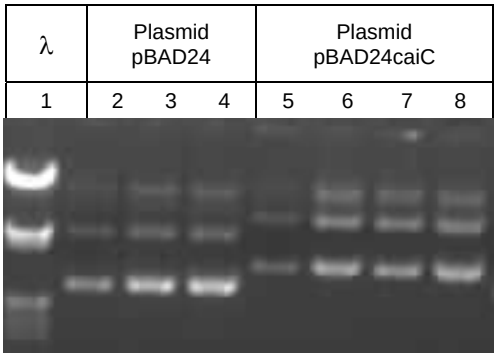

Figure. 2: Agarose electrophoresis gel of isolated plasmids. Plasmids from transformed *E. coli* LMG194 which were separated using a 1% agarose gel in TAE buffer. Lane number 1 corresponds to Lambda DNA/EcoRI+HindIII Marker.

In order to check the effect of the recombinant process growth and L(-)-carnitine production were evaluated. Wild type and engineered strain were cultured in LB medium supplemented with crotonobetaine 50 mM and arabinose 0.2 % as inducers of expression of *cai* operon and *caiC* gene, respectively. The cell growth was higher in wild type whereas the biotransformation yield reached by engineered strain was 15-fold higher than wild type. Moreover, different concentrations of inducer (0.0001–1.0%, w/v) were analyzed in order to optimize L(-)-carnitine production process. As mentioned above cell growth and biotransformation were evaluated. In the case of CaiC, high L(-)-carnitine productivities were attained even at the lowest L-arabinose concentrations assayed. In addition, L(-)-carnitine specific productivity was enhanced compared with the wild type host strain (14.5 mM, 29% yield). Further, the production of L(-)-carnitine by *E. coli* O44K74 under the same experimental conditions was even lower (8.5 mM, 17% yield) than in the CaiC overexpressing strain. Finally, arabinose 0.15 % was selected to be supplemented in subsequent assays (Fig. 3).

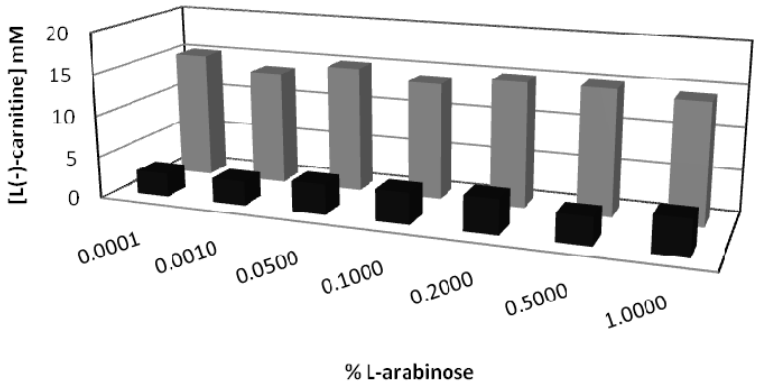

Figure 3. The effect of L-arabinose concentration on L(-)-carnitine production. Experiments were performed in LB medium supplemented with 50 mM crotonobetaine under anaerobic conditions. Bars represent the L(-)-carnitine concentration after 48 h by wild type (■) and overexpressing strain (■).

The presence of alternative electron sinks can inhibit the formation of the by-product, γ-butyrobetaine [2, 8], therefore, the effect of supplementing the medium with fumarate was studied. It was found that the addition of 0-4 g l^{-1} fumarate to the growth medium enhanced L(-)-carnitine production for all the strains assayed. Maximum production was obtained at 2 g l^{-1} (Fig. 4). Yields increased by 10-fold in CaiC overexpressing strain (41.2%), respect to the low yield obtained (3.8%) with the control *E. coli* LMG194 strain.

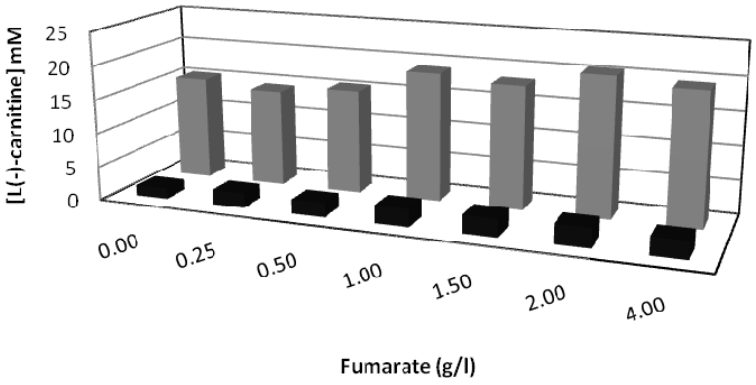

Figure 4. The effect of fumarate concentration on L(-)-carnitine production. Experiments were performed in LB medium supplemented with 50 mM crotonobetaine under anaerobic conditions. Bars represent the L(-)-carnitine concentration after 24 h by wild type (■) and overexpressing strain (■).

2. Effect of precursor coenzyme A addition.

CaiC is involved in coenzyme A transfer between substrate and products and in the synthesis of CoA-derivatives of trimethylammonium compounds [22]. Therefore, the engineered strain was used to determine the effect of the pool of coenzyme A derivatives on the biotransformation of L(-)-carnitine. In order to determine whether intracellular levels of coenzyme A were limiting for the activation of trimethylammonium compounds, a sufficient concentration to be saturating of pantothenate (a precursor of coenzyme A biosynthesis) was added to the culture

medium and both cell growth and L(−)-carnitine production were analyzed. As expected the results showed that the presence of a higher amount of available coenzyme A increased L (-)-carnitine production (Fig. 5).

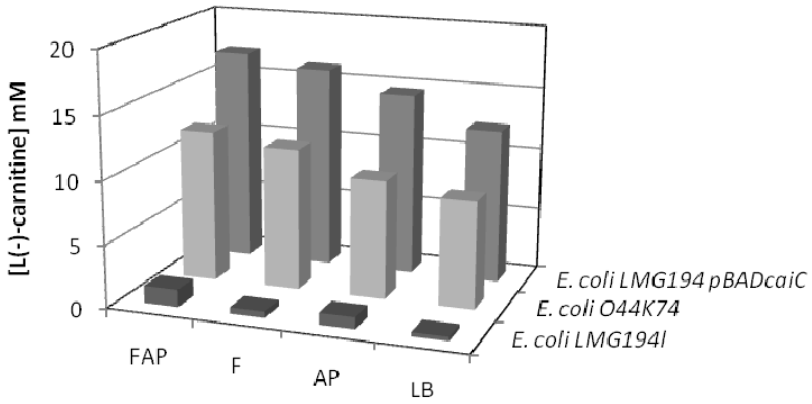

Figure 5: Effect of pantothenate on L(-)-carnitine production of *E. coli* LMG194, *E. coli* 044K74 and *E. coli* LMG194 pBAD*caiC* strains. Experiments were performed under anaerobic conditions in LB medium supplemented with 50 mM crotonobetaine (LB), and the addition of 5 mM pantothenate (AP), 12.5 mM fumarate (F) and 5 mM pantothenate + 12.5 mM fumarate (FAP). L(-)-carnitine refers to 24 h of incubation.

In fact, productivity rose from 1.8 to 5.3 (mmol $gDCW^{-1}$) in the wild type strain and from 85.2 to 97.9 (mmol $gDCW^{-1}$) in the CaiC overexpressing strain. In the case of *E. coli* O44K74, L(−)-carnitine production also increased, although the specific productivity was lower (from 37.5 to 32.7 mmol $gDCW^{-1}$). In addition, the final yield and specific productivities were much lower than those obtained using the strain overexpressing CaiC. Moreover, the fermentative metabolism was analyzed and it was observed that the specific production of acetate decreased in all the strains, reflecting the distinct composition in the intracellular acetyl-CoA/CoA ratio under these conditions.

3. Effect of acetate and pyruvate.

Acetate and pyruvate are related to the central metabolism, as a consequence their addition could modify the intracellular acetyl-CoA/CoA ratio. When cells were grown in the presence and absence of acetate and pyruvate as carbon sources, whose assimilation involves acetyl-CoA, a decrease in L(−)-carnitine production was observed with both the wild type *E. coli* LMG194 and the engineered *E. coli* LMG194 pBAD*caiC* strains (Fig. 6). In the case of *E. coli* LMG194 pBAD*caiC* cells grown in the presence of pyruvate, the decrease in the production of L(−)-carnitine was greater (50% decrease) than in acetate-grown cells (24% decrease). In addition, L(−)-carnitine was produced at a lower rate during exponential growth by cells grown in the presence of pyruvate. Interestingly, for the acetate-grown cells, the decrease was only observed in the early stationary phase (data not shown).

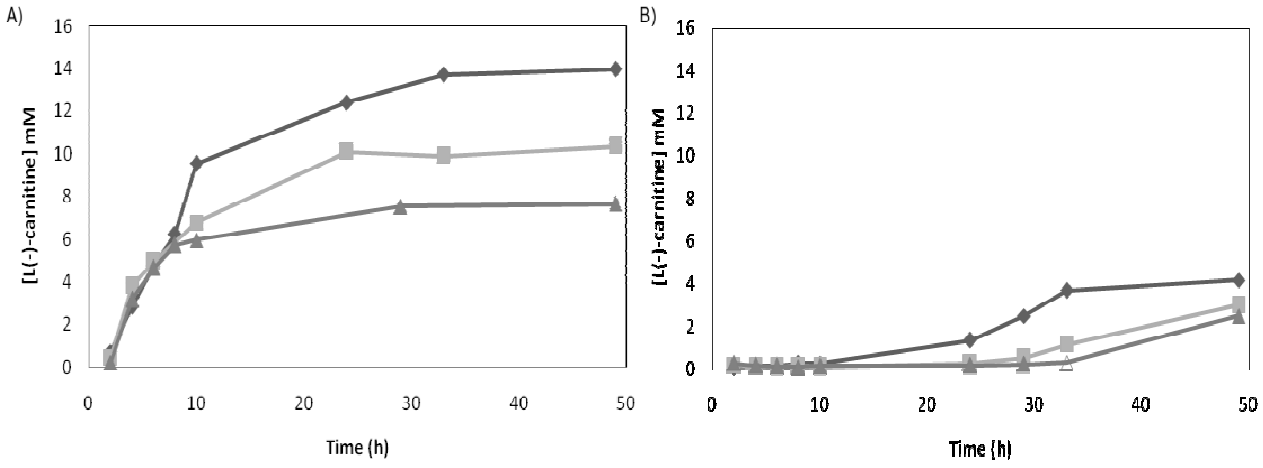

Figure 6. Effect of acetate and pyruvate on the L(-)-carnitine production. *E. coli* LMG194 (—◆—), *E. coli* O44K74 (—■—) and *E. coli* LMG194 pBAD*caiC* (—▲—) were anaerobically grown in presence of 50 mM crotonobetaine supplemented with acetate (A) and pyruvate (B).

Analysis of anaerobic central carbon metabolites revealed that, while pyruvate was being consumed during exponential growth, acetate levels increased as a result of anaerobic metabolism. Only after the cells had reached the stationary phase of growth did the acetate levels fall significantly (data not shown). This correlation between substrate consumption and the inhibition of L(−)-carnitine production seems quite likely to be due to the build-up of high intracellular concentrations of acetyl-CoA, thus limiting the amount of free coenzyme A.

4. Role of CaiC in the secondary metabolism.

CaiC was originally proposed to be a betaine:CoA ligase on the basis of sequence similarities [7]. Nevertheless, to date, no experimental evidence supporting this statement has been provided. A database search revealed the existence of several bacterial sequences with high similarity to CaiC, many of them belonging to the group of Enterobacteria (*Escherichia, Shigella, Salmonella, Proteus*) and annotated as a probable crotonobetaine/carnitine:CoA ligase because of their homology with the gene of *E. coli* O44K74 (Table 3). However, none of them has been functionally characterized yet. In addition, high homology is observed with other bacterial and eukaryotic proteins. In all cases, the most notable structural features of these sequences are the presence of specific AMP and CoA binding sites, both of which would be necessary for the putative ligase activity.

Enzyme modified method [17] was carried out verifying CoA:carnitine-ligase activity proposed for CaiC. The presence of CaiC led to formation of a new compound whereas the peak of CoA was decreasing. Moreover, ATP was confirmed as an essential compound for CaiC activity. In fact, AMP was identified in the reaction medium (data not shown). The enzyme activity assay was performed using a cell-free extract of the mutant strain *E. coli* BW25113 Δ*caiC* [16] in order to verify that CaiC is responsible of that activity. In spite of fact that *caiC* knockout strain was grown under anaerobiosis and in the presence of crotonobetaine to induce the

expression of the *cai* operon, the reaction was not detected. In contrast, the wild type strains *E. coli* LMG194 and *E. coli* BW25113, under these experimental conditions, expressed CaiC activity. Finally, when the *caiC* knockout strain was transformed with the pBAD*caiC* plasmid, the enzyme activity was restored. Thus, CaiC was demonstrated to be the sole protein able to catalyse the formation of the compound detected (identified as L(-)-carnitinyl-CoA).

On the other hand, several compounds very closely related to L(-)-carnitine (L(-)-carnitine, D(+)-carnitine, crotonobetaine and γ-butirobetaine) were employed to determined the substrate specificity of CaiC by CoA-ligase assays. All of them were used by CaiC as expexted since they have been described to be involved in the L(-)-carnitine metabolism in *E. coli* [2, 8]. These observations strongly suggest that CaiC is a highly specific betaine:CoA ligase.

Table 3. Study of homology. Sequences with high similarity to caiC was provided and compared by data base and blast tool.

ORGANISM	PROTEIN	REFERENCE	- Log (E-value)
Escherichia coli O157:H7	Probable crotonobetaine/carnitine-CoA ligase	(Perna et al., 2001; Hayashi et al., 2001)	141
Shigella dysenteriae serotype 1 (strain Sd197)	Probable crotonobetaine/carnitine-CoA ligase	(Yang et al., 2005)	141
Shigella sonnei (strain Ss046)	Probable crotonobetaine/carnitine-CoA ligase	(Yang et al., 2005)	141
Escherichia coli 044K74	Probable crotonobetaine/carnitine-CoA ligase	(Eichler et al.,1994)	140
Salmonella typhimurium	Probable crotonobetaine/carnitine-CoA ligase	(McClelland et al., 2001)	113
Salmonella choleraesuis	Probable crotonobetaine/carnitine-CoA ligase	(Chiu et al., 2005)	112
Bos taurus (Bovine)	Acids transporter 1	(Ordovas et al., 2005)	93
Proteus sp. (strain LE138)	Probable crotonobetaine/carnitine-CoA ligase	(Engemann *et al*., 2005)	88
Desulfotomaculum reducens MI-1	Crotonobetaine/carnitine-CoA ligase	(Copeland *et al*., unpublished results)	62
Rhodopseudomonas palustris BisB5	AMP dependent synthetase and ligase	(Copeland et al., unpublished results)	24
Bacillus cereus (strain ATCC 10987)	Putative long chain fatty acids-CoA ligase	(Rasko et al., 2004)	18
Bordetella parapertussis	Putative acetyl-CoA synthetase	(Parkhill et al., 2003)	17
Mus musculus (mouse)	Long chain fatty acids transporter 1 (Slc27a1)	(Schaffer y Lodish , 1994)	17
Caenorhabditis elegans	Hypothetical protein	(C. elegans Sequencing Consortium, 1998)	17

5. Dual functionality of CaiC.

To determine the degree of reversibility of the reaction, the betainyl:CoA ligase activity assay using L(-)-carnitine, crotonobetaine or γ-butyrobetaine was carried out. Following the incubation, an aliquot was analysed to determine whether the reaction had taken place and was complete. Once the first betainyl-CoA was formed, it was used as the substrate for a CoA-transferase (betaine exchange) activity assay. For that, a second (different) betaine and ATP were added to the mixture of the first reaction. Following incubation, the reaction mixture was analysed (Fig. 7).

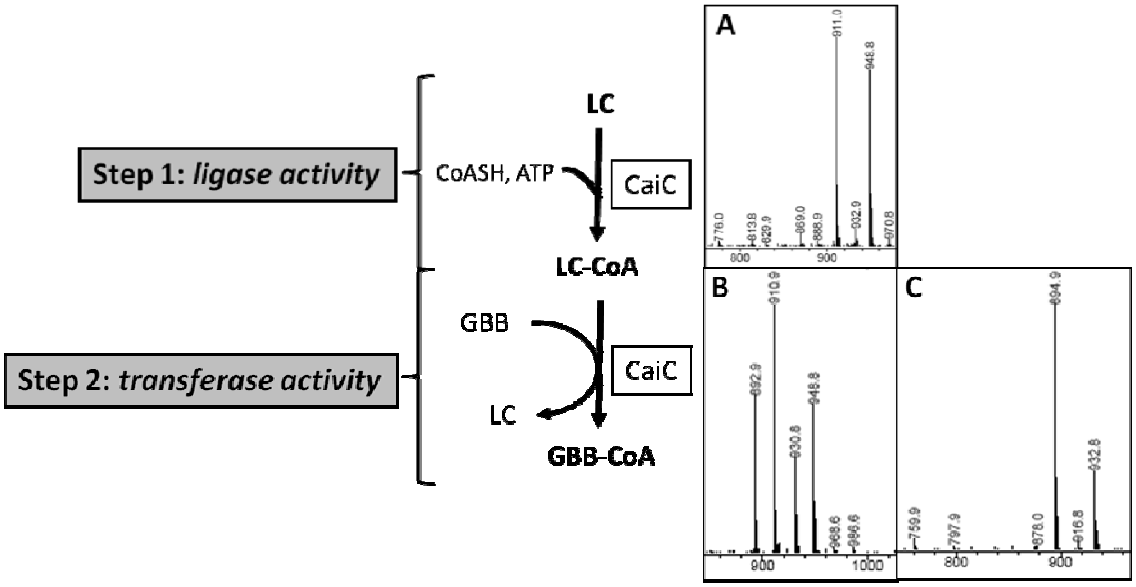

Figure 7. Example of dual activity of CaiC. Experiments are described in the Materials and Methods section. Samples were taken after finishing ligase and transferase activities. Subsequently, samples were analyzed by HPLC-ESI-MS. Mass spectra of peaks corresponding to A) L(-)-carnitinyl-CoA (LC-CoA) obtained in the first step, B) mixture of L(-)-carnitinyl-CoA and γ-butyrobetainyl-CoA (GBB-CoA) obtained in the second and C) γ-butyrobetainyl-CoA to relate the peaks of B).

With the pairs L(-)-carnitine/γ-butyrobetaine and crotonobetaine/γ-butyrobetaine, whose derivatives have sufficiently different retention times, two peaks could be observed by HPLC, while ESI-MS confirmed that transference of CoA moiety between betaines had occurred. This indicated that CaiC was also able to exhibit an in vitro CoA-transferase-like activity, similar to that described for CaiB. When control experiments were performed using *E. coli* BW25113 Δ*caiB* pBAD*caiC*, the same results were obtained.

DISCUSSION

The overexpression of CaiC in *E. coli* LMG194 converted this poorly producing strain into an overproducing one, indicating that this is a controlling factor for the production of L(-)-carnitine. Furthermore, it should be noted that yields and specific productivities were higher than those of the overproducing strain *E. coli* O44K74 under all tested conditions (Figs. 3 and 5), representing an important point of departure for the development of novel metabolic engineering approaches for strain improvement. It seems likely that post-translational effects would account for suboptimal in vivo expression of CaiC. In fact, the presumed Shine-Dalgarno (ribosome-binding) sequence of *caiC*, located at -5 from the ATG start codon, is the shortest (only three nucleotides, GAA) in the cai operon [7], which might explain this fact (Table 4).

Table 4. Shine–Dalgarno sequence of genes of *cai* operon and distance to start codon.

Gene	Shine-Dalgarno sequence	Distance (pb)
caiT	GGAA	7
caiA	AAGAGG	7
caiB	AGGAG	6
caiC	GAA	5
caiD	GAAAGAA	10
caiE	AGAAG	11
caiF	GGAG	5

The addition of fumarate to the growth medium allows a further increase in production. Previous results in our group demonstrated an activation of the glyoxylate shunt and an increase in the production of acetate after a pulse of fumarate within the reactor [10]. This suggests an increased flux of acetyl-CoA

towards the production of acetate and a feeding of the anaplerotic pathways through the glyoxylate shunt.

Since coenzyme A is the cofactor for CaiC, alteration of the intracellular level of coenzyme A and acetyl-CoA was studied. CoA and its thioesters are important regulators of several key enzymes in the intermediary metabolism: total CoA levels result from the balance of biosynthesis and degradation [23] while acetyl-CoA depends on the cellular metabolic state [12]. During the biotransformation of trimethylammonium compounds, a higher demand for coenzyme A is to be expected. Pantothenate kinase (PanK) is the rate-controlling step in the biosynthesis of coenzyme A [24]), although supplementation with pantothenate has been shown to increase the coenzyme A content [12]. The addition of pantothenate under biotransformation conditions increased L(-)-carnitine specific productivity in the CaiC overexpressing strains, respectively. The build-up of betainyl-CoAs would not inhibit bacterial PanK, which has been reported to be more effectively inhibited by free CoA than by its thioester derivatives [23]. Thus, the total concentration of CoA derivatives can be engineered, provided that free CoA remains sufficiently low (Fig. 1). While pyruvate is a highly energetic and readily assimilable carbon source, the metabolization of acetate only occurs in stationary phase cells. Acetate is taken up by the reversible and low-affinity Pta–Ack pathway and by the high-affinity and irreversible Acs pathway [25]. Regardless of the pathway followed, the addition of pyruvate or acetate to the medium alters the metabolism of acetyl-CoA, representing a simple and effective way of engineering intracellular cofactor levels. L(-)-carnitine production was largely inhibited in growth and biotransformation experiments in the presence of pyruvate and acetate, illustrating the importance of a functionally active glyoxylate shunt and the role of the acetyl-CoA/CoA ratio [8]. In addition, in the case of pyruvate grown cells, this inhibitory effect reflected the build up of a large intracellular pool of acetyl-CoA, reducing the availability of free-coenzyme A for the biotransformation. In addition, this hypothesis is also supported by the fact that the inhibition coincided with the consumption of pyruvate and acetate as carbon source.

Conceived as the rational modification of intracellular coenzyme pools, confactor engineering (CE) is a very promising strategy for the modification of metabolic fluxes [11, 26]. The main feature of CE is that it directly affects the coenzyme pools common to a great number of enzymes from different pathways and, thus, a global response is to be expected. The first works on CE focused on the redox balance [26, 27] and the acetyl-CoA/CoA ratio [12] as controlling parameters of the central metabolism. In this work, we have attempted for the first time to improve a bioprocess based on secondary metabolism by applying the principles of CE. The metabolism of L(−)-carnitine is taken as model system since this occurs at the level of coenzyme A [22]. Furthermore, the link between central carbon and carnitine metabolisms resides in the ATP and acetyl-CoA, and strong in vivo alteration of the energetic cofactor pools has been observed in pulse experiments [8, 10].

Despite the betaine:CoA ligase activity of CaiC activity had previously been suggested on the basis of sequence similarities [7], such activity has been demonstrated for the first time in this work. CaiC showed a high degree of correspondence with betaine:CoA ligases belong to the group of Enterobacteria such as *E. coli* O157:H7, *Shigella dysenteriae*, *Salmonella* serotype *Typhimurium* or *Proteus sp*. LE138 [28]. Moreover, high similarities are also found with proteins from less related bacteria, such as *Bacillus cereus*, *Rhodopseudomonas palustris* or *Desulfotomaculum reducens*, or even more complex organisms, such as *Caenorhabditis elegans*, *Mus musculus* or *Bos taurus*. It is noteworthy that none of these proteins has been functionally characterized yet.

CaiC is responsible for activating L(-)-carnitine and its derivatives showing a high degree of specificity. Moreover, *caiC* knockout did not showed an ability to perform the biotransformation of crotonobetaine into L(-)-carnitine, underlining that this activity is required for proper functioning. Moreover, a betaine exchange (CoA-transferase) activity, which had not been described before for CaiC, was observed *in vitro*. However, this second activity might not be relevant for the *in vivo*

biotransformation, as a Δ*caiB* mutant strain was not able to produce L(-)-carnitine from crotonobetaine, even when CaiC was overexpressed.

Thus enzyme activity redundancy does not occur in vivo, and the role of CaiC is most likely to be related to the activation of trimethylammonium compounds, while CaiB would transfer the CoA moiety. Moreover, CaiC activity has been demonstrated to be dependent on ATP (this work). Despite these energetic considerations, the overexpression of CaiC led to the increased production of L(-)-carnitine in *E. coli*.

Finally, although CaiC was active on D(+)-carnitine and γ-butyrobetaine, this was not a sufficient condition for them to be used as substrates for the biotransformation involving growing *E. coli* LMG194 cells. In the case of γ-butyrobetaine, the irreversibility of crotonobetaine reductase activity explains why no L(-)-carnitine was produced. Moreover, the racemization of D(+)-carnitine was only possible using resting cells, as inhibition of crotonobetaine reductase activity (CaiA) under aerobic conditions has been shown to enhance this biotransformation [29]. Further experiments should be performed to optimize the biotransformation conditions.

CONCLUSIONS

A bacterial betaine:CoA ligase has been characterized for the first time. CaiC was shown to be a highly specific CoA ligase, also exhibiting *in vitro* CoA-transferase activity. However, the *in vivo* activity of CaiC was dedicated to the synthesis of betainyl-CoAs. Finally, the importance of CaiC as a bottleneck in the L(-)-carnitine biotransformation process is highlighted, while its overexpression opens up new perspectives on the application of strain development and metabolic engineering strategies to improve L(-)-carnitine production by bacteria. Moreover, the availability of free coenzyme A affects L(−)-carnitine metabolism. Not only the activation of trimethylammonium compounds, but also central metabolism, regulating the acetyl-CoA/CoA ratio, are crucial. Taken together, these data suggest the activation of trimethylammonium compounds as the main control point in the biotransformation. In addition, optimization of L(−)-carnitine production depends on the engineering of both primary and secondary metabolisms, especially in the co-regulation of acetate metabolism and the glyoxylate shunt. Further experiments are being undertaken in order to unravel the intricate regulatory network underlying these observed mechanisms.

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CHAPTER 2

Metabolic engineering for high yielding L(-)-carnitine production in Escherichia coli.

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Arense P, Bernal V, Charlier D, Luis Iborra J, Foulquie-Moreno MR, Canovas M: **Producción de L(-)-carnitina mediante cepas de *Escherichia coli* modificadas genéticamente**. Spanish patent P201230867.

ABSTRACT

L(-)-carnitine production has been widely studied because of its beneficial properties on various diseases and dysfunctions. Enterobacteria possess a specific biotransformation pathway which can be used for the enantioselective production of L(-)-carnitine. Although bioprocesses catalyzed by enzymes or whole cells can overcome the lack of enantioselectivity of chemical methods, current processes still have severe disadvantages, such as the low yields, side reactions and the need of high catalyst concentrations and anaerobic conditions for proper expression of the biotransformation pathway. Additionally, genetically engineered strains so far constructed for L(-)-carnitine production are based on plasmids and, therefore, suffer from segregational instability.

In this work, a stable, high yielding strain for L(-)-carnitine production from low cost substrates was constructed. For that aim, a metabolic engineering strategy was implemented in a multiple mutant for use in both growing and resting cells systems. The effect of mutations on gene expression and central metabolism was analyzed, in order to further characterize the productivity constraints of the wild type and the overproducer strains. Precise deletion of genes which encode proteins of central and carnitine metabolisms were performed. Specifically, flux through the TCA cycle was increased by deletion of *aceK* (which encodes a bifunctional kinase/phosphatase which inhibits isocitrate dehydrogenase activity) and the synthesis of the by-product γ-butyrobetaine was prevented by deletion of *caiA* (which encodes a crotonobetainyl-CoA reductase). Both mutations led to improve the L(-)-carnitine production by 20 and 42%, respectively. Moreover, the highly regulated promoter of the *cai* operon was substituted by a constitutive artificial promoter increasing the biotransformation rate, even under aerobic conditions. Resting cells of the BW Δ*aceK* Δ*caiA* p37*cai* strain produced 59.6 mmol $l^{-1} \cdot h^{-1}$ of L(-)-carnitine, doubling the productivity of the wild type strain. In addition, almost

total conversion was attained in less than two hours without concomitant production of the side product γ–butyrobetaine.

L(-)-carnitine production has been enhanced by strain engineering. Metabolic engineering strategies herein implemented allowed obtaining a robust and high yielding *E. coli* strain. The new overproducer strain was able to attain almost complete conversion of crotonobetaine into L(-)-carnitine with growing and resting cells, and even under aerobic conditions, overcoming the main environmental restriction to carnitine metabolism expression. So far, this is the best performing L(-)-carnitine production *E. coli* strain described.

INTRODUCTION

Worldwide, the demand of L(-)-carnitine [R(-)-3-hydroxy-4-trimethylaminobutyrate] is increasing due to its multiple applications as pharmaceutical and nutraceutical product, hence the need of developing more efficient production methods. One of the main constraints of these processes is the enantioselectivity requirement. Chemical synthesis yields a racemic mixture of D,L-carnitine, which cannot be administered to patients and has to be resolved to specifically obtain the active enantiomer [1-4]. The natural enantioselectivity of microbial and enzymatic biotransformations offers an advantage over classical chemical synthesis. Several biological processes have been developed for the production of L(-)-carnitine from non-chiral precursors [5-12] especially using strains belonging to the genera *Escherichia* and *Proteus.* At the industrial level, Lonza exploits a non-disclosed proprietary strain belonging to a genus branching between *Agrobacterium* and *Rhizobium* and close to *Rhizobium meliloti* [13].

Crotonobetaine (dehydrated D,L-carnitine) and D(+)-carnitine are by-products from the chemical L(-)-carnitine production process (Fig. 1A), which can be transformed into L(-)-carnitine. This enantioselective biotransformation has the potential to enhance the overall economic and environmental viability of the chemical synthesis process. In this respect, the L(-)-carnitine metabolism in *E. coli* has been widely studied and characterized [5, 11, 14-16] because of its role in anaerobic respiration, and in stress survival, especially in osmoprotection [17-19]. *E. coli* is able to transform crotonobetaine into L(-)-carnitine through a series of sequential steps. Substrates and products are transported by a specific membrane antiporter (CaiT) [20]. All biochemical steps occur at the level of coenzyme A thioester derivatives: activation of betaines involves a carnitine-CoA ligase (CaiC), which synthesizes the ester derivatives at the expense of ATP hydrolysis, and a crotonobetainyl-CoA:carnitine CoA-transferase (CaiB) which inexpensively exchanges the CoA moiety between betaines [16, 21-23]. The enantioselective hydration is catalyzed by a crotonobetainyl-CoA hydratase (CaiD) [16, 22]. As a side

reaction, crotonobetaine can be reduced to γ-butyrobetaine by means of a crotonobetainyl-CoA reductase (CaiA) [16, 24], a process known as carnitine respiration which is inhibited by electron acceptors such as oxygen or fumarate (Fig. 1B).

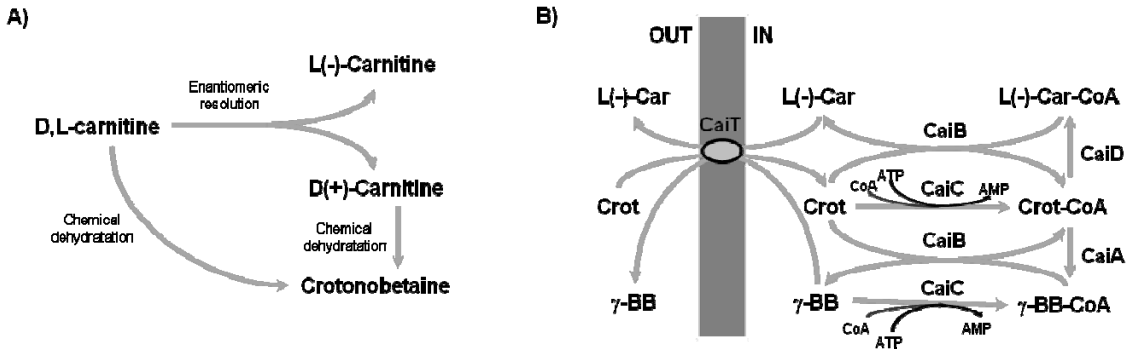

Figure 1. Synthesis of L(-)-carnitine. (A) Chemical synthesis of L(-)-carnitine and crotonobetaine. (B) Metabolism of trimethylammonium compounds in *E. coli*. Biotransformation of crotonobetaine into L(-)-carnitine. Abbreviations: L(-)-Car: L(-)-carnitine; Crot: crotonobetaine; γ-BB: γ-butyrobetaine; CaiT: L(-)-carnitine/crotonobetaine/γ-butyrobetaine protein transporter; CaiB: crotonobetainyl-CoA:L(-)-carnitine CoA-tranferase; CaiC: L(-)-carnitine, crotonobetaine or γ-butyrobetaine CoA-ligase; CaiD: crotonobetainyl-CoA hydratase. Adapted from [5].

All the activities, necessary for the metabolism of trimethylammonium compounds, are encoded by two divergent structural operons: *caiTABCDE*, which encode the carnitine biotransformation enzymes [25], and *fixABCX*, which encodes putative flavoproteins involved in anaerobic carnitine respiration [26, 27]. A specific transcriptional activator (CaiF) is expressed from an adjacent ORF located in the genome upstream the *cai* operon [28]. Both *cai* and *fix* operons are expressed from a common intergenic promoter-operator region, which is tightly regulated by cAMP-CRP, FNR, and CaiF. Expression of *caiF* is activated by cAMP-CRP and FNR, the

major regulator of gene expression under anaerobic conditions [29, 30]. So far, L(-)-carnitine production by *E. coli* is carried out in anaerobic conditions to induce the expression of the *cai* operon, as described in the current model for the regulation of carnitine metabolism [5, 11, 15]. Briefly, the transcription of *caiF* is activated in the absence of oxygen by the global regulator of the transition from aerobic to anaerobic growth, FNR, which modulates the expression of hundreds of genes [29, 31]. In the presence of trimethylammonium compounds (D,L-carnitine or crotonobetaine), the CaiF protein promotes the expression of the carnitine metabolism. Therefore, the *cai/fix* operons are not expressed in the presence of oxygen and in the absence of the proper inducers [29, 30].

With the aim of developing a combined and sustainable chemical-biotechnological process for industrial production of L(-)-carnitine, we have previously reported several strategies to enhance the biotransformation of inexpensive substrates such as crotonobetaine or D(+)-carnitine. Previous approaches focused on the optimization of biotransformation conditions using high cell concentrations in cell recycle reactors, immobilized cell systems or resting cells [6, 14, 32]. Up to 40-60% crotonobetaine conversion was obtained with the wild type, non pathogenic, *E. coli* O44K74 strain [32, 33], and 60-70% using recombinant *E. coli* strains overexpressing either the carnitine-CoA ligase or the crotonobetainyl-CoA hydratase genes (encoded by *caiC* and *caiD*, respectively) [34, 35].

The major drawbacks of previous processes are the low conversion yields and the production of the side-product γ-butyrobetaine. Although volumetric productivities were high, the conversion yields could be improved, since the presence of excess (non-transformed) substrates and by-products in the biotransformation media seriously hinders downstream processing. Moreover, the *cai/fix* operons are only expressed under anaerobic conditions, with a concomitant decreased energetic efficiency, and the need to supplement the medium with fumarate in order to inhibit the carnitine respiration pathway. In addition, using plasmid-transformed strains in

large scale productions presents several drawbacks such as the dependence on expensive synthetic inducers and antibiotics. Moreover, plasmids can be lost as a result of inefficient segregation between daughter cells and the high metabolic burden imposed by the maintenance of this extra genetic material [14, 36]. Overall, all these constraints further restrict the economics of the bioprocess, preventing its implementation in an industrial scale.

This work aims at improving L(-)-carnitine production in *E. coli* by strain optimization techniques. The strategies here implemented intend to overcome the major drawbacks previously exposed. All modifications will be performed at the chromosomal level in order to obtain genetically stable, marker-free, high-yielding strains.

MATERIAL AND METHODS

1. Strains and plasmid.

The wild type strain *E. coli* BW25113 [*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$] was obtained from the Keio collection [37]. The mutant strains constructed in the present study (Table 1) were obtained as described below. The strains were stored in 50% glycerol at -80ºC.

Standard *E. coli* cultures for molecular biology work were performed in Luria-Bertani broth (LB). Antibiotics (ampicillin 100 μg mL^{-1}, kanamycin 30 μg mL^{-1}, chloramphenicol 30 μg mL^{-1}) were added whenever necessary.

The plasmids pKD46 (Red helper plasmid, Ampicillin resistance), pKD3 (containing a FRT-flanked chloramphenicol resistance (*cat*) gene), pKD4 (containing a FRT-flanked kanamycin resistance (*kan*) gene), pCP20 (expressing FLP recombinase activity) [38, 39], and pKD-Cre (expressing Cre recombinase activity) were obtained from Prof. Dr. J-P Hernalsteens (Vrije Universiteit Brussels, Belgium). The chloramphenicol resistant (cat) gene flanked by loxP sites and the priming P1 and P2 sites was cloned into pBlueScript using *XbaI* and *BamHI* restrictions sites.

Table 1. List of bacterial strains used in this work.

Strain	References	Genotype	Short name
E.coli BW25113	Keio collection, Baba et al. [51]	lacIq rrnBT14 ΔlacZWJ16 hsdR514 ΔaraBADAH33 ΔrhaBADLD78	BW25113
E. coli BW25113 Δ*aceK*	This work	[BW25113] ΔaceK	BW Δ*aceK*
E. coli BW25113 Δ*aceA*	This work	[BW25113] ΔaceA	BW Δ*aceA*
E. coli BW25113 Δ*aceAK*	This work	[BW25113] ΔaceAK	BW Δ*aceAK*
E. coli BW25113 Δ*caiA*	This work	[BW25113] ΔcaiA	BW Δ*caiA*
E. coli BW25113 Δp*caiF caiF*-p8	This work	[BW25113] ΔpcaiF::caiF-p8	BW p8*caiF*
E. coli BW25113 Δp*cai cai*-p37	This work	[BW25113] Δpcai::cai-p37	BW p37*cai*
E. coli BW25113 ΔaceK Δp*cai cai*-p37	This work	[BW25113] ΔaceK Δpcai::cai-p37	BW Δ*aceK* p37*cai*
E. coli BW25113 Δ*aceK* Δ*caiA* Δp*cai cai*-p37	This work	[BW25113] ΔaceK ΔcaiA Δpcai::cai-p37	BW Δ*aceK* Δ*caiA* p37*cai*

2. Strain engineering: gene knock-out *and promoter* knock-in *strategies.*

Standard molecular biology protocols were used [40]. Knockout mutants were constructed by successive deletion of specifically targeted genes or regulatory regions using the method of Datsenko and Wanner [39]. Targeted sequences were PCR-amplified using specifically designed primers (see Supplementary material) and transformed into pKD46-carrying cells. Mutants were selected for either kanamycin or chloramphenicol resistance. The pCP20-encoding FLP recombinase protein or pKD-Cre-encoding Cre recombinase protein was used to excise the antibiotic-resistance cassette. For the mutation of both *cai/fix* and *caiF* promoters, the promoter knock-in method was used [41]. The specific strategy consisted in the replacement of the respective endogenous promoter sequences by synthetic promoters. Knock-in mutants were constructed from these deletion strains. Two constitutive promoters with different strength were tested for the tuning strategy: p37 (strong) and p8 (weak). These synthetic promoters have been previously described [42] (Fig. 4). The mutant strains constructed are listed in Table 1. All constructions were checked by PCR and DNA sequencing.

3. Culture conditions.

For the biotransformation of L(-)-carnitine from crotonobetaine, a pre-culture was grown at 37 ºC aerobically using LB medium, pH adjusted to 7.5 with KOH prior to autoclaving (LB). The cultures were inoculated with 3% (v/v) of an overnight grown pre-culture. Cultures were grown under both aerobic and anaerobic conditions at 37 ºC in LB medium supplemented with 50 mM crotonobetaine as substrate (LB-CB). In some cultures, fumarate 12.5 mM was added acting as electron acceptor and as inhibitor of the reaction catalyzed by the crotonobetainyl-CoA reductase (CaiA). Batch anaerobic assays were performed in 100 mL vessels with 60 mL working volume under nitrogen atmosphere and magnetic stirring. Aerobic assays were performed in 250 mL erlenmeyer flasks with 50 mL working volume in a rotary shaker (150 rpm). The experiments were performed in triplicate.

4. Resting cells.

For the resting cell assays, anaerobic cultures in LB medium with 5 mM of crotonobetaine, used as inducer of *cai* operon, were harvested at the end of the exponential growth phase, centrifuged at 16,000xg for 10 min, and washed twice with 67 mM potassium phosphate buffer, pH 7.5. Cells were resuspended in 50 mM potassium phosphate buffer, pH 7.5 with 50 mM crotonobetaine and incubated at 37ºC in erlenmeyer flasks in a rotary shaker (150 rpm). All experiments were performed at least in triplicate and under sterile conditions.

5. Analytical procedures.

Cell growth was followed by optical density (OD) at 600 nm with a spectrophotometer (Novaspec II; Pharmacia-LKB, Sweden) and converted to dry cell weight (DWC). For L(-)-carnitine and extracellular metabolite analysis, cell-free supernatant was obtained by centrifugation at 19,000xg for 10 min. L(-)-carnitine concentration was determined with an enzymatic assay [32].

γ-Butyrobetaine was determined by HPLC [32] with a Spherisorb-NH_2 column (3 µm, 4.6 x 150 mm) supplied by Waters (Barcelona, Spain). The isocratic mobile phase was acetonitrile/H_3PO_4 0.005 M pH 5.5 (65/35) at a flow rate of 1 mL min^{-1}. For the analysis of fermentation products (acetate, ethanol, formate, fumarate, pyruvate, and succinate), a cation exchange Aminex HPX-87H column supplied by BioRad Labs (Hercules, CA) was used. The isocratic mobile phase was 5 mM H_2SO_4 at a flow rate of 0.5 mL min^{-1}. A HPLC system from Shimadzu (Kyoto, Japan) was used. The effluent was monitored using diode array and refractive index detectors (Shimadzu, Kyoto, Japan).

6. RNA isolation and quantitative PCR.

RNA was isolated at mid-exponential phase, when L(-)-carnitine production rate was maximum. The cultures were pelleted by centrifugation at 15,000×*g* at 4ºC for 30 s. Total RNA was isolated by Qiagen Rneasy®Mini Kit (QIAGEN Ibérica,

Madrid, Spain). Additionally, DNaseI digestion of the isolated RNA was performed using the RNase-free DNase Set (QIAGEN Ibérica, Madrid, Spain) to avoid DNA interferences during PCR steps. RNA quality and quantity were evaluated by microfluidic capillary electrophoresis on an Agilent 2100 Bioanalyzer (Agilent Technologies, PaloAlto, CA) using Agilent RNA 6000 Pico kit. The primers used in this work were designed using the PrimerExpress® Software v3.0 (Applied Biosystems, FosterCity, CA) and ordered from Sigma–Aldrich Co (St.Louis, USA) (see Supplementary materials). The *dnaA* (encoding the multifunctional initiator of chromosome replication and transcriptional regulator) and *polA* genes (encoding the DNA polymerase I) were used as HKG. Quantitative PCR was performed in a 7300 Real-Time PCR System (Applied Biosystems,Foster City, CA) using PowerSYBR®Green PCR Master Mix (Applied Biosystems, Foster City, CA). Samples were run in triplicate. Raw data were transformed into threshold cycle (Ct) values. Relative gene expression was calculated by the comparative Ct method (ΔΔCt). Experiments were performed in triplicate.

7. Statistical analysis of data.

The statistical analyses were carried out using R (version 2.15.1). A one-way ANOVA was applied to determine the differences among different conditions and strains. A Tukey test was also carried out to ascertain the significant differences between data pairs. The threshold p-value chosen for statistical significance was $p<0.05$.

RESULTS

1. Strain engineering for L(-)-carnitine production.

On the basis of previous knowledge, four strategies were designed to enhance L(-)-carnitine production in *E. coli*, dealing with either central or secondary metabolism: (i) altering the glyoxylate shunt/TCA cycle flux ratio at the isocitrate node, (ii) avoiding the side reduction of crotonobetaine to γ-butyrobetaine (carnitine respiration pathway), and (iii) enhancing the expression of the L(-)-carnitine operon structural (*caiTABCDE*) or regulatory genes (*caiF*) to relieve repression by aerobic conditions. All modifications were performed in *E. coli* BW25113, in which L(-)-carnitine productivity is in the same order of magnitude as in the well characterized *E. coli* O44K74 strain. To determine the effect on L(-)-carnitine production, the strains were cultured anaerobically in LB-CB medium at 37ºC (Fig. 2).

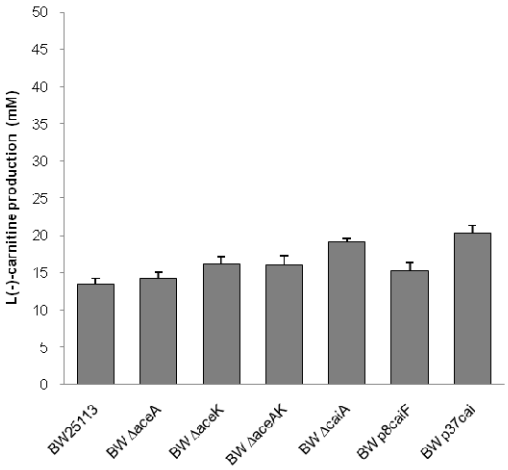

Figure 2. L(-)-carnitine production in the engineered *E. coli* strains. Experiments were performed under anaerobic conditions in LB medium supplemented with 50 mM crotonobetaine (LB-CB). L(-)-carnitine titers reached a maximum after 12 h (upon entrance to stationary phase).

First, as regards the modification of central metabolism, the genes encoding isocitrate lyase (*aceA*) and isocitrate dehydrogenase phosphatase/kinase (*aceK*) were deleted. The *aceK* knockout strain (devoid of post-translational control of isocitrate dehydrogenase) showed an improvement in the production of L(-)-carnitine (20%), while deletion of *aceA* (encoding the first enzyme of the glyoxylate shunt) only had a slight effect (6%). No further improvement was observed in the *aceAK* double mutant, indicating that deregulation of the TCA cycle is more relevant than lowering the glyoxylate shunt flux.

Second, to avoid the reduction of crotonobetainyl-CoA into γ-butyrobetainyl-CoA (Fig. 1), the *caiA* gene (encoding the crotonobetainyl-CoA reductase) was deleted. This modification led to an improvement in L(-)-carnitine production of 42% (Fig. 2). In fact, the side-reaction was effectively suppressed since no γ-butyrobetaine was detected in the supernatant of any of the Δ*caiA* strains assayed (results not shown).

Furthemore, the expression of the carnitine metabolism structural (*cai* operon) and regulatory (*caiF*) genes was tuned using artificial promoters, in order to ensure a constitutive expression of the carnitine metabolism and relieve the repression under aerobic conditions. When the endogenous promoters were replaced by constitutive artificial promoters, L(-)-carnitine production increased 51% and 20% in the BW p37*cai* and BW p8*caiF* strains, respectively (Fig. 2).

To further study these strains with single modifications, growth and L(-)-carnitine production rates were determined in anaerobic cultures in LB-CB medium in the absence (Fig. 3A) and presence of fumarate (Fig. 3B) (used as an alternative electron acceptor [24, 33]). In the absence of fumarate, statistically significant differences in the specific L(-)-carnitine production rates were observed between the assayed strains. The highest L(-)-carnitine titers were achieved by the BW p37*cai* and BW Δ*caiA* strains (21.7 and 20.7 mM respectively) (Fig. 3A). On the other hand, when fumarate was added, BW Δ*caiA* reached the highest production,

28.7 mM ($p<0.001$) (Fig. 3B) and a similar 20-30% increase in the specific growth rate was observed for all the strains with single modifications.

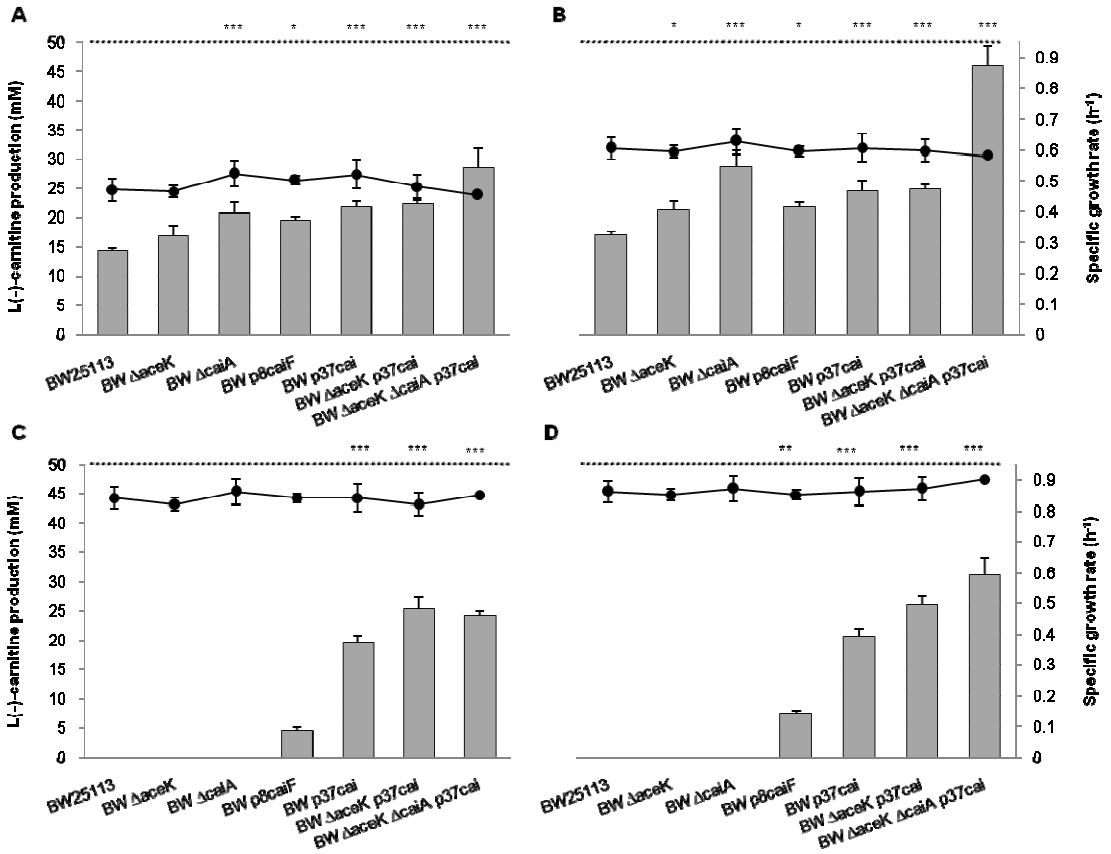

Figure 3. Effect of the mutations of central and secondary metabolism on L(-)-carnitine production. Experiments were performed in LB medium supplemented with 50 mM crotonobetaine (LB-CB) in the absence and presence of fumarate and under aerobic and anaerobic conditions. Anaerobic cultures: (A) LB-CB medium and (B) LB-CB medium supplemented with 12.5 mM fumarate. Aerobic cultures: (C) LB-CB medium and (D) LB-CB medium supplemented with 12.5 mM fumarate. Bars represent the L(-)-carnitine yield after 24 h and dots represent the specific growth rate. Discontinuous line indicates the maximum production (corresponding to 50 mM L(-)-carnitine). Adjusted p-values for ANOVA ($p<0.001$) and Tukey test of L(-)-carnitine production, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

These results allowed us to select the mutations that positively affected L(-)-carnitine production. To further enhance productivity, all modifications, namely, deletion of *aceK* and *caiA* and replacement of the *cai* promoter, were implemented in the same strain. Given its close proximity to the *cai* operon promoter, the *caiA* gene was deleted combining FRT and loxP sites in order to avoid the deletion of the contiguous *caiT* gene (Fig. 4A). As expected, the mutations did not affect growth significantly.

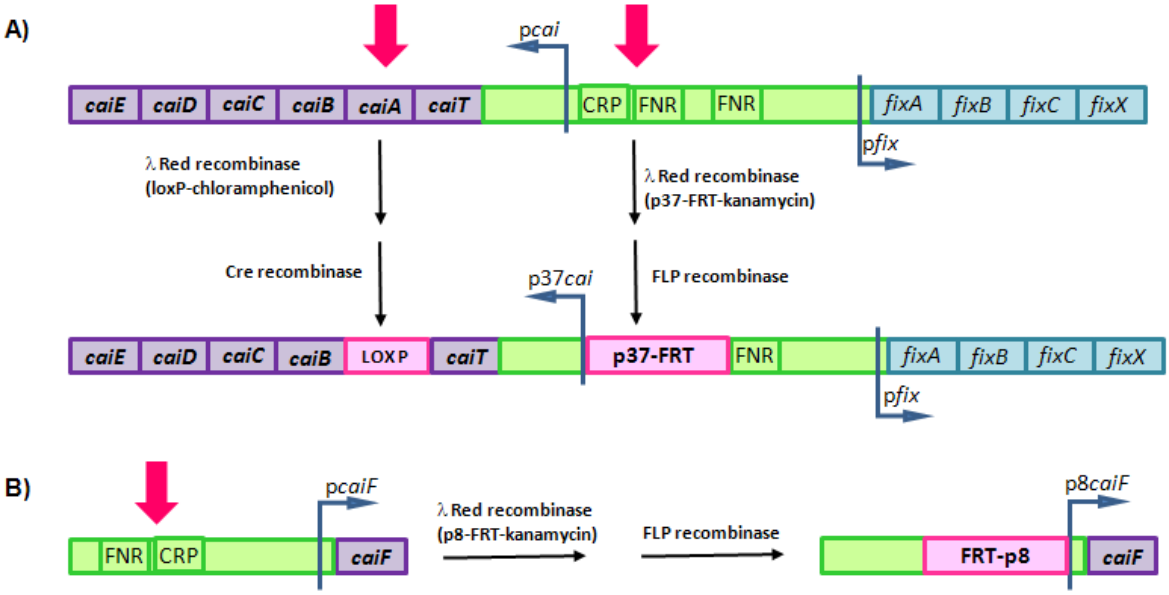

Figure. 4. Strain engineering strategy followed to improve L(-)-carnitine production in *E. coli* BW25113. (A) Deletion of the *caiA* gene and replacement of the endogenous *cai* operon promoter and regulatory sequences (FNR and CRP binding sites) by the p37 artificial promoter. (B) Replacement of the endogenous *caiF* promoter and a close regulatory region (FNR and CRP binding sites) by the p8 artificial promoter.

Nevertheless, the specific L(-)-carnitine production rate was higher than in the single mutants. In order to check the effect of the mutations in the BW Δ*aceK* Δ*caiA* p37*cai* strain compared to the wild type, expression of genes belonging to the *cai* operon was analyzed by qRT-PCR. Upstream (*caiT*) and downstream (*caiB* and *caiC*) genes of the deleted *caiA* were analyzed in order to assess whether a polar effect appeared due to this deletion. As expected, the constitutive promoter increased the expression of the carnitine metabolism genes. However, *caiB* and *caiC* exhibited a lower level of expression in the Δ*caiA* strains (Table 2), indicating that the *caiA* deletion exerts a polar effect. In addition, the p37 promoter also led to higher expression of the divergent *fix* operon (Table 2). This finding is not surprising, since it is known that the *cai* and *fix* operons are expressed from a common intergenic control region [29].

Table 2. Relative gene expression in the engineered strains growing anaerobically on LB-CB medium supplemented with 12.5 mM fumarate. For each gene, the transcription level of that gene in the wild type strain was used as reference to normalize the data. Relative gene expression in the control strain is, therefore, taken as 1. The results are the averages of three independent measurements of each gene/condition in three independent experiments.

Strains	*caiT*	*caiB*	*caiC*	*fixA*
BW Δ*caiA*	1.443 ± 0.20	0.86 ± 0.21	0.81 ± 0.06	1.32 ± 0.19
BW Δ*aceK* p37*cai*	3.00 ± 0.16	3.08 ± 0.24	2.18 ± 0.20	1.95 ± 0.17
BW Δ*aceK* Δ*caiA* p37*cai*	3.23 ± 0.17	1.76 ± 0.10	1.47 ± 0.12	1.84± 0.10

Furthermore, the single, double (BW Δ*aceK* p37*cai*), and triple (BW Δ*aceK* Δ*caiA* p37*cai*) mutants and the wild type strain were grown in the absence (Fig. 3A) and presence of fumarate (Fig. 3B) under anaerobic conditions in LB-CB medium (Fig. 3A, B). The L(-)-carnitine yield was used to compare their abilities for the biotransformation of crotonobetaine into L(-)-carnitine. The combination of all three

mutations contributed to the highest increase of specific L(-)-carnitine production rate (which doubled in both absence and presence of fumarate) and yield (reaching 70% and 92% of conversion, respectively). The highest titer obtained was 46 mM L(-)-carnitine with the BW Δ*aceK* Δ*caiA* p37*cai* strain (Table 2, Fig. 3B).

2. Biotransformation under aerobic conditions.

As described before, it might be highly desirable to produce L(-)-carnitine under aerobic conditions with engineered strains that constitutively overexpressed the *caiF* gene and the *cai* operon. Tuning gene expression with artificial oxygen-independent promoters should allow to reach this goal and to overcome the limitations exhibited by the wild type strain [41, 42].

Indeed, the engineered strains produced L(-)-carnitine under aerobic conditions when either the endogenous promoters p*caiF* or p*cai* were replaced by the constitutive promoters p8 or p37, respectively. As expected, the wild type, BW Δ*aceK,* and BW Δ*caiA* strains were not able to produce L(-)-carnitine (Fig. 3C, D).

The presence or absence of fumarate did not affect the maximum specific growth rate, being 0.84 h^{-1} for cultures without fumarate and 0.86 h^{-1} for cultures supplemented with fumarate (Fig. 3C and D, respectively). Moreover, the L(-)-carnitine yield was higher in the strains that harbored the p37-promoter upstream the *cai* operon. Nevertheless, the activity of the promoter p8 was sufficient to activate the expression of carnitine metabolism and to produce L(-)-carnitine aerobically in the BW p8*caiF* strain. On the other hand, the addition of fumarate to cultures enhanced L(-)-carnitine production as occurred in anaerobic cultures.

In spite of the fact that the strain with the highest specific L(-)-carnitine production rate was BW Δ*aceK* p37*cai*, the strain BW Δ*aceK* Δ*caiA* p37*cai* showed the highest L(-)-carnitine yield when reached the stationary phase in the presence of

fumarate ($p<0.001$), obtaining 31.2 mM L(-)-carnitine. For p37-mutants, the biotransformation yield ranged between 40-60%.

Altogether, the modifications performed on *E. coli* BW25113 allowed aerobic L(-)-carnitine production, although titers obtained were lower than under anaerobic conditions (Fig. 3).

3. Biotransformation with resting cells.

Further experiments were carried out to test the biotransformation capacity of resting cells. Resting cells assays with the wild type and BW Δ*aceK* Δ*caiA* p37*cai* (the best performing strain in growth assays) were performed.

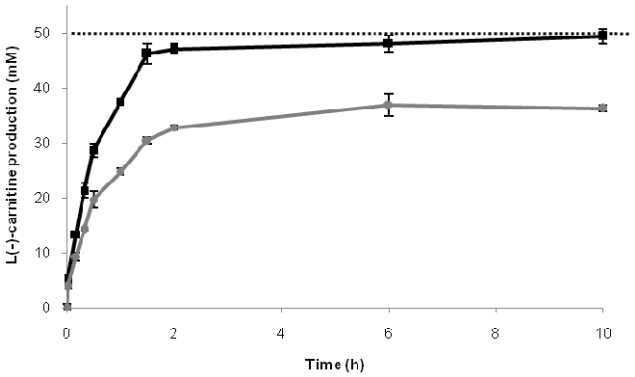

Figure. 5. L(-)-carnitine production by resting cells. The performance of the BW25113 (wild type) (grey) and BW25113 Δ*aceK* Δ*caiA* p37*cai* (black) strains is compared. Discontinuous line indicates the maximum production (corresponding to 50 mM L(-)-carnitine). Resting cell experiments were performed in phosphate buffered 50 mM crotonobetaine, as explained in the Methods section.

Resting cells conditions enhanced L(-)-carnitine production of both strains. Moreover, the productivities were 28.5 and 59.6 mmol $l^{-1} \cdot h^{-1}$ for the wild type and the BW Δ*aceK* Δ*caiA* p37*cai* mutant, respectively. In addition, the mutant strain was able to reach almost 100% conversion in less than two hours (Fig. 5), which is the highest conversion ever reported for L(-)-carnitine producing *E. coli* strains.

4. Effect of mutations on control points of the central metabolism.

The wild type and the BW Δ*aceK* Δ*caiA* p37*cai* mutant were selected to study the changes imposed by the genetic modifications on central metabolism. To this end, seven metabolites were analyzed: succinate, pyruvate, fumarate, lactate, acetate, ethanol, and formate. Pyruvate, lactate, and ethanol did not show any important change and only low concentrations or traces were detected under the culture conditions assayed. Therefore, the metabolic study was focused on acetate, which is the main metabolic product of *E. coli* and can be considered as readout of the energetic state of the cells, and on succinate, which is a product of the mixed acid fermentation pathway of *E. coli* and also results from fumarate respiration (Table 3).

In anaerobic cultures supplemented with fumarate, the wild type and the mutant strain exhibited a similar behavior. The maximum concentration of succinate and acetate coincided with the end of the exponential phase, when the culture broth was completely depleted of fumarate (12.2 and 13.9 mM succinate, and 10.2 and 16.4 mM acetate for the wild type and the overproducer strain, respectively). Acetate decreased slightly during the stationary phase. The results obtained in the assays without fumarate were similar to those observed before, being the concentration reached for every metabolite lower. Formate was only detected in the exponential phase, increasing steeply during the first ten hours of culture, especially in the mutant strain. It was not detected in the stationary phase of cultures, which indicates the activity of the formate hydrogen-lyase (Fhl) enzyme under these conditions. Thus, the central metabolism of *E. coli* strains was focused on maintaining suitable levels

of ATP and free coenzyme A, which are a limiting factor in the biotransformation process.

On the other hand, the strains grown aerobically showed similar patterns in the metabolite profiles, although differences were found in the levels reached in the stationary phase. At the beginning of the stationary phase, succinate and acetate achieved their maximum concentration (8.3 and 18.1 mM, respectively, for the mutant; 7.4 and 12.5 mM, respectively, for the control strain), emphasizing the importance of the acetate metabolism. Moreover, acetate decreased drastically in the later stationary phase, indicating the activity of the acetate scavenging systems [43-45]. The acetate production flux highlights the need of maintaining the acetyl-CoA/CoASH ratio depending on cellular demand. Without fumarate, the production of acetate showed a pattern similar to the one described above, but its concentration was half that in the presence of fumarate.

Table 3. Metabolic performance of wild-type (BW25113) and engineered (BW Δ*aceK* Δ*caiA* p37*cai*) *E. coli* strains during L(-)-carnitine production in anaerobic and aerobic conditions. Specific production/consumption rates of L(-)-carnitine and the main extracellular metabolites were calculated during the early exponential phase of cultures. All rates are expressed in mmol g^{-1} h^{-1}. Pairwi€se statistical comparison of the parameters assessed for both strains was perfomed with ANOVA and Tukey tests. Adjusted p-values are indicated as follows: *p<0.05, **p<0.01, ***p<0.001..

Strain and conditions	$q_{L\text{-}car}$	q_{Suc}	$-q_{Fum}$	q_{Acet}	q_{EtOH}	q_{Form}
Anaerobic cultures						
LB-CB						
BW25113	9.30 ± 0.12	8.43 ± 0.22	---	15.71 ± 0.28	7.23 ± 0.20	4.21 ± 0.14
BW Δ*aceK* Δ*caiA* p37*cai*	18.87 ± 0.23***	7.20 ± 0.17***	---	17.44 ± 0.16***	2.49 ± 0.12***	12.96 ± 0.42***
LB-CB + fumarate (12.5 mM)						
BW25113	14.23 ± 0.15	13.89 ± 0.54	17.74 ± 0.26	13.15 ± 0.36	4.8 ± 0.32	N.D.
BW Δ*aceK* Δ*caiA* p37*cai*	27.75 ± 0.17***	10.31 ± 0.32***	19.51 ± 0.41*	16.44 ± 0.21***	3.95 ± 0.27***	N.D.
Aerobic cultures						
LB-CB						
BW25113	---	N.D.	---	9.49 ± 0.23	0.43 ± 0.05	---
BW Δ*aceK* Δ*caiA* p37*cai*	6.15 ± 0.10***	N.D.	---	7.79 ± 0.12***	1.17 ± 0.08*	---
LB-CB + fumarate (12.5 mM)						
BW25113	---	3.32 ± 0.11	9.42 ± 0.24	5.74 ± 0.10	0.15 ± 0.02	---
BW Δ*aceK* Δ*caiA* p37*cai*	7.02 ± 0.07***	2.95 ± 0.08	6.82 ± 0.17***	8.31 ± 0.14***	1.26 ± 0.06***	---
ANOVA	1.30E-20***	1.70E-25***	8.56E-21***	3.68E-18***	3.40E-23***	1.38E-29***

DISCUSSION

This work demonstrates the successful construction of *E. coli* strains engineered for high yield production of L(-)-carnitine. For the first time, almost the highest theoretical yield of L(-)-carnitine production is shown using both growing and resting cells systems. Biotransformation yields ranging from 40 to 95% were obtained in the growing cells system, while conversion was almost complete using resting cells. The strain optimization strategy presented here intended to overcome the major drawbacks previously identified. The modifications introduced had a cumulative effect on strain performance, improving yield and productivity without affecting growth or physiology of the bacteria. In fact, the best results were obtained with the strain carrying the combination of several modifications (BW Δ*aceK* Δ*caiA* p37*cai*), which had a growth profile similar to that of the control strain.

Previous works demonstrated that the optimization of the biotransformation process depends on the engineering of both primary and secondary metabolisms and its regulation [15, 18, 34, 46, 47]. Knockout mutants on the glyoxylate shunt genes *aceK* and *aceA* showed an increase in L(-)-carnitine production [34], underlining the importance of the TCA cycle flux for L(-)-carnitine production. However, its impact on the productivity was small when compared to the overexpression of enzymes performing the biotransformation such as CaiC and CaiB [21, 34, 35] or the whole *cai* operon (this work).

The replacement of the promoter of the *caiF* gene with the constitutive p8 promoter enhanced L(-)-carnitine production under aerobic and anaerobic conditions. CaiF acts as a specific regulatory protein for the activation of carnitine metabolism binding to two 11-bp inverted repeat half-sites separated by 13 bp in the intergenic region of the two operons *caiTABCDE* and *fixABCX*. The expression of *caiF* is activated by cAMP-CRP and FNR (the main transcriptional regulator under anaerobic conditions). Therefore, the expression of the trimethylammonium compounds metabolism is prevented in the presence of oxygen and/or glucose [28-

30]. Transcriptional repression was overcome after promoter replacement in BW p8*caiF*, although this was not sufficient for optimal performance of carnitine metabolism, especially under aerobic conditions. Various reasons can respond for this observed effect. For instance, although the FNR binding site located at -55.5 bp in the *caiF* promoter was replaced, the presence of further putative half sites for FNR binding is known, whose role in *caiF* expression has not been studied. In addition, the *cai* promoter is also regulated by FNR (two sites at -90.5 and -41.5 bp, respectively) and ArcA (four sites located at -101, -91, +28, and +50 bp) [48], and CaiF overexpression could not be enough for optimal expression under aerobic conditions.

The most remarkable improvement was obtained by tuning the expression of the *cai* operon and deleting *caiA* gene. Replacement of the endogenous promoter by the constitutive promoter p37 enhanced L(-)-carnitine production under anaerobic and aerobic conditions, relieving the *cai* operon from the regulatory effect of oxygen and CaiF. Importantly, the formation of the side-product γ-butyrobetaine was effectively avoided by deleting *caiA*, redirecting crotonobetaine towards L(-)-carnitine production [16, 24]. This deletion led to a 25-60% enhancement in L(-)-carnitine production in the BW Δ*caiA* strain and a 30-87% enhancement in the BW Δ*aceK* Δ*caiA* p37*cai* strain. This is the best performing strain obtained, achieving over 95% conversion of the substrate in a growing system, and almost 100% of conversion in a resting cells system.

When the effect of the metabolic engineering strategy on the physiology of the bacteria was assessed, several metabolic changes between the wild type and the BW Δ*aceK* Δ*caiA* p37*cai* strain were observed. The main observation under the anaerobic biotransformation conditions was that energy producing processes such as fumarate respiration [49, 50] and acetate and formate production [43, 45, 51-54] were enhanced in the mutant. This supports that L(-)-carnitine production is highly dependent on the energetic state of cells [15]. Interestingly, although fumarate was originally used as electron acceptor and inhibitor of the crotonobetainyl-CoA

reductase activity (CaiA) [15, 24, 33], media supplementation with this energetic substrate enhanced growth and L(-)-carnitine production, even in Δ*caiA* strains. Therefore, ATP generation through the reduction of fumarate, similarly to aerobic oxidative phosphorylation, is a major mechanism of energy generation in anaerobic conditions [15, 47, 50].

Moreover, increased production of acetate (the end-product of the major energy producing anaerobic pathway of *E. coli*) and formate (the end product of pyruvate-formate lyase) was not observed in aerobic cultures, in which energy is mostly produced by respiration and acetate production occurs exclusively as a result of an overflow metabolism [43-45].

It is important to emphasize that the engineered strains created in this work were able to carry out the biotransformation under aerobic conditions, while no L(-)-carnitine was produced by wild type *E. coli*. The best performing strain was BW Δ*aceK* Δ*caiA* p37*cai*, with 65% of conversion. Moreover, the L(-)-carnitine production yield was 4-fold higher than that reported in a previous work under aerobic conditions [33, 34]. The high growth rate and the low biotransformation rate of the BW Δ*aceK* Δ*caiA* p37*cai* strain avoided that total conversion of crotonobetaine under aerobic conditions, since stationary phase of cultures was reached before conversion was complete. This suggests that other limitations in central metabolism may occur. A plausible explanation for the observed differences between both conditions could be the availability of coenzyme A, as shown in previous works [15, 34, 47]. Probably, the higher activity of the central metabolism and the consequent demand of acetyl-CoA to be used in different pathways increased, thus reducing the level of free coenzyme A.

Summarizing, we have successfully engineered *E. coli* for efficient, high-yielding L(-)-carnitine production from an inexpensive substrate (such as crotonobetaine). A stable engineered strain was obtained, which does not depend on the use of expensive inducers (since the p37 and p8 promoters used are constitutive)

or antibiotics (since all modifications are chromosomal, stable and antibiotic marker-free). In addition, fast transformation was attained almost quantitatively, therefore with improved downstream processing. Exploitation of this biotransformation metabolism using an engineered *E. coli* strain in high-density reactors is a feasible and economically viable strategy for the implementation of L(-)-carnitine production processes at the industrial scale.

CONCLUSIONS

L(-)-carnitine production in *E. coli* based growing and resting cells systems has been successfully improved. Multiple stable mutations introduced in a single strain enhanced production without reducing cell viability or affecting specific growth. Furthermore, the biotechnological process was improved and allowed nearly 100% conversion in a short period of time, thereby, reducing time of transformation and simplifying downstream processing. Moreover, the main restriction to aerobic expression of the carnitine metabolism was eliminated. Therefore, this study presents a successful strain improvement strategy by means of gene deletion and promoter replacement and contributes to get further insights into the secondary metabolism of trimethylammonium compounds in *E. coli*.

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CHAPTER 3

Metabolic adaptation of Escherichia coli to long-term exposure to salt stress.

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ABSTRACT

The aim of this work was to understand the relevance of central carbon metabolism in salt stress adaptation of *Escherichia coli.* The cells were grown on anaerobically in batch and continuous reactors at different NaCl concentrations using glycerol as a carbon source. Enzyme activities of the main metabolic pathways, external metabolites, ATP level, L(-)-carnitine production and the expression level of the main genes related to stress response were used to characterize the metabolic state under the osmotic stress. The results provided the first experimental evidence of the important role played by central metabolism adaptation and cell survival after long-term exposure to salts tress. Increased glycolytic fluxes and higher production of fermentation products indicated the importance of energy metabolism. Carbon fluxes under stress conditions were controlled by the decrease in the isocitrate dehydrogenase/isocitrate lyase ratio and the phosphoenolpyruvate carboxykinase/phosphoenolpyruvate carboxylase ratio, and the increase in the phosphotransferase/acetyl-CoA synthetase ratio. Altogether, the results demonstrate that, under salt stress, *E. coli* enhances energy production by substrate-level phosphorylation (Pta–Ack pathway) and the anaplerotic function of the TCA cycle, in order to provide precursors for biosynthesis. The results are discussed in relation with the general stress response and metabolic adaptation of *E. coli*.

INTRODUCTION

Single microorganisms can adapt in a reversible regulatory process to several environmental and bioprocess-related stresses [1]. In *Escherichia coli*, when the osmolarity of the culture medium increases, osmoadaptation occurs [2]. To avoid dehydration, the osmotic pressure inside the cells increases [3], certain solutes being accumulated to high concentrations, either by uptake from the medium or de novo synthesis [4, 5]. These osmoregulatory solutes include K+, aminoacids (e.g. glutamate, proline), polyalcohols (e.g. trehalose, glycerol) and other zwitterionic organic solutes (e.g. betaines such as glycinebetaine and carnitine) [5, 6]. The global effects of the stress response have been analyzed by using DNA microarray technology or proteomics [7-9]. Most of these works focused on the short-term responses to moderate salt concentrations (0.3–0.4 M) under aerobic conditions, however, osmoadaptation is a time-dependent process which is fundamentally different under aerobic or anaerobic conditions [8].

Despite the research efforts devoted to unraveling the responses displayed upon exposure to osmotic stress [10-12], the mechanisms through which the central and energy metabolism of bacteria adapt to such conditions remain largely unstudied. In fact, the rearrangement of energy metabolism in response to high osmolarity reflects the higher energy needs [13] and the mechanisms of adaptation to this stressful environment. The protective role of carnitine (trimethylammonium compound) against osmotic up-shocks in *E. coli* has been extensively analyzed [14-17]. Further, trimethylammonium compounds are substrates of an anaerobic secondary metabolic pathway that has been used for L(-)-carnitine production using *E. coli* strains [14, 18-20]. Salt stress enhances the uptake and biotransformation of trimethylammonium compounds in resting cells [7, 21]. Experimental evidence on the important role played by salt stress on the energetic state and the main metabolic pathways of cells during the biotransformation was provided. In fact, the short-term (up to 120 min) effects of salt stress perturbations underlined the important role of the glyoxylate shunt, the TCA cycle and the acetate pathways. In addition, studies

with wild type and engineered strains confirmed that the bioprocess is controlled by the availability of ATP and acetyl-CoA/CoA [19, 22].

To optimize this bioprocess, it is necessary to understand the pathways involved in the biotransformation in the presence or absence of NaCl, since substrates (crotonobetaine and D(+)-carnitine) and product (L(-)-carnitine) of the biotransformation (Fig. 1) are osmoprotectants. The lack of available information is especially relevant concerning the long-term effects on the central metabolism of salt stress exposure; a deeper insight into metabolic adaptations under osmotic stress becomes necessary for strain improvement.

The aim of this work is to contribute to the understanding of the interaction of the osmoprotection mechanisms with primary and secondary (focusing on L(-)-carnitine production) metabolisms after long-term exposure to salt stress. In order to shed light on the mechanisms of metabolic adaptation of cells, the response was studied at four salt concentrations (NaCl molarity: 0.085-control, 0.3-medium, 0.5-high and 0.8-veryhigh) in a chemostat. Cellular metabolism was characterized by following the production of L(-)-carnitine and fermentation products, the ATP level, selected enzyme activities (TCA cycle, glyoxylate shunt, anaerobic metabolism, glycolysis, anaplerosis and oxaloacetate regeneration) and the expression of genes related to the osmoadaptative response. The results allowed gaining an insight into the metabolism of *E. coli* and the general stress response and adaptation of in the presence of salt.

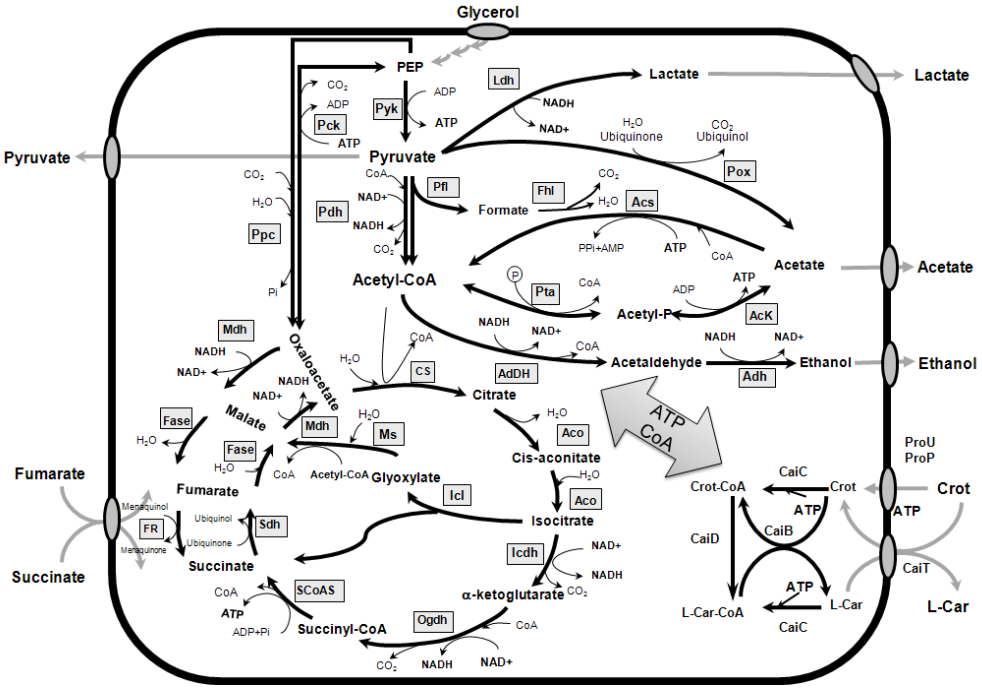

Figure 1. Central and carnitine metabolisms of *E. coli*. Both metabolisms are linked at the level of cellular cofactors, mainly ATP and coenzyme-A and its derivatives [19, 23]. AcK: Acetate kinase, Acs: Acetyl-CoA synthetase, CS: Citrate syntase; Icdh: Isocitrate dehydrogenase, Icl: Isocitrate lyase, Ldh: Lactate dehydrogenase, PEP: Phosphoenolpyruvate, Pck: PEP carboxykinase, Pcc: PEP carboxylase, Pfl: Pyruvate formate lyase: PyK: Pyruvate kinase and Pta: Phosphotransacetylase, Pdh: Pyruvate dehydrogenase, Fase: Fumarase, Mdh: Malate dehydrogenase, MS: Malate synthase, Aco: Aconitase, FR: Fumarate reductase, Sdh: Succinate dehydrogenase, SCoAS: Succinyl-CoA synthetase, Ogdh: Oxoglutarate dehydrogenase (EcoCyc-MetaCyc, [24]). Anaerobic metabolism of L(-)-carnitine in *E. coli*. CaiT: L(-)-carnitine/γ-butyrobetaine/crotonobetaine protein transporter, CaiB: acetyl-CoA/CoA transferase, CaiC: crotonobetaine/L(-)-carnitine/γ-butyrobetaine CoA ligase, CaiD: enoyl-CoA hydratase, Crot: crotonobetaine, L-car: L-carnitine, Crot-CoA: crotonobetainyl-CoA, L-Car-CoA: L-carnitinyl-CoA, ProU and ProP: non-reversible trimetylammonium compounds carrier induced by osmotic stress.

MATERIAL AND METHODS

1. Bacterial strain and culture media.

The overproducer strain *E. coli* O44K74 (DSM8828), which contains the complete cai and fix operons, was used. The strain was stored in 20 % glycerol at −80 ◦C. All experiments were performed using glycerol as carbon source and crotonobetaine as substrate of the biotransformation and inducer of carnitine metabolism [14, 19, 20]. The standard complex medium (CM) used contained (g/L): bacteriological peptone, 20; NaCl, 5; glycerol (carbonsource), 12.6; crotonobetaine, 4; and fumarate, 2. The NaCl concentration was altered as stated in the text. The pH of the media was adjusted to 7.5 with 1 M KOH prior to autoclaving.

2. Batch and continuous reactor operation

2.1. Growth of the bacteria.

The different *E. coli* strains were grown under the conditions stated in the text. The cultures were inoculated with a 3% (v/v) pre-culture, using the complex medium. The cells were grown at 37 ◦C, under anaerobiosis.

2.2. Chemostat reactor operation.

Experiments were performed in BiostatB (Braun Biotech International GMBH, Melsungen, Germany) reactors equipped with temperature, pH and oxygen probes, using pump controls for continuous operation. A 2 L culture vessel with 1.8–2.0 L working volume was used. Strict anaerobiosis was maintained to avoid repression of the carnitine metabolism [14] by bubbling nitrogen. The culture was inoculated as explained and continuous operation was started by feeding with the complex medium at a dilution rate of 0.1 h^{-1}. At the steady state, 0.5 g/L biomass dry weight was reached. Then, the feeding was switched to another medium containing the new NaCl concentration (0.3, 0.5 and 0.8M, respectively). Samples for metabolites and enzyme activities were withdrawn from the reactor during the long-

term experiments. Samples were immediately centrifuged at 16,000×g. The rotor was precooled at −20 ◦C. Supernatants were used to determine external metabolites, whereas pellets were used for enzyme activities and ATP content.

3. Enzyme assays.

For enzyme activity assays, reactor bulk liquid samples were withdrawn and centrifuged at 16,000×g at 4 ◦C. The supernatant was removed and cells were resuspended in the extraction buffer (65 mM potassium phosphate, pH 7.5). Cells were sonicated on ice for 6 cycles (10 s each), at 10 μm amplitude. The extract was centrifuged for 15 min at 16,000×g and 4 ◦C to remove cell debris and the supernatant was used for subsequent activity measurements. Protein content was determined by the method of Lowry et al. [25]. The enzyme activities were determined spectrophotometrically in a Synergy TM HT Multi-Mode Microplate Reader (Bio-Tek,Winooski, VT) with 200 μL total reaction volume. Enzyme activity was defined as μmol of substrate consumed per minute and mg of protein (U/mg). All the enzyme activity assays were taken from previous literature and optimized for the conditions and media used in this study. Isocitrate dehydrogenase (Icdh) [26], isocitrate lyase (Icl) [27], acetyl-CoA synthetase (Acs), phosphotransacetylase (Pta) [28], pyruvate dehydrogenase complex (Pdh) [29], malate dehydrogenase (Mdh) [30], pyruvate oxydase (Pox) [31], pyruvate kinase (Pyk), phosphoenolpyruvate carboxykinase (Pck), phosphoenolpyruvate carboxylase (Ppc) [32], were measured. See Supplementary Material for details.

4. Substrate consumption for growth and biotransformation processes.

L(-)-Carnitine concentration was determined enzymatically with the carnitine acetyl transferase method [33]. Bacterial growth was followed spectrophotometrically at 600 nm, using a Novaspec II from Pharmacia-LKB, (Uppsala, Sweden), and converted to dry weight accordingly.

5. Metabolite analysis.

The glycerol, acetate, fumarate, lactate, ethanol, pyruvate and succinate in the reactor bulk were determined by HPLC. A cation exchange Aminex HPX-87H column, supplied by BioRad Labs (Hercules, USA) was used. The isocratic mobile phase was H_2SO_4 5 mM at a flow rate of 0.5 mL/min and 42 ◦C. The effluent was monitored using a refractive index detector.

6. Determination of metabolic culture parameters.

In the batch cultures, the specific consumption/production rates were calculated in the mid-exponential phase of growth, using the following equation:

$$q_A = \pm \frac{1}{X} \cdot \frac{d[A]}{dt} \tag{1}$$

where qA represents the specific rate of substrate consumption or product formation, X is the amount of cells in the interval of time considered, and d[A]/dt is the slope of the representation of [A] vs. time. Negative values indicate consumption. In the chemostat reactors, metabolite specific production rates were calculated according to:

$$q_A = \frac{[A]_{ss}}{X_{ss}} \cdot D \tag{2}$$

and for substrates (glycerol, fumarate) specific consumption rates:

$$q_S = \frac{[S]_0 - [S]_{ss}}{X_{ss}} \cdot D \tag{3}$$

where [A] and [S] are the concentrations of metabolites/substrates (in mmol L^{-1}), X is the concentration of cells (in gDW L^{-1}) and D is the dilution rate at which the reactor is operated (in h^{-1}). The subscripts 0 and ss refer to the initial and steady state concentrations, respectively. Negative values indicate consumption.

7. Determination of ATP content.

The energy content per unit of cell (ATP level) and the NADH/NAD^+ ratio were determined throughout the experiments. Measurements were performed in a microplate spectrophotometer Synergy-HT (Bio-Tek,USA). For ATP determination, the HSII bioluminescence assay kit from Boëhringer (Mannhein, Germany) was used in the conditions stated by the manufacturer, and after cell lysis with DMSO. The cell content was determined after biomass optical density transformation to dry weight.

8. RNA isolation and RT-PCR.

RNA was isolated at mid-exponential phase. The cultures were pelleted by centrifugation at 15,000×g at 4 ◦C for 30 s. Total RNA was isolated by Qiagen Rneasy® Mini Kit (QIAGEN Ibérica, Madrid, Spain). Additionally, Dnase I digestion of the isolated RNA was performed using the Rnase-Free Dnase Set (QIAGEN Ibérica, Madrid, Spain) to avoid DNA interferences during PCR steps. RNA quality and quantity were evaluated by micro fluidic capillary electrophoresis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) using Agilent RNA 6000 Pico kit. The primers used in this work were designed using the Primer Express® Software v 3.0 (Applied Biosystems, FosterCity, CA) and ordered from Sigma–Aldrich Co (St.Louis, USA). The dnaA and rrsA genes (encoding transcriptional dual regulator and 16S ribosomal RNA, respectively) were used as internal control for relative quantification. Quantitative PCR was performed in a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) using Power SYBR® Green PCR Master Mix (Applied Biosystems,Foster City, CA). PCRs were

run in triplicate. Raw data were transformed in to threshold cycle (Ct) values. Relative gene expression was calculated by the comparative Ct method (ΔCt). Experiments were performed in triplicate.

RESULTS AND DISCUSSION

1. Salt adaptation of the metabolism of E. coli in batch cultures.

The wild type *E. coli* O44K74 strain was grown in batch in the presence of three different NaCl concentrations (0.085 (control medium), 0.5 and 0.8 M NaCl); growth and production of external metabolites were determined and several alterations were observed.

Table 1. Comparison of metabolic performance of parent and adapted *E. coli* O44K74 in batch culture system. Specific rates of glycerol and fumarate consumption and succinate, ethanol and acetate production are expressed in mmol·(g_{DW}·h)$^{-1}$. The study was performed as explained in Materials and Methods.

NaCl (M)	***E. coli* O44K74 (control)**				
	Succinate*	**Fumarate***	**Ethanol***	**Acetate***	**Glycerol***
0.085(Control)	21.96±0.12	12.81±0.23	1.78±0.10	12.77±0.19	6.46±0.10
0.5	19.25±0.15	21.11±0.25	1.90±0.12	8.94±0.15	8.35±0.28
0.8	14.76±0.11	15.40±0.15	5.25±0.10	5.25±0.28	3.80±0.05

NaCl (M)	***E. coli* O44K74AS (adapted)**				
	Succinate*	**Fumarate***	**Ethanol***	**Acetate***	**Glycerol***
0.085(Control)	23.66±0.35	17.25±0.65	2.56±0.10	13.07±0.12	10.40±0.72
0.5	23.90±0.72	24.98±1.55	2.29±0.22	12.04±0.76	16.76±0.87
0.8	18.85±0.25	20.53±0.95	4.65±0.32	10.71±0.25	6.67±0.40

* In mmol·(g_{DW}·h)$^{-1}$

Specific fumarate consumption rate increased with the concentration of salt in the medium; succinate was secreted paralleling fumarate consumption and its specific production decreased with salt concentration (Table 1). This is important since, under anaerobiosis, fumarate acts as electron acceptor and inhibitor of the crotonobetaine reductase enzyme [14, 19], enhancing the biotransformation of trimethylammonium compounds. The specific L(-)-carnitine productivity decreased with the concentration of salt, as already reported for growing cells [10].

2. Salt adaptation of the metabolism of E. coli in continuous cultures.

To get a further insight into the metabolic determinants of adaptation to long-term exposure to salt stress in *E. coli*, osmotic up-shift experiments were performed. In a previous work, short-term metabolic responses to an osmotic up-shift pulse were identified [10]. In this work, the *E. coli* O44K74 strain was grown in a chemostat under standard salt conditions (control, 0.085 M NaCl) and, once that the steady state was reached, the standard medium was switched to a hyperosmotic medium, thus creating a NaCl gradient. The new steady state was reached 40–60 h after the medium switch. The perturbations were carried out by a gradual osmotic up-shift, so that cells could adapt and long-term responses to stress could be ascertained. In order to discriminate concentration-dependent responses, three salt concentrations were assayed for the osmotic up-shifts (steady state concentrations of 0.3, 0.5 and 0.8 M NaCl, respectively), corresponding to moderate, high and very high salt stress. The metabolism was followed during the up-shift until the new steady state was reached. The effect of salt started to be noticeable soon after the medium switch. Biomass decreased as a function of the NaCl concentration, the highest concentration leading to an eightfold decrease compared to that of the control (Fig. 2A). On the other hand, the specific glycerol consumption rate increased fivefold at the highest NaCl concentration.

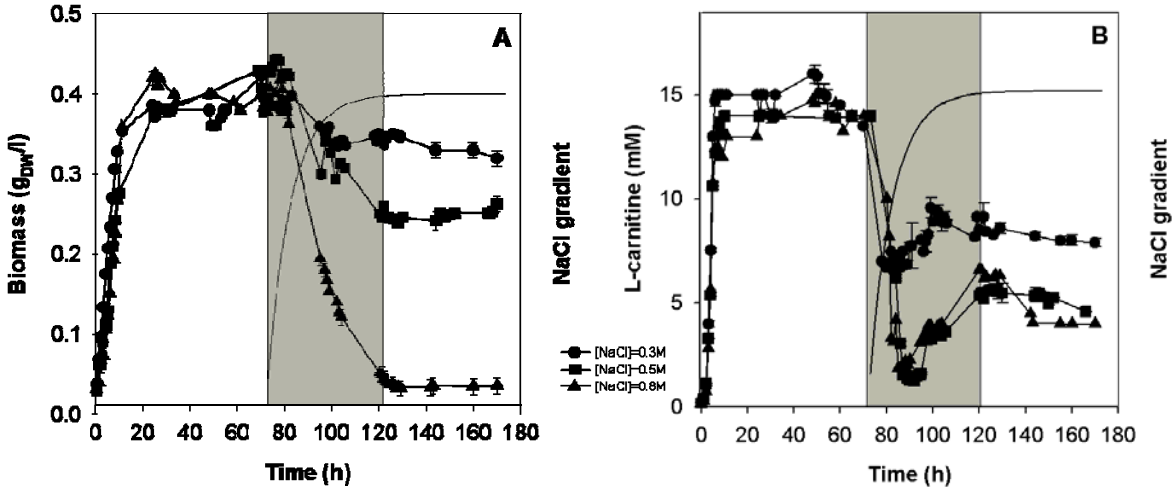

Figure 2. Effect of long term salt stress exposure on growth and L(-)-carnitine production of *E. coli* O44K74 (wild type), after switching the NaCl concentration from 0.085 (control) to 0.3, 0.5 and 0.8 M NaCl concentration. A) Biomass B) L(-)-carnitine production. (●) 0.3, (■) 0.5 and (▲) 0.8 M NaCl.

2.1. Long-term response of central metabolism to osmotic up-shift in a chemostat.

The long-term cellular adaptation to salt allowed us to disclose concentration-dependent responses. Important differences were observed in the energy and fermentation pathways, with the central pathways rearranging towards enhanced efficiency. Following the NaCl up-shift and parallel to the decrease in biomass content, a salt concentration-dependent increment in the production of all metabolites was observed (Fig. 3). Succinate and acetate specific productivities increased when the NaCl concentration rose from 0.3 to 0.8M (Fig. 3). Similar behavior was also observed with ethanol and lactate, however, such differences were not so pronounced since cells preferred to use the fumarate present in the medium as electron acceptor to achieve redox balance (Fig. 1).

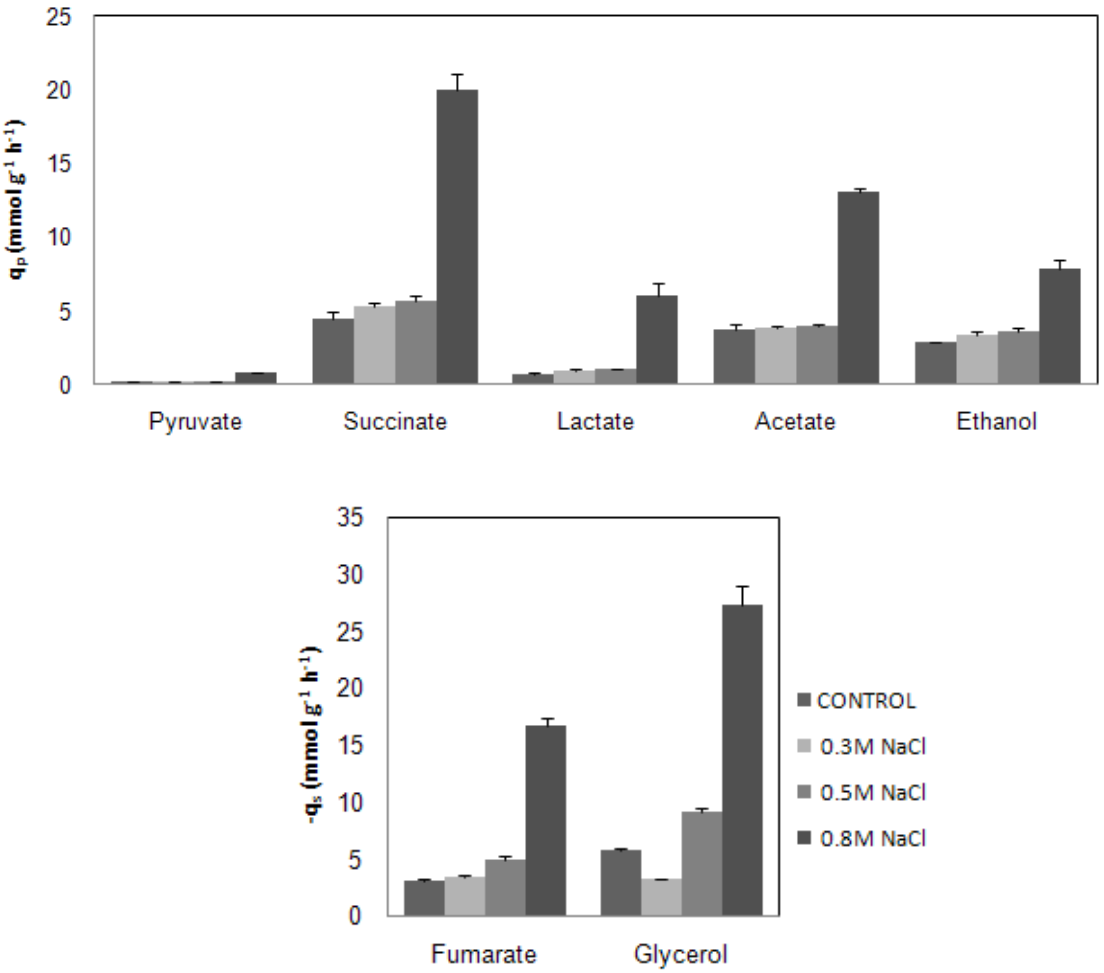

Figure 3. Specific rates of metabolite production (q_P) or substrate consumption ($-q_S$) in *E. coli* O44K74 (wild type). Experiments were performed in a chemostat fed at D=0.1 h^{-1}, using complex medium (control, 0.085 M NaCl). Once the steady state was reached, NaCl concentration was switched to 0.3, 0.5 and 0.8 M NaCl. See the text for details of experimental set up. All rates are expressed in mmol·(g_{DW}·h)$^{-1}$.

Low fumarate concentrations are an indicator of an active anaerobic respiration pathway. Residual (non-consumed) fumarate were only observed in the 0.8 M NaCl up-shift experiment, indicating that fumarate respiration could be partially inhibited by salt stress, as already described [2]. At the lower salt

concentrations (control and 0.3 M), the bulk concentration of succinate was higher than the initial concentration of fumarate in the medium (data not shown), which evidences that succinate was produced from both anaerobic respiration and fermentation pathways. However, with increasing salt concentration, the amount of succinate produced was increasingly reduced, probably as a result of the partial inhibition of fumarate respiration. Moreover, at the highest salt concentration, more time was needed to achieve the new steady state, an increment that was related with the higher effect of salt on cellular physiology and the higher energy requirement to maintain cellular homeostasis (Fig. 4).

2.2. L(-)-Carnitine production after osmotic up-shifts in chemostat reactors.

Similarly to what was found in batch experiments, specific L(-)-carnitine production decreased as NaCl concentration increased (Fig. 2B). This suggests that either salt stress inhibits the biotransformation or trimethylammonium compounds (L(-)-carnitine, crotonobetaine and/or γ-butyrobetaine) accumulate within the cells as osmoprotectants, or even both effects occur simultaneously. On the other hand, the cellular ATP content sharply increased to nearly fivefold the steady state level coinciding with the NaCl up- shifts to 0.5 and 0.8 M, an effect which was not observed at 0.3 M NaCl (Fig. 4). This transient effect may be consequence of the energy demand to protect the cell against the osmotic shock, while ATP levels restored once that the steady state was reached.

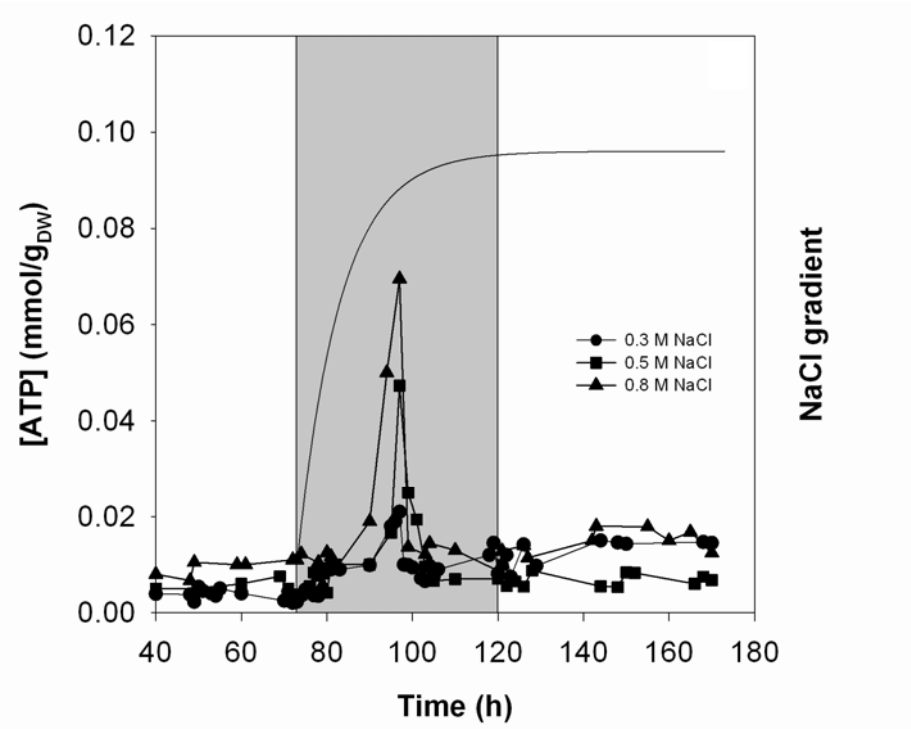

Figure 4. Effect of long term salt stress exposure of *E. coli* O44K74 (wild type) on cellular energy: ATP cell content along osmotic up-shifts. Once that cultures reached the steady state, medium feeding was switched from 0.085 (control) to 0.3 (●), 0.5 (■) and 0.8 M (▲)NaCl concentration respectively.

2.3. Enzyme activities during the chemostat up-shift of NaCl concentrations.

The changes in the main enzyme activities of central metabolism are depicted in Fig. 5 for standard and salt stress conditions at the different steady state NaCl concentrations considered.

The enzymes study reveals the main cellular responses on the energetic and intermediary metabolism after long-term NaCl exposure. Pyk and Pdh, rendering pyruvate and acetyl-CoA, respectively, control energy yields during growth. The increase in Pyk activity is in agreement with increased glycerol consumption and suggests that glycolytic rates increased as a consequence of higher energy needs under stress. On the other hand, Pta and Acs, which are part of acetate metabolism, showed opposite behavior (Fig. 5).

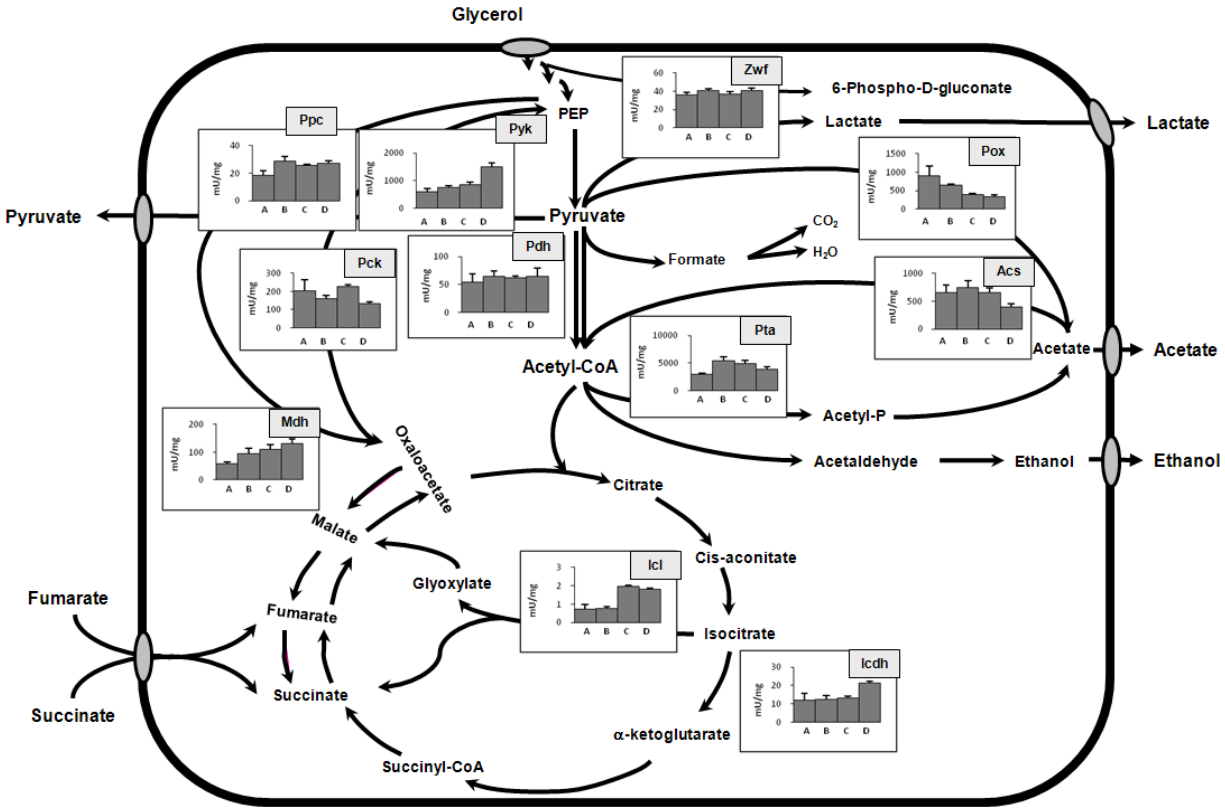

Figure 5. Enzyme activity levels in 0.085 M (A) and after long term experiment 0.3 (B), 0.5 (C) and 0.8 M (D) exposure**.** Central metabolism of *E. coli*. Acs: Acetyl-CoA synthetase, Icdh: Isocitrate dehydrogenase, Icl: Isocitrate lyase, Mdh: Malate dehydrogenase, Pdh: Pyruvate dehydrogenase, Pox: Pyruvate oxidase, Zwf: Glucose 6-phosphate-1-dehydrogenase, PEP: Phosphoenolpyruvate, Pck: PEP-carboxykinase, Ppc: PEP-carboxylase, Pyk: Pyruvate kinase and Pta: Phosphotransacetylase. (EcoCyc-MetaCyc, 44).

Under anaerobiosis, acetyl-CoA is transformed to acetate (forming ATP) by the Pta–Ack pathway [34]; acetate generated by this and other pathways (such as PoxB) can also be recycled back to acetyl-CoA through Acs [35]. After long-term exposure to salt, Acs and PoxB activities decreased with respect to the control, underlining the relative importance of the Pta–Ack pathway. This would permit for increased energy synthesis in order to with stand the selective pressure imposed and explains the increased rates of acetate formation (Fig. 3). This also reveals the alteration of regulatory mechanisms, since RpoS (the main regulator of cellular stress response) is up-regulated under stress conditions [11, 12] and alters energy management [36]. The transcription of *acs* has been determined to be negatively affected by the σ^S factor (RpoS) [37], which is in agreement with our findings. On the other hand, PoxB showed a threefold decrease at the highest NaCl concentration, which is some what surprising since its expression depends on the stress-related σ^S factor RpoS [38]. Altogether, the higher glycolytic and Pta–Ack pathway rates, and the repression of Acs revealed increased energy needs under salt stress. In the first minutes after an osmotic up-shift several membrane-dependent functions stop and respiration temporarily ceases. Accordingly, the increased acetate production (Figs. 3 and 5) allows the generation of more ATP and may be considered as a consequence of the inhibition of anaerobic respiration [2, 39], explaining the transient increase in ATP content observed during the first hours of the NaCl up-shift (Fig. 4A). This steep increase in ATP has already been described as an early response to salt stress and has been proposed to be due to substrate-level phosphorylation reactions [10, 40]. Despite this, in the steady states, the energetic state of cells was balanced by cellular maintenance (Fig. 4A). In fact, the maintenance coefficients for cellular integrity increase with the medium's osmolarity [13]. The TCA cycle and the glyoxylate shunt compete for isocitrate. Fluxes around this node are controlled by post-translational inactivation of Icdh by the Icdh-kinase/phosphatase (encoded by *aceK*), thus increasing the relative activity of Icl [19, 26]. Icl (*aceA*) and Mdh (*mdh*) activities increased with salt concentration (Fig. 5), confirming the higher demand for metabolic intermediates and biomass building blocks in the presence of salt

stress; consequently, the Icdh/Icl ratio decreased. High expression of Icl in cell recycle reactors and in nutrient-deprived resting cells indicates that the glyoxylate cycle is related to stress situations [19, 23]. Furthermore, although it has been said that the glyoxylate bypass and the Acs pathway are coordinately regulated through IclR to allow growth on acetate [41, 42], this was not observed in our work. Under saline conditions, the glyoxylate bypass was probably devoted to consume acetyl-CoA formed from glycerol, thus increasing the biosynthetic activity of cells (Figs. 3 and 5). Moreover, the in vivo activity of IclR depends on the intracellular pools of glyoxylate, pyruvate and PEP [24, 43, 44]. The changes observed in the enzyme activities related to the pyruvate node would suggest the feasibility of pyruvate accumulation in the high salt concentration medium, explaining the incremented glyoxylate shunt activity through inhibition of IclR (Fig. 6). Finally, as regards the anaplerotic/gluconeogenic pathways controlled by Pck and Ppc, contrary effects were also shown, since under salt stress the Ppc relative activity increased 1.5-fold, and Pck decreased to almost half the control. Under anaerobic conditions, the TCA cycle functions to provide biosynthetic precursors to the cells, and the altered steady state activities of Icl, Ppc and Pck, controlling anaplerosis and gluconeogenesis (Fig. 5) demonstrate the effect of salt stress on cellular needs for anaplerotic intermediaries and energy. Higher fluxes in the central energy-producing and anaplerotic pathways have also been found in *Corynebactetium glutamicum* when exposed to increased osmolarity [13]. These pathways allow replenishing the OAA pool needed for biosynthesis. Taken together, the results presented demonstrate the involvement of the biosynthetic pathways in the adaptation to salt, since are required for the production of cellular structural components, even when low biomass yields were observed.

3. In vivo metabolic evolution: adaptation to salt stress.

To ascertain whether long-term adaptation of *E. coli* cells to salt stress could lead to altered biocatalytic capabilities, *E. coli* O44K74 was cultured in a chemostat for 400 h (ca. 60 generations) at a dilution rate of 0.1 h^{-1} and a concentration of 0.8 M NaCl. Once the cells reached the new steady state, they were diluted and transferred to agar plates (CM supplemented with 0.8 M NaCl). The isolated colonies were grown in fresh medium to the mid-exponential growth phase before they were stored in glycerol at −80 ◦C. The adaptation process was favoured by the conditions of the assay: use of complex medium [9], presence of osmoprotectants (glycerol and crotonobetaine) [45, 46] and anaerobiosis [8]. On the other hand, the osmotic up-shift was carried out when the culture had reached the steady state, and it is known that halted growth leads to the acquisition of multiple resistance mechanisms, since several resistance and tolerance genes are regulated by the σ^S factor (RpoS) [47]. Other authors have reported in vivo evolution of *E. coli* after a lower number of generations and under less stringent conditions [48] . The *E. coli* strain adapted to high salt concentration thus selected will be referred as *E. coli* O44K74AS (adapted strain). The performance of primary and secondary metabolisms of the control and adapted strains were compared in batch cultures at three different salt concentrations: 0.085 (control), 0.5 and 0.8 M NaCl. In Table 2, the specific growth and L(-)-carnitine production parameters are depicted for both strains. The results showed that growth rate was slightly higher for the adapted strain than for the control. Moreover, the adapted strain clearly showed a more active metabolic pattern than the control when grown under standard conditions (Table 1). Fumarate was up-taken more rapidly by the adapted strain under standard conditions than by the wild type strain (control). This behavior resembles that of cells exposed to osmotic stress. Furthermore, the adapted strain produced higher levels of fermentation products (namely acetate, succinate and ethanol) than the parent strain, thus revealing further ATP synthesis and a higher rate of redox cofactors regeneration. When grown under stress conditions (0.5 and 0.8 M NaCl), the specific metabolite

consumption/production rates were higher for the adapted *E. coli* strain than for the control (Table 1). This was especially evident for glycerol consumption and fumarate reduction. This is important since it indicates that, although the adaptation to salt stress is rapidly triggered in the parent strain, stress response in the adapted strain was more intense. Moreover the metabolic mechanisms of adaptation to high salt were constitutively up-regulated in the adapted strain, even under non-stress conditions (Table 1).

Table. 2. Comparison of adapted and parent *E. coli* O44K74 strains in batch systems. Samples for L(-)-carnitine were taken at the beginning of the stationary phase. The study was performed as explained in Materials and Methods.

NaCl (M)	***E. coli* O44K74 (control)**		
	μ (h^{-1})	**$q_{L\text{-}car}$ ($mmol \cdot (g_{DW} \cdot h)^{-1}$)**	**Maximum Biomass[a] ($g_{DW} \cdot L^{-1}$)**
0.085 (Control)	0.326±0.020	13.32±1.00	0.568±0.010
0.5	0.335±0.025	8.04±0.02	0.484±0.010
0.8	0.142±0.025	0.83±0.01	0.174±0.020

NaCl (M)	***E. coli O44K74AS (adapted)***		
	μ (h^{-1})	**$q_{L\text{-}car}$ ($mmol \cdot (g_{DW} \cdot h)^{-1}$)**	**Maximum Biomass[a] ($g_{DW} \cdot L^{-1}$)**
0.085 (Control)	0.518±0.047	15.04±1.20	0.564±0.010
0.5	0.323±0.011	7.60±0.020	0.341±0.020
0.8	0.363±0.049	0.79±0.010	0.119±0.010

E. coli O44K74AS were obtained by strain selection after 400 h of culture in a chemostat at 0.8 M NaCl.

[a] Values refer to ca. 24 h of culture.

3.1. Gene expression analysis.

To further understand the observed alterations, gene expression analysis by means of qRT-PCR was performed. The 24 genes analyzed were selected based on their implication on central metabolism and the response to salt stress. RNA was isolated from both control and adapted strains in standard (CM) and salt-stressed cultures (CM 0.8 M NaCl). The wild type strain in the control medium was selected as the state of reference to analyze relative gene expression (Fig. 6). Both strains showed a downregulation in the genes of carnitine metabolism under stress conditions. The transcription of the more important sigma factor involved in the stress response, *rpoS*, was up-regulated in both strains in high salt media and, interestingly, also in the adapted strain under standard conditions, which substantiates the fact that stress mechanisms are constitutively active in this strain. The expression of three other transcription factors analyzed (*crp*, *fruR* and *arcA*) was down-regulated in both strains. Regarding other genes which usually appear in the stress situation and encode proteins such as porins and other membrane proteins (*ompC*/*ompF* and *proV*), their expression was up-regulated in the stressed cultures. The genes belonging to acetate metabolism showed opposite expressions, *pta* was induced while *poxB* was repressed. In the adapted strain, the relative weight of the glyoxylate pathway augmented under stress conditions. Finally, although the adapted strain showed a higher expression of most of the genes analyzed in the control conditions, surprisingly, some genes related to protection against stress were down-regulated (*relA* and *dps*). Taken together, these results indicate a higher metabolic activity and, particularly, higher glycolytic fluxes. Thus, (i) the wild type strain rapidly adapts to high salt concentration, and (ii) under standard conditions, the metabolism of the adapted strain is constitutively similar to that exhibited under stress (including the repression of L(-)-carnitine metabolism, as observed at the transcript level) (Table 2, Fig. 6). These results are in good agreement with the up-regulation of RpoS (Fig. 6) [11, 36] and with long-term adaptation to salt stress observed in chemostat experiments (Figs. 2–5), namely the activation of Pta, the repression of Acs (Fig. 5) and the higher ATP requirements. The increase shown in

the expression of the main regulatory factor under stress conditions *rpoS* in the adapted strain correlates with its metabolic profile.

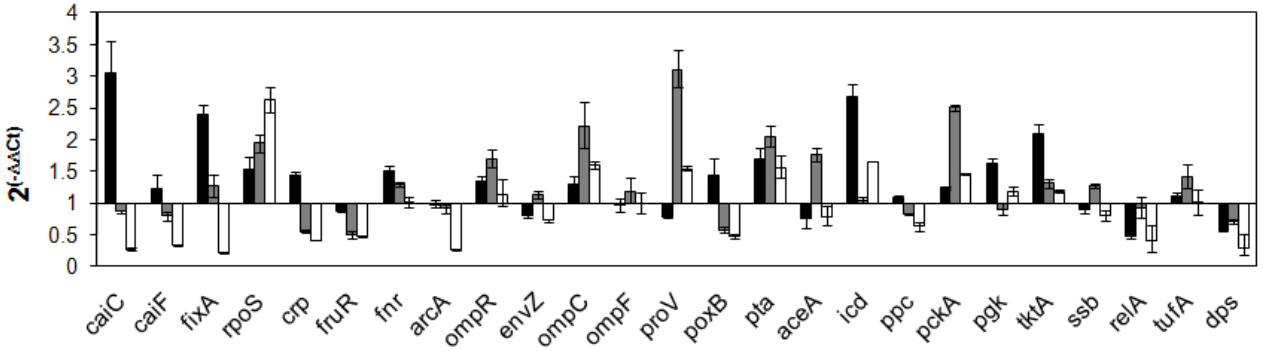

Figure 6. Relative gene expression in *E. coli* O44K74 (wild type) and *E. coli* O44K74AS (adapted strain) growing on control (0.085 M NaCl) and stressed (0.8 M NaCl) cultures. For each gene, the transcription level of that gene in the wild type strain growing on the control medium was used as reference to normalize the data. Relative gene expression of the control strain in the control conditions is, therefore, taken as 1. The relative gene expression level of the wild type strain growing on stressed conditions (grey) and the adapted strain growing on control (black) and stressed conditions (white) are shown. The results are the averages of three independent measurements of each gene/condition in triplicate, independent experiments.

On the other hand, the response to stress in both strains points to a connection with the repression of the regulator transcriptional factor *crp*, *fruR* and *arcA*. The growth of cells under salt stress in batch and chemostat reactors provoked a decrease in the trimethylammonium compounds biotransformation capacity (Table 1 and Fig. 2B), pointing to their accumulation as osmoprotectants to maintain cellular homeostasis [5, 10, 49]. In a previous work, changes in the differential expression/functionality of the transport systems involved in osmoprotection and biotransformation were correlated with salt stress [21]. In vivo evolution of *E. coli* under salt stress conditions led to enhanced up-regulation of osmoadaptation

mechanisms while down-regulating the trimethylammonium compounds biotransformation pathway.

4. Metabolic determinants of salt stress adaptation.

Interestingly, this work confirms the importance of the glyoxylate shunt and acetate metabolism during long-term exposure to salt and upon stress adaptation. The results obtained in the salt up-shifts (Fig. 5) point to crucial changes around the isocitrate (glyoxylate shunt and TCA cycle) and OAA (TCA cycle, gluconeogenesis and anaplerotic pathways) nodes. Similar results were observed in a gene expression analysis carried out by Rozen [9]. However, subtle changes are missed in gene expression studies because of the stringent limits imposed for data analysis; our approach, following metabolite profiles and enzyme activities, complement these works. Moreover, in several halophiles the Ppc enzyme is conserved while the anaplerotic reactions catalyzed by Pck and oxaloacetate decarboxylase are missing [50]. On the other hand, the glyoxylate shunt is observed in many extremophiles [51, 52]. This is important since indicates that the responses to salt stress observed in *E. coli*, might be conserved in microorganisms adapted to stringent growth conditions, namely, halophiles. Finally, it should be emphasized that the analysis of enzymes illustrates the response of the metabolism of *E. coli* to high salt concentrations. In fact, the enzyme activities measured herein mirror the actual metabolic function and account for the presence of effectors. The results highlight the demand for energy and metabolic intermediaries to maintain cellular structures. *In vivo*, metabolite pools are efficiently equilibrated by the corresponding enzymes, since operate near equilibrium [53], and the altered enzymes reflect the alteration in growth and environmental conditions, driving to altered flux distributions. Finally, the analysis of the transcriptome and the distribution of fluxes of central carbon and carnitine metabolisms and the RpoS regulatory network under the different production environments should be studied. Part of this work is being undertaken by our group to shed light on the salt stress response on the complete *E. coli* metabolism.

CONCLUSION

As a result of long-term exposure to NaCl, the metabolism of *E. coli* adapts to stress conditions. In fact, when subjected to *in vivo* evolution, an adapted *E. coli* strain showed altered metabolic capabilities, even in the absence of salt. This study shows that gene expression, enzyme activities and cofactor levels are altered as a result of long-term exposure to osmotic stress, these alterations being concentration-dependent. The cellular state is regulated by the Icdh/Icl, Pta/Acs and Pck/Ppc ratios and Pdh and Pyk activeties. The energy metabolism of *E. coli* cells (acetate production and the Pta–Ack pathway) and the gluconeogenic and anaplerotic pathways (Pck, Ppc and glyoxylate shunt) were affected, thus indicating the important role played by the TCA cycle for the production of biosynthetic intermediaries, as well as the cellular needs of energy to sustain metabolite transport, cell structure and growth as well as an adequate production of biosynthetic precursors. Finally, data here presented indicate that there arrangement of the metabolic fluxes distribution plays an important role in cell survival and adaptation. The participation of the TCA cycle, glyoxylate cycle and anaplerotic pathways (providing cellular intermediates) and glycolysis and the acetyl-CoA/acetate metabolism (providing energy) in survival to salt stress has been demonstrated.

APPENDIX A

NOMENCLATURE

aceBAK	glyoxylate shunt operon
Ack	acetatekinase
Acs	acetyl-CoA synthetase
ArcAB	regulator of the anaerobic/aerobic metabolism
cAMP	cyclicAMP
TCA	tricarboxylic acids cycle
CHR	crotonobetaine hydration reaction (CaiD:CaiB)
CRR	crotonobetaine reduction reaction (CaiA:CaiB)
CRP	Catabolic Repressor Protein receptor (cAMPreceptor)
CS	citrate synthase
ETC	electrontransport chain
fixABCX	operon necessary for crotonobetaine reduction in *E. coli*
FNR	transcriptional regulator under anaerobiosis
H-NS	histone protein
Icdh (*icd*)	isocitrate dehydrogenase
Icl (*aceA*)	isocitrate lyase
IcIR	repressor of Icl and activator of Acs
Ldh	lactate dehydrogenase
MS	malate synthase
Mdh	malate dehydrogenase
OAA	oxaloacetate
PEP	phosphoenolpyruvate
Pck (*pckA*)	PEP-carboxykinase
Ppc (*ppc*)	PEP-carboxylase
Pfl	pyruvate formate lyase
Pyk	pyruvate kinase
Pta (*pta*)	phosphotransacetylase
PoxB (*poxB*)	pyruvateoxidase

σ^S or RpoS (*rpoS*)	Sigma subunit of RNApolymerase, up-regulated upon entry into stationary phase, and various stresses
caiTABCDE	carnitine operon
CaiT	carnitine/crotonobetaine/γ-butyrobetaine transporter
CaiA	crotonobetainyl-CoA reductase
CaiB	CoA-transferase
CaiC (*caiC*)	crotonobetaine/D,L-carnitine/ γ -butyrobetaine:CoA ligase
CaiD	enoyl-CoAhydratase
CaiE	protein of unknown function
CaiF (*caiF*)	transcriptional activator of *cai* operon
fixA	probable flavoprotein subunit required for anaerobic carnitine metabolism
crp	CRP transcriptional dual regulator
fruR	FruR transcriptional dual regulator
fnr	FNR transcriptional dual regulator
arcA	ArcA transcriptional dual regulator
ompR	OmpR transcriptional dual regulator
envZ	osmolarity sensorprotein EnvZ
ompC	outer membrane porin OmpC
ompF	outer membrane porin OmpF
proV	ATP-binding component of transport system for glycine, betaine and proline
pgk	phosphoglycerate kinase
tktA	transketolase
ssb	ssDNA-binding protein
relA	GDP pyrophosphokinase/GTP pyrophosphokinase
tufA	elongation factor Tu (TufA)
dps	global regulator, starvation conditions

APPENDIX B

1. Materials and Methods: Enzyme assays.

The enzyme activity assays were optimized for the conditions and media used in this study. Enzyme activity was defined as μmoles of substrate consumed per minute and mg of protein (U/mg). In each case, reactor bulk liquid samples were withdrawn and centrifuged at 16,000xg at 4°C. The supernatant was removed and cells were resuspended in the extraction buffer (65 mM potassium phosphate, pH 7.5). Cells were sonicated in ice for 6 cycles (10 s each), at 10 μm amplitude, with a probe of 1 cm diameter. The extract was centrifuged for 15 min at 16,000xg and 4°C to remove cell debris and the supernatant was used for subsequent activity measurements. Protein content was determined by the method of Lowry et al. [25]. The enzyme activities were determined spectrophotometrically in a Synergy™ HT Multi-Mode Microplate Reader (BIO-TEK, Winooski, VT) with 200 μl total reaction volume.

Isocitrate dehydrogenase (Icdh). The method was that of Bennet and Holms [26]. The assay conditions were as follows: 65 mM potassium phosphate buffer, pH 7.5, 5 mM $MgCl_2$, 2.4 mM $NADP^+$, 15 mM D,L-sodium isocitrate . The increase in NADPH absorbance at 340 nm (ε_{NADPH} = 6.22 $mM^{-1}cm^{-1}$) was followed at 37 °C. One unit of enzyme activity was that required to generate 1μmol of NADPH per min.

Isocitrate lyase (Icl). The assay was that described by Dixon and Konberg [27], using: 65 mM potassium phosphate buffer, 5 mM $MgCl_2$, 20 mM phenylhydrazine, 15 mM D,L-sodium isocitrate. The reaction was followed by the increase in absorbance at 324 nm due to the reaction of the glyoxylate generated with phenylhydrazine to form a hydrazone ($\varepsilon_{complex}$ = 17 $mM^{-1}cm^{-1}$). One unit of enzyme activity was taken as that needed to generate 1 μmol of adduct (glycoxylic acid phenylhidrazone) per min.

Acetyl-CoA synthetase (Acs). The method used was that established by Brown et al. [28]. The reaction mixture was: 100 mM Tris-HCl buffer, pH 7.8, 5 mM D,L-acid

malic, 20 mM ATP, 2.5 mM $MgCl_2$, 0.1 mM CoASH, 3 mM NAD^+, 0.5 U malate dehydrogenase, 0.25 U citrate synthase, 100 mM sodium acetate. The reaction was followed by the increment in NADH absorbance at 340 nm (ε_{NADH} = 6.22 $mM^{-1}cm^{-1}$) and 45°C. Enzyme activity unit was defined as the enzyme required for the generation of 1 μmol of NADH per min.

Phosphotransacetylase (Pta). The method used was that of Brown et al. [28]. The assay mixture contained: 250 mM Tris-HCl buffer, pH 7.8, 10 mM D,L-acid malic, 1 mM $MgCl_2$, 0.5 mM CoASH, 3 mM NAD^+, 0.5 U malate dehydrogenase, 0.25 U citrate synthase, 10 mM acetyl phosphate. The reaction was followed as the increment in NADH absorbance at 340 nm (ε_{NADH} = 6.22 $mM^{-1}cm^{-1}$) and 37°C, one unit being taken as the enzyme required for the generation of 1 μmol of NADH per min.

Pyruvate dehydrogenase complex (Pdh). The method was that of Brown et al. [29]. The reaction mixture was: 65 mM potassium phosphate buffer, pH 8, 1 mM $MgCl_2$, 0.5 mM thiamine pyrophosphate (TPP), 5 mM sodium pyruvate, 5 mM cysteine, 1 mM NAD+, 0.1 mM coenzyme A. The reaction was followed as the decrease NADH absorbance at 340 nm (ε_{NADH} = 6.22 $mM^{-1}cm^{-1}$) and 37°C, one unit being taken as the enzyme required for the generation of 1 μmol of NADH per min.

Malate dehydrogenase (Mdh) The method was that of Park et al. [30], with minor modifications. The assay was performed with 65 mM potassium phosphate buffer pH 7.5, with 0.5 mM NADH, and 0.1 mM oxaloacetic acid as substrate. The reaction was followed as the decrease in NADH absorbance at 340 nm (ε_{NADH} = 6.22 $mM^{-1}cm^{-1}$) and 37°C, one unit being taken as the enzyme required for the oxidation of 1 μmol of NADH per min.

Pyruvate oxydase (Pox). A previously described method [31] with minor modifications. The reaction was assayed using 65 mM potassium phosphate buffer pH 6, 5 mM $MgCl_2$, 0.25 mM thiamine pyrophosphate (TPP), 2.5 mM potassium ferrocyanide**,** 50 mM pyruvate. The reaction was followed as the increase in

potassium ferricyanide absorbance at 405 nm ($\varepsilon_{ferricyanide}$ = 0.093 $mM^{-1}cm^{-1}$) and 37^{o}C. One enzyme activity unit was taken to be the enzyme required to generate 2 μmol of ferricyanide per min.

Pyruvate kinase (Pyk). The method was that of Peng and Shimizu [32] with minor modifications. The reaction mixture contained: 50 mM Bis-Tris buffer, pH 6.5, 25 mM $MgCl_2$, 10mM KCl, 0.25 mM DTT, 0.5 mM NADH, 0.25mM ADP, 0.5 U lactate dehydrogenase (LDH), 2.5 mM phosphoenol pyruvate. The reaction was followed as the decrease in NADH absorbance at 340 nm (ε_{NADH}=6.22 $mM^{-1}cm^{-1}$) and 37^{o}C, one unit being taken as the enzyme required for the oxidation of 1μmol of NADH per min.

Phosphoenol*pyruvate *carboxykinase (Pck). The method was that of Peng and Shimizu [32], with minor modifications. The reaction mixture contained: 50 mM Bis-Tris buffer, pH 6.5, 2.5 mM DTT, 10 mM $MgCl_2$, 75 mM $NaHCO_3$,0.25 mM NADH, 1 U malate dehydrogenase (MDH), 5mM ADP, 10 mM phosphoenol pyruvate. The reaction was followed as the decrease in NADH absorbance at 340 nm (ε_{NADH}=6.22 $mM^{-1}cm^{-1}$) and 37^{o}C, one unit being taken as the enzyme required for the oxidation of 1 μmol of NADH per min.

Phosphoenol*pyruvate *carboxylase (Ppc). The method was that of Peng and Shimizu [32], with minor modifications. The assay was performed with 100 mM Tris-HCl buffer, pH 8, 5 mM $MgCl_2$, 10 mM $NaHCO_3$,10 mM DTT, 0.5 mM NADH, 1 U malate dehydrogenase (MDH), 2.5 mM phosphoenol pyruvate. The reaction was followed as the increase in NADH absorbance at 340 nm (ε_{NADH}=6.22 $mM^{-1}cm^{-1}$) and 37^{o}C, one unit being taken as the enzyme required for the oxidation of 1 μmol of NADH per min.

2. Materials and Methods: Primers used for real time PCR. The primers used in this work were designed using the Primer Express® Software v3.0 (Applied Biosystems, Foster City, CA) and ordered from Sigma-Aldrich (Sigma-Aldrich Co., St. Louis, USA). The *dnaA* and *rrsA* genes (encoding transcriptional dual regulator and 16S ribosomal RNA, respectively) were used as internal control for relative quantification.

Group	*Gene*	Forward primer	Reverse primer
Internal control	*dnaA*	5′-TGGCGAAAGAGCTGACTAACC-3′	5′-ACGGCAGGCATGAAGCA-3′
	rrsA	5′-CCTTACGACCAGGGCTACACA-3′	5′-CACTTTATGAGGTCCGCTTGCT-3′
Sigma factors	*rpoS*	5′- CGCACTGCGTGGAGATGTC-3′	5′-CCATAACGGCGGGCAAT-3′
Transcription factors	*crp*	5′-CCGGACGGTATGCAAATCA-3′	5′-AGAATGCGTCCCACGGTTT-3′
	fruR	5′-GGACGCACACGTTCTATTGGT-3′	5′-TAGTTAGCGATGCGGGTATAGCT-3′
	fnr	5′-ACCTGTCCCGTCGTTTTGC-3′	5′-TCATCGTCAGGCGGAATTC-3′
	arcA	5′-ATGCAGACCCCGCACATT-3′	5′-ACTTTTCAACGTGTTGCGTGTT-3′
PPP	*tktA*	5′-GACGCAGTACAGAAAGCCAAATC-3′	5′-GGAAATCACGCCACAGGACTT-3′
Stress response	*ompR*	5′-GCGCTGACGACTACATTCCAA-3′	5′-GCAGCACCGCACGGATA-3′
	envZ	5′-GGCGCAACACTATGAATTCTTAAG-3′	5′-TGACCTCAACGCGCACTTC-3′
	ompC	5′-GCGCTGAAAACGAAAACAACT-3′	5′-GAAAGAACCCACATCCTGGAATT-3′
	ompF	5′-AACATCTACCTGGCAGCGAACTA-3′	5′-GCCGCTGGTGTTTGTAAATTTATT-3′
	proV	5′-AGGTGCGCAGAAAAAAGATTG-3′	5′-TGTCCAGCACGGTCATATGC-3′
Glycolysis	*pgk*	5′-GCGAGAAGAAAGACGACGAAAC-3′	5′-TCCATTACGAACACGTCACACA-3′
Carnitine metabolism	*caiC*	5′-GGCGACAAGGTTGCACTACA-3′	5′-AGCCCGAACCAGCAAAAGA-3′
	caiF	5′-GCGCCTGCGCAATAACA-3′	5′-CAGGAATACGCGGCGTCTT-3′
	fixA	5′-CTGCCTGCGGTTGTTGCT-3′	5′-CATACCTGGACGGGCTTTTTC-3′
Acetate metabolism	*pta*	5′-TCGCAAACTACCACGCTAACA-3′	5′-AGACTGGGCAAACTGGTGCTT-3′
	poxB	5′-GCCCTGCGCGGTAAAGA-3′	5′-TTAACCCGGTCATTCCAACATC-3′
TCA cycle	*icd*	5′-GGCACAAGGCAAGAAGATCAC-3′	5′-AGGGATAATCGGATTTTCAGGAA-3′
Glyoxylate shunt	*aceA*	5′-AGCCTCGGCGCACTG-3′	5′-TAGACTGCTTCAATACCCGCTTTC-3′
Gluconeogenesis and anaplerosis	*pckA*	5′-GTCTTTCCGTCCGTTTCATCAC-3′	5′-CTCGGGCGAATAAACATGT-3′
	ppc	5′-TAACCGAACAGGGCGAGATG-3′	5′-AGGCTGCTGACGGTGATTTC-3′
Synthesis and protection	*ssb*	5′-GGCGCAGTTGCCAACATT-3′	5′-TCTCGCCGGTCGCTTTATC-3′
	relA	5′-TCTACGCACCGCTGGCTAAC-3′	5′-GGAGGTAACGGAAGCAGTAATCTT-3′
	tufA	5′-GCAGAGTGGGAAGCGAAAATC-3′	5′-GTCAATCGCACGCTCTGGTT-3′
	dps	5′-TCCGACAGCGAGAAAAAAGC-3′	5′-TCAAAGAAAGATCAATAAACTGGATAACC-3′

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CHAPTER 4

Integration of fluxomics, metabolomics and trascriptomics to describe anaerobic long-term salt adaptation of E. coli using glycerol as C-source.

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ABSTRACT

The response of a microorganism to an environmental stress follows two well-known phases, short-term or shock and long-term adaptation. The former has been extensively studied in high-osmotic conditions, especially using transcriptomics, whereas the latter seems not to have been considered in depth. This is important bearing in mind that glycerol has started to be used as carbon source and it is highly contaminated with NaCl, which could interfere with certain bioprocesses if no additional purification procedures are undertaken. Indeed, glycerol is produced massively as a by-product of the biodiesel and bioethanol industry and it is replacing other classical C-sources. Among microorganisms, *Escherichia coli* has served as a model organism for decades using synthetic media and aerobic conditions, even though this bacterium is a common gut inhabitant accustomed to high osmolality, nutrient-rich medium and anaerobic conditions. It is therefore surprising that even few works have been performed using these conditions. In this work the anaerobic long term adaptation of E. coli was analyzed using chemostat cultures with a gradual osmotic upshift. When the stationary state was reached with standard salt concentration (0.085 M NaCl), it was switched to a higher concentration of NaCl which was maintained until a new stationary state. Two final concentrations of NaCl (0.5 M and 0.8 M NaCl) were assayed each leading to different cell survivals. Metabolomic and fluxomic profiles of all the stationary states were obtained and the transcriptional evolution from one to another was monitored. A perfect correlation was found between the different –omic technologies, supporting a broader picture of E. coli adaptation to long-term high salinity conditions. A common concentration-dependent pattern was found, based on (i) increasing ATP-generating fluxes such as glycerol consumption or succinate and formate production, and (ii) diminishing ATP-consumption processes. Among the latter, it is important to highlight the complete depletion of amino acids from the medium, which could reduce their de novo biosynthesis. On the other hand, marked differences were observed including a

switch in fermentation pattern and a strong alteration of the redox state as confirmed by flux and transcriptomic analyses.

INTRODUCTION

Escherichia coli shows osmoadaptation when the osmolarity of the culture medium increases [1], an ability of fundamental importance for their growth and survival. Some compatible solutes may alleviate the inhibitory effects of high osmolarity when they are present in the culture medium, suggesting that they accumulate in high concentrations, either though uptake from the medium or through *de novo* synthesis [2, 3]. These are called osmoprotectant compounds and among the most important are the betaines, which have been widely studied [4-6]. The adaption process depends on several factors besides the composition of the medium including the state of growth [7-9], and the presence of osmoprotectants (glycerol and crotonobetaine) [10, 11] and oxygen [12]. Despite the fact that the strategies followed to maintain cellular integrity and physiological capabilities have certain similarities, they also show several differences, depending on the magnitude of stress. Moreover, the time of exposure to osmotic stress has been seen to promote differential genetic expression [12]. The response to short-term exposure to NaCl has been thoroughly studied unlike long-term adaptation. In general, the short-term response is fast and temporary, returning to initial state after the perturbation [13]. Moreover, a general response has been described in several works, namely a rapid loss of water (plasmolysis), loss of turgor, diminished respiration, increased intracellular ATP concentration and the cytoplasmic pH, a large increase in the rate of uptake and the amount of cytosolic K^+, including the accumulation of glutamate [7]. On the other hand, the response to long-term exposure involves a different cellular physiological state which adapts to the new conditions. In fact, the research performed in a previous work involving long-term high osmolarity pointed to the *in vivo* evolution of *E. coli* with altered metabolic capabilities [9], as has been reported in other types of stress [14].

E. coli is a highly adaptable bacterium which is able to survive and multiply in diverse and sometimes hostile environments. In fact, it is a natural inhabitant of the mammalian intestine so that it withstands diverse conditions of growth depending

on the diet of its host. Moreover, this adaptability has begun to be exploited to enhance production in industrial processes, in which cells are exposed to large changes in osmolarity, primarily as a result of the composition of the medium and product accumulation [15, 16].However, few experimental assays have been developed in conditions close to the natural environment of *E. coli* (high osmolarity, complex medium and anaerobic conditions). Both rich medium and anaerobically grown cells are of special importance in the osmoadaptation process because they allow cells to acquire favorable genetic and metabolic features to overcome osmotic upshift [12, 17]. Moreover, it has been recently demonstrated that fumarate is the most important electron acceptor for *E. coli* to colonize the mouse intestine [18]. However, very few studies can be found regarding bioprocesses using anaerobic conditions and using fumarate as electron acceptor, even though these conditions are closer to the natural environment of *E. coli* and fumarate improves both cell growth and productivity in anaerobic conditions, for example, during L(-)-carnitine biotransformation [19, 20] and 1,3 propanediol biosynthesis [21]. As regards high osmolarity, the intestine may present NaCl concentrations of 0.3 M or even higher [22] and, although osmoprotectans molecules such as L(-)-carnitine are also present, hardly any reports have described high osmolarity with osmoprotectans [23].

The price of glycerol has fallen drastically since it is generated as a by-product of the biodiesel and bioethanol production [24] and it is increasingly used as C-source for several bioproceses [25]. However, crude glycerol is highly contaminated with ash, whose principal component is NaCl [26]. Therefore, high quantitates of NaCl could be present in crude or even processed glycerol. However, few studies using glycerol as C-source have taken this into account and some have used crude glycerol [27] even though NaCl may affect productivity, as has been reported in the case of polyhydroxyalcanoate synthesis [28]. Moreover, the use of osmoprotectants, such as L(-)-carnitine or crotonobetaine (a by-product of L(-)-carnitine chemical synthesis) could alleviate the harmful effects of high NaCl concentrations [13]. To our knowledge, few works have dealt with the anaerobic cultivations of *E. coli* compensated with an electron acceptor such as fumarate, and

where the high concentratios of NaCl is alleviated with an osmoprotectant such as crotonobetaine. Moreover, glycerol and both fumarate and crotonobetaine are inexpensive to obtain [27, 29, 30] and can be transformed into high value compounds (lactate, ethanol, formate, succinate and L(-)-carnitine).

The many technologies applied have contributed to increasing our knowledge of the adaptive response. Proteomics and transcriptomics, which use a global approach, have identified groups of genes involved in the osmoadaptation process [31]. However, further research should be carried out measuring the level of internal and external metabolites and monitoring their evolution through flux analysis. Moreover, other mechanisms involved in the distribution of metabolic fluxes such as posttranscriptional control, enzyme kinetics and allosteric control, could exert changes or adjustments in the metabolism that would not be reflected at transcriptomical or proteomical level [32]. The results obtained at metabolic level may identify metabolic targets which can be modified in order to enforce desirable cellular properties and improve biotechnological processes. In this sense, the amino acid metabolism has been deeply investigated because of the role they play as protective osmolytes to relieve the effect of environmental stresses [33, 34]. Particularly, in *E. coli*, metabolic assays have focused on the osmoprotector effect and the advantages produced by the uptake of individuall amino acids. Commonly, proline and glycine have been the most studied amino acids related to the osmoprotection, with emphasis placed on the influence of the medium and the transporter mechanism [35-38]. However, while the effect of the presence of individual amino acids in the medium has been deeply investigated [3, 39, 40], there is little information on the effect of complex mixtures (the most common situation to real environmental conditions). Osmotic adaptation has been analyzed in aerobic conditions using minimal medium, although this is hardly a common condition in nature. Besides, there is a lack of information regarding the effect of a complex medium of high osmolarity in the presence of an osmoprotectant, even though peptone has been demonstrated to be more efficient than glycine-betaine in enhancing the growth of *E. coli* in high osmolarity media [38].

In this work, continuous cultures of *E.coli* O44K74, which were supplemented with a gradually increasing concentration of NaCl to reach 0.5 and 0.8 M, were analyzed to evaluate the response to high and very high osmotic stress. During long-term exposure to the new conditions (> 100 h), cells underwent a set of changes, which can be considered as an osmoadaptation process effect. In order to estimate the critical modifications undergone to overcome stress and to develop tolerance to salt, the metabolism was examined at several levels using different techniques (metabolomics, fluxomics and transcriptomics). The internal and external metabolites and energetic cofactors showed an altered pattern with respect to that observed in control conditions (0.085 M NaCl). Surprisingly, all amino acids of the culture medium were taken up as soon as the salt concentration started to increase. Furthermore, important changes in redox cofactors were assessed.

MATERALS AND METHODS

1. Bacterial strains and growth conditions.

The stable overproducer strain *E. coli* O44K74 (DSM 8828), which contains the complete *cai* and *fix* operons, was used. The strain was stored in 20% glycerol at –80ºC. The standard complex medium (CM) used contained (g/L): bacteriological peptone, 20; NaCl, 5; glycerol (carbon source), 12.6; crotonobetaine, 4; and fumarate, 2. The NaCl concentration was altered as stated in the text. The pH of the media was adjusted to 7.5 with 1 M KOH prior to autoclaving [41, 42].

Experiments were performed in Biostat B (Braun Biotech International GMBH, Melsungen, Germany) reactors equipped with temperature, pH and oxygen probes, using pump controls for continuous operation. A 2 L culture vessel with 1.8 L working volume was used. Strict anaerobiosis was maintained by bubbling nitrogen. The culture was inoculated as explained and continuous operation was started by feeding with the complex medium at a dilution rate of 0.1 h^{-1}. At the steady state, 0.5 g/L biomass dry weight was reached. Then, the feeding was switched to another medium containing the new NaCl concentration (0.5 and 0.8 M, respectively).

2. Adaptation of E. coli in steady state chemostats to osmotic up-shift at two different salt concentrations.

E. coli cells were grown in chemostats with a dilution rate of 0.1h^{-1}. The stationary state was taken to have been reached when OD measurements were constant for at least 5 residential times (50 h), t=0 h. Afterwards, NaCl was increased to reach 0.5 M (high) or 0.8 M (very high) in separate reactors as previously described (see above). As shown in Figure 1, a second steady state was reached in both reactors about 50 h after NaCl addition. Samples were harvested when both steady states had clearly been established (t=0 h and 75 h after NaCl addition); for transcriptomics, an additional sample (29 h after NaCl addition) was harvested to

cover not only both stationary states, but also the transition phase between them. Cells adapted to 0.8 M NaCl were harvested and further used for fermentation profile studies at high NaCl concentration in batch. The strain was named *E. coli* O44K74 AS (Adapted Strain).

3. Time-course of exometabolite concentrations.

Samples for the analysis of external metabolites were withdrawn from the reactor during the long-term experiments every hour and were immediately centrifuged at 16,000 x g. The rotor was at 4^{o}C. Supernatants were used to determine external metabolites.

Organic acids (acetate, ethanol, fumarate, pyruvate, and succinate) were determined by an HPLC system from Shimadzu (Kyoto, Japan) with a cation exchange Aminex HPX-87H column supplied by BioRad Labs (Hercules, CA). The isocratic mobile phase was 5 mM H_2SO_4 at a flow rate of 0.5 mL min^{-1}. The effluent was monitored using a refractive index detector (Shimadzu, Kioto, Japan).

Amino acids determination was carried out as endometabolome analysis (see below).

4. Endometabolome analysis.

Endometabolome analysis has been performed by method described in [43] (Appendix A).

5. Time-course microarray experiment design.

Cells were grown as described in the main text in the Material and Methods section using continuous reactors. The stationary state was confirmed after 5 residential times (50 h) when no modification in the biomass concentration was observed, after which the samples were harvested (t=0). From this moment, the medium was switch to another with higher NaCl concentration (0.5 M and 0.8 M).

Samples were harvested at different time points to cover not only the new stationary state (confirmed previously at t=75 h), but also the transition phase (t = 29 h).

5.1. RNA extraction and microarray sample preparation.

At every time point described above, the cultures were pelleted by centrifugation at 15000xg at 4°C for 30 s. Total RNA was isolated by QiagenRneasy® Mini Kit (QIAGEN Ibérica, Madrid, Spain). Additionally, Dnase I digestion of the isolated RNA was performed using the Rnase-Free Dnase Set (QIAGEN Ibérica, Madrid, Spain) to avoid DNA interferences during PCR steps. RNA quality and quantity were evaluated by microfluidic capillary electrophoresis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) using Agilent RNA 6000 Pico kit. The GeneChip*E. coli* Genome 2.0 array of the Affymetrix system was used to compare gene expression of *E. coli* O44K74 during the osmoadaptation process. The processing of extracted RNA, cDNA labeling, hybridization, and slide-scanning procedures were performed according to the manufacturer's instructions (Affymetrix).

5.2. Microarray data analysis.

The statistical analysis was performed using the Bioconductor suite [44] in R [45]. Affymentrix data files were processed with affy package [46]. All of the microarrays were pre-processed simultaneosly using Robust Multichip Average (RMA), as described by Irizarry et al. [47]. Afterwards, differentially expressed genes were identified from the time-course experiment using the maSigPro package [48]. A quadratic regression model was used for the gene selection with a P-value cut-off of 0.05, adjusted with Benjamini and Hochberg's method [49]. Furthermore, the fold-change in expression had to exceed a factor of 2 compared with the initial steady state values for genes to be considered differentially expressed.

5.3. Clustering.

Genes previously found differentially expressed were represented with a heatmap using package gplots [50]. Moreover, hierarchical clustering analysis was carried out using the complete agglomeration method from the stats package [45].

5.4. GO enrichment analysis.

Based on the different clusters found in the previous section, genes were analysed for gene term enriched analysis with a conditional hypergeometric test algorithm included in the GO stats Bioconductor package [51]. P-value cut-off was set at 0.05. GO terms in the three categories, namely, Biological Process (BP), Molecular Function (MF) and Cellular Component (CC) were included.

6. Metabolic flux analysis.

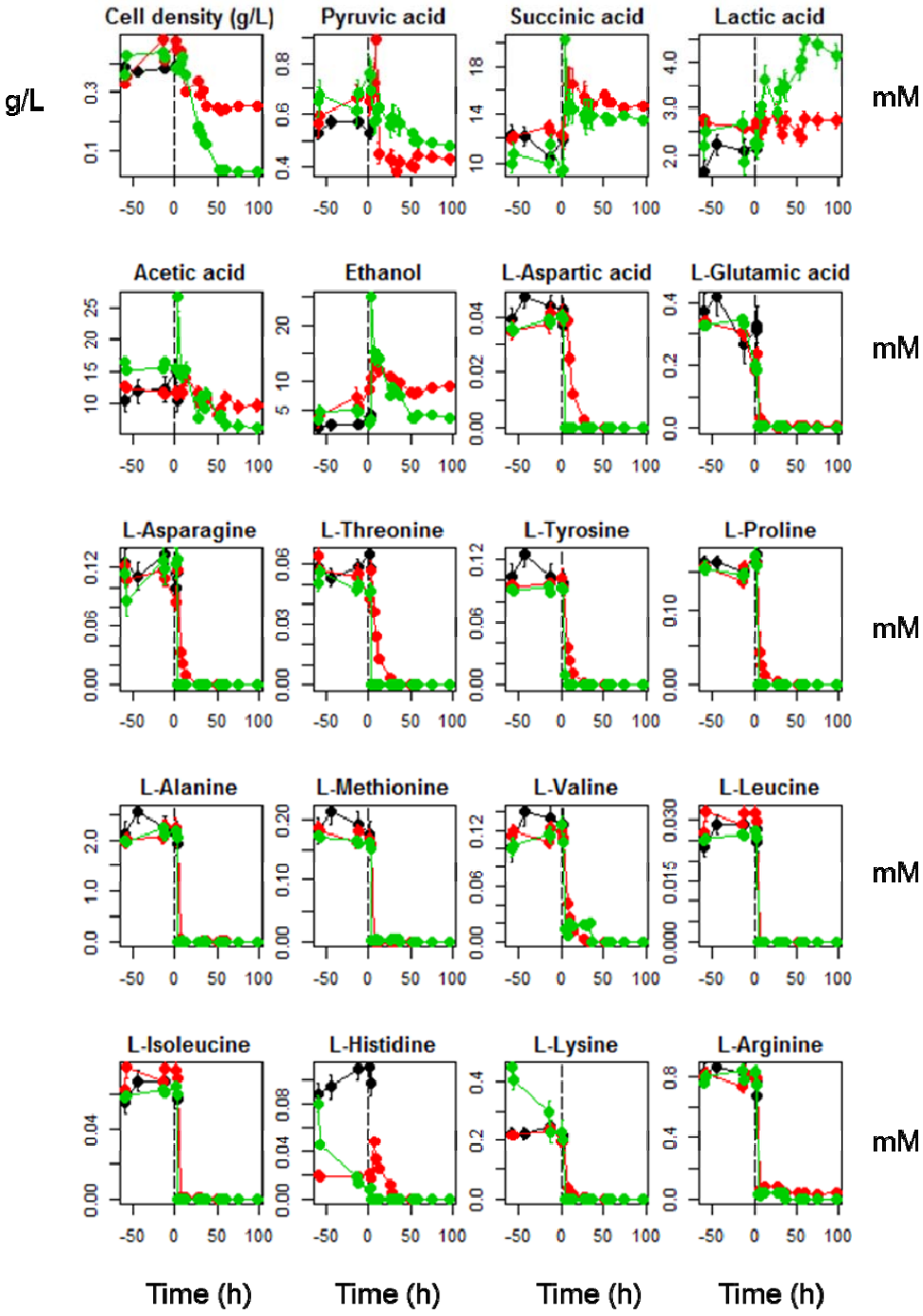
A previously developed *E. coli* large-scale stationary model [30], including a model of carnitine metabolism [19] and anaerobic conditions was used. The software used was Insilico Discovery (Insilico Biotechnology AG, Stuttgart, Germany), version 2.00, which allowed us to perform the Metabolic Flux Analysis for the different conditions applying simplex algorithm for the estimation of metabolic fluxes by linear programming [52]. The model has 13 degrees of freedom, which can be solved with experimental fluxes, which were obtained taking into account that the dilution rate was kept constant at 0.1 h^{-1} and measuring the concentration of the following extracellular metabolites, as shown above: (i) glycerol, fumarate and crotonobetaine in the feeding medium, (ii) fermentation products (ethanol, pyruvate, acetate and lactate) as well as crotonobetaine derivates (L(-)-carnitine and γ-butyrobetaine) in the effluent medium. Pro U and biomass (T.bio) fluxes were determined as previously described [30].

RESULTS

2.1 Exometabolite evolution of the adaptation to osmotic up-shift at high (0.5 M NaCl) and very high (0.8 M NaCl) salt concentrations.

To establish the effect of the increase in the salt concentration (0.5 and 0.8 M NaCl), the evolution of the main fermentative metabolites and fourteen amino acids was followed as well as the cell density (Figure 1). Before t = 0, the salt concentration was kept at basal value (0.085 M) until the stationary state was confirmed during five residential times. The results pointed to a good reproducibility in the three reactors assayed. Afterwards, the inlet NaCl concentration was increased to 0.5 and 0.8 M in two different reactors. Consequently, a new stationary state was reached and confirmed during five residential times (t =75 h). Cell density decreased in both conditions, drastically so at the very high salt concentration. As regard the fermentation pattern, pyruvate and acetate decreased, whereas succinate increased in both conditions. On the other hand, the lactate level strongly increased only at 0.8 M NaCl, whereas ethanol increased only at 0.5 M, indicating different adaptations to these extreme conditions.

In general, all the amino acids in the medium reached a steady state during the system evolution at control NaCl concentration (before t = 0), indicating a constant uptake rate. Surprisingly, as the medium with high or very high salt concentrations was added, the level of all the measured amino acids (Asp, Glu, Asn, Thr, Tyr, Pro, Ala, Met, Val, Leu, Iso, His, Lys and Arg) decreased to depletion. Moreover, this effect was more dramatic at 0.8 M than at 0.5 M NaCl (see Asp, Asn, Thr, Tyr, Pro and Val levels in Fig. 1), emphasizing the effect of the salt concentration on this response.


g/L
Cell density (g/L)
Pyruvic acid
Succinic acid
Lactic acid
mM
Acetic acid
Ethanol
L-Aspartic acid
L-Glutamic acid
mM
L-Asparagine
L-Threonine
L-Tyrosine
L-Proline
mM
L-Alanine
L-Methionine
L-Valine
L-Leucine
mM
L-Isoleucine
L-Histidine
L-Lysine
L-Arginine
mM
Time (h)
Time (h)
Time (h)
Time (h)

2. Metabolic flux analysis of the long-term adaptation to osmotic up-shift at two different salt concentrations with a large-scale stationary E. coli model.

It was difficult to extract any firm conclusions from the above experiments since the cellular concentration was modified while the glycerol consumption rate kept constant (results not shown). In order to compare the metabolic flux redistribution in the different salt conditions, a large-scale stationary *E. coli* model was used to perform a Metabolic Flux Analysis at the three above mentioned states (control, 0.5 and 0.8 M NaCl). In order to deal with the 13 degrees of freedom of this model, 14 fluxes were experimentally determined (see Materials and Methods section) and summarized in Table 1. The experimental fluxes perfectly matched those obtained with the model, confirming the correlation between the model results and the experimental data. Large-scale stationary *E. coli* model results are summarized in Fig. 2 to show a general overview of the fluxes, considering the initial stationary state with control NaCl concentration and the subsequent stationary states at 0.5 and 0.8 M NaCl. Moreover, metabolic fluxes were normalized using the glycerol income flux to compare them during the different steady states. The principal outcome of this analysis was the evident redistribution of the main fermentation pathways: (i) the proportion of glycerol converted to lactate more than doubled when a final concentration of 0.8 M NaCl was reached, whereas it remained constant at 0.5 M; (ii) the acetate yield decreased for both NaCl concentrations; (iii) the proportion of glycerol transformed into ethanol strongly increased at 0.5 M, but decreased at 0.8 M NaCl. Surprisingly, pyruvate conversion to acetyl-CoA through PDH decreased when the NaCl concentration increased to 0.5 M and was completely abolished at 0.8 M NaCl. On the other hand, the pyruvate formate lyase (PYRFL) reaction flux correspondingly increased at 0.8 M NaCl.

Table 1. Experimental and theoretical fluxes obtained in the steady states of continuous reactors of *E. coli* with different concentrations of NaCl. Metabolic fluxes were normalized using the glycerol income flux and the experimental as expressed as mean ± standard deviation.

Transformation	Without NaCl		0.5 M NaCl		0.8 M NaCl	
	Experimental Flux	Theoretical Flux	Experimental Flux	Theoretical Flux	Experimental Flux	Theoretical Flux
Feed glycerol	5.36 ± 0.12	5.36	5.82 ± 0.13	5.82	7.91 ± 0.17	7.91
Feed fumarate	0.49 ± 0.04	0.49	0.53 ± 0.04	0.53	0.72 ± 0.06	0.72
Feed CR	1.96 ± 0.04	1.96	2.13 ± 0.04	2.13	2.90 ± 0.06	2.90
Efflluent glycerol	4.36 ± 0.13	4.36	4.82 ± 0.31	4.82	6.91 ± 0.43	6.91
Effluent LC	0.42 ± 0.08	0.42	0.21 ± 0.04	0.21	0.29 ± 0.06	0.29
Effluent CR	0.25 ± 0.05	0.25	0.98 ± 0.05	0.98	1.67 ± 0.07	1.67
Effluent GBB	1.27 ± 0.05	1.27	0.94 ± 0.05	0.94	0.92 ± 0.07	0.92
Ex.ethanol	0.28 ± 0.04	0.28	0.39 ± 0.03	0.39	0.23 ± 0.02	0.23
Ex.pyruvate	0.03 ± 0.00	0.03	0.02 ± 0.00	0.02	0.03 ± 0.00	0.03
Ex.fumarate	0.01 ± 0.00	0.01	0.01 ± 0.00	0.01	0.10 ± 0.04	0.10
Ex.acetate	0.54 ± 0.09	0.54	0.42 ± 0.04	0.42	0.42 ± 0.05	0.42
Ex.lactate	0.11 ± 0.00	0.11	0.11 ± 0.01	0.11	0.24 ± 0.01	0.24
Pro U	0.15 ± 0.00	0.15	0.10 ± 0.00	0.10	0.09 ± 0.00	0.09
T.bio	6.27E-04 ± 0.78E-05	6.27E-04	4.26E-04 ± 0.00E-04	4.26E-04	0.58E-04 ± 0.00E-04	0.58E-04

Taking all these results together, it seems that cells reached a new stationary state at 0.5 M NaCl by reducing NADH formation, which was accomplished by decreasing the flux through NADH-forming pathways, such as PDH, and increasing regeneration reactions as ethanol fermentation. Similarly, the generation of NADH decreased to a greater extent when the NaCl concentration was higher (0.8 M), although the regeneration of NAD changed from ethanol to lactate production.

It is generally accepted that the TCA cycle is repressed in anaerobic conditions; however, as our group has reported previously [9], the glyoxylate pathway flux increased at 0.8 M NaCl. The results obtained from the flux analysis corroborate this (Fig. 2).

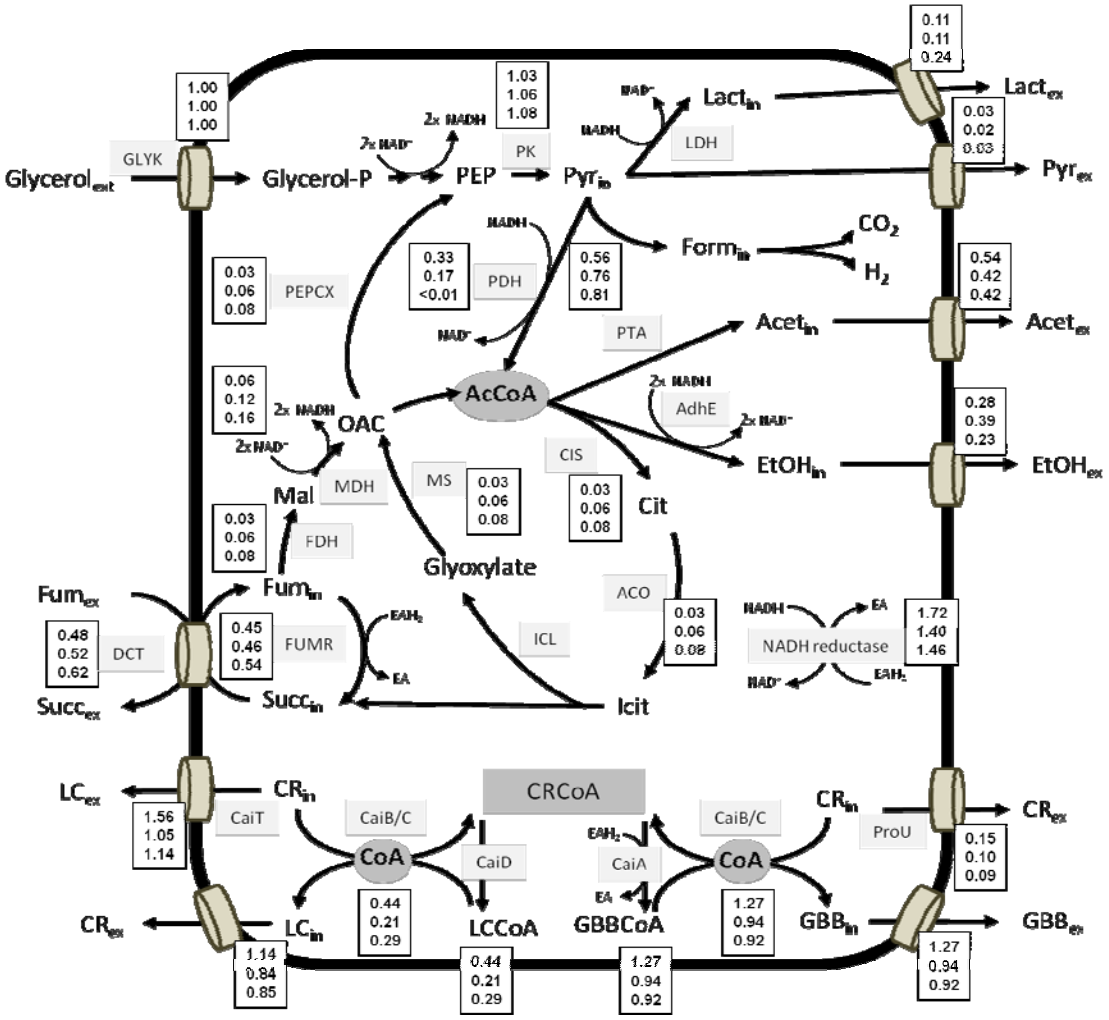


Figure 2. Summarised Metabolic Flux Analysis obtained at the steady states of continuous reactors of *E. coli* with different concentrations of NaCl (see Materials and Methods). Metabolic fluxes were normalized using the glycerol income flux. Data in the box correspond to steady state at 0.085 (control), 0.5 and 0.8 M, respectively. Abbreviations are given in Appendix B.

3. Transcriptional evolution of the adaptation to osmotic up-shift at high (0.5 M NaCl) and very high (0.8 M NaCl) salt concentrations.

Out of more than 10.000 genes represented on the microarrays, as few as 114 genes were significantly changed ($p \leq 0.05$ and at least 2-fold alteration) in the 0.5 M reactor, whereas 229 changned in 0.8 M reactor (Fig. 3). A relevant number of genes were commonly modified (49), as shown in Fig. 3. This fact suggested a common expression strategy to different NaCl concentrations.

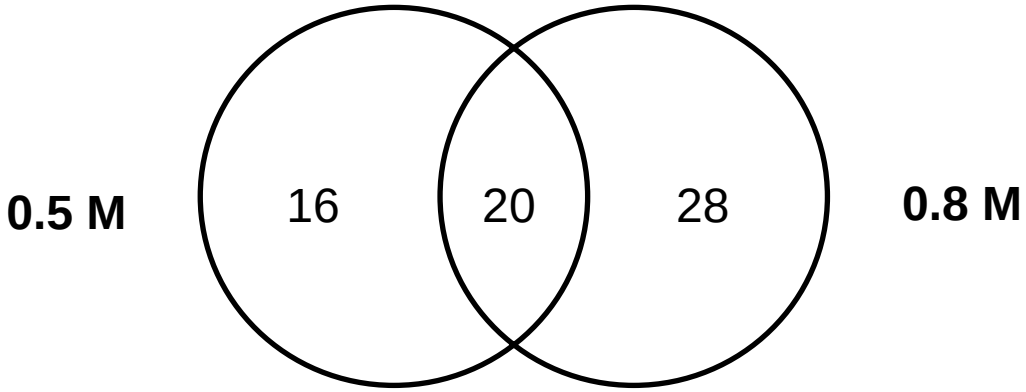
Although common expression strategy was found for both 0.5 and 0.8 M of NaCl, some differences were of note. The common strategies included: (i) inhibition of flagellum development, as well as the concentration-dependent upregulation of (ii) *de novo* nucleotides metabolism, (ii) amino acid and (iii) sulphur transport and metabolism. On the other hand, only at very high NaCl concentration (0.8 M): (i) pyrimidine biosynthesis and (ii) the alcohol dehydrogenase expression were inhibited, while the (iii) lactate dehydrogenase, (iv) formate dehydrogenase and (v) fumarate transport were upregulated.

3.1 Significantly changed genes common to both NaCl concentrations (0.5 M and 0.8 M)

Interestingly, a pattern of gene expression was conserved (Fig. 3), with 20 genes significantly downregulated and 29 genes significantly upregulated in both conditions. Most of the downregulared genes were altered with similar fold change and less than 5-fold repression. GO enrichment analysis (Table 2) identified two downregulated genes which were related to locomotion (*cheW, flgK*) (complete analysis can be found in an additional file). In contrast, several upregulated genes were induced more than 5-fold and with noticeable differences according to the final salt concentration. For example, almost all the glycine betaine transporter genes (*proVWX)* were strongly upregulated, some of them more than 10 times at 0.5 M and more than 60 times at 0.8 M (Table 3), as was

the case for the purine biosynthetic process genes (*purK, purE*) which were upregulated 3 times at 0.5 M and more than 10 times at 0.8 M. Furthermore, two genes related with sulphur assimilation and transport were significantly upregulated (*cysJ and cysP)* more strongly at 0.8 M NaCl.

Downregulated genes



Upregulated genes

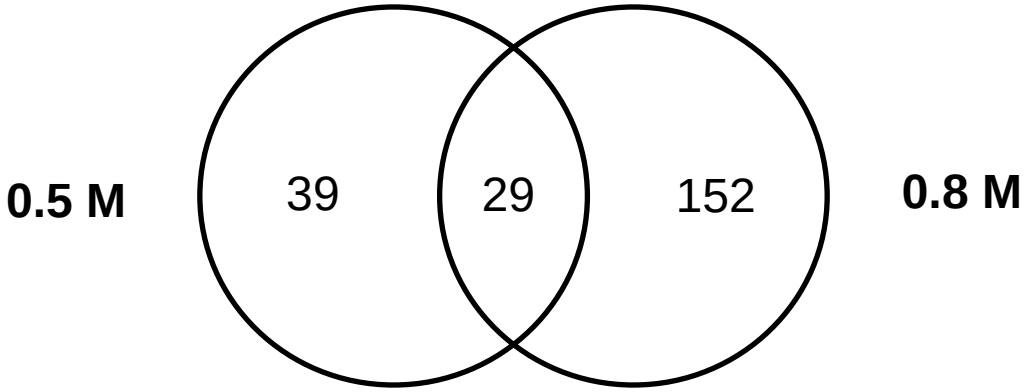


Figure 3. Number of genes significantly changed ($p \leq 0.05$ and at least 2-fold alteration) of *E. coli* in the long-termadaptation to medium and high NaCl concentrations (0.5 and 0.8 M) illustrated in a Venn diagram.

Table 2: Selected Enriched Gene Ontology groups for common genes of *E. coli* in the long-termadaptation to high and very high NaCl concentrations (0.5 and 0.8 M).

GO number	GO name	Count[1]	%[2]	P-value	Gene Ecocyc Ids
	Long-term downregulated Genes				
Biological Process					
GO:0040011	locomotion	2	3.08	1.93E-02	*cheW, flgK*
	Long-term upregulated Genes				
Biological Process					
GO:0006865	amino acid transport	4	4.08	5.11E-04	*pheP, proW, proV, proX*
GO:0000103	sulfate assimilation	1	16.67	2.37E-02	*cysJ*
GO:0009168	purine ribonucleoside monophosphate biosynthetic process	2	8.50	2.00E-03	*purK, purE*
Molecular Function					
GO:0008271	secondary active sulfate transmembrane transporter activity	1	16.67	2.79E-02	*cysP*

[1]Number of found genes for each GO term.
[2]Coverage of the total number of genes for this GO term.

Table 3: Selected common genes of *E. coli* in the long-termadaptation to high and very high NaCl concentration (0.5 and 0.8 M).

Ecocyc Ids	Gene Title	P-value		Fold-Change	
		0.5 M	0.8 M	0.5 M	0.8 M
	Long-term downregulated Genes				
cheW	purine-binding chemotaxis protein	1.75E-02	1.81E-02	-6.03	-7.57
flgK	flagellar hook-associated protein FlgK	2.57E-02	2.66E-03	-2.19	-3.25
	Long-term upregulated Genes				
phe	phenylalanine transporter	1.91E-03	1.56E-03	2.84	3.35
proV	glycine betaine transporter ATP-binding subunit	3.45E-02	1.69E-02	9.59	26.62
proW	glycine betaine transporter membrane protein	9.43E-03	2.03E-03	14.77	83.95
proX	glycine betaine transporter periplasmic subunit	2.38E-02	3.80E-03	11.26	68.25
cysJ	sulfitereductase subunit alpha	2.18E-02	2.23E-03	2.04	11.64
purE	phosphoribosylaminoimidazole carboxylase	1.52E-02	3.90E-03	4.28	18.70
purK	phosphoribosylaminoimidazole carboxylase	9.02E-03	3.40E-03	3.24	10.18
cysP	thiosulfate transporter subunit	8.28E-03	5.00E-03	5.73	8.94

3.2 Significantly changed genes specific to high NaCl concentration (0.5 M).

Clustering analysis (Fig. 4) showed that 36 genes were significantly downregulated, whereas 68 genes were significantly upregulated at the second steady state (Fig. 4, Clusters I and II, respectively). Although a selection of GO terms is shown in Table 4, the complete GO enrichment analysis is included in an additional file.

Surprisingly, most of the upregulated GO terms in the enrichment analysis were described in the common genes section with additional genes, which could suggest a general response independent of the salt concentration.

Table 4: Selected Enriched Gene Ontology clusters of *E. coli* in the long-termadaptation to high NaCl concentration (0.5 M).

GO number	GO name	Count[1]	%[2]	P-value	Gene Ecocyc Ids[3]
	Cluster I. Long-term downregulated Genes				
Biological Process					
GO:0040011	locomotion	2	3.08	3.85E-02	***cheW***, *flgK*
	Cluster II. Long-term upregulated Genes				
Biological Process					
GO:0006865	amino acid transport	4	4.08	5.95E-03	*pheP*, ***proW***, ***proV***, ***proX***
GO:0009168	purine ribonucleoside monophosphate biosynthetic process	2	11.74	7.01E-03	*purK*, *purE*

[1]Number of found genes for each GO term.

[2]Coverage of the total number of genes for this GO term.

[3]Genes are underlined if they are up/down regulated with a fold-change of more than 10 and bold text indicates a fold change of more than 5.

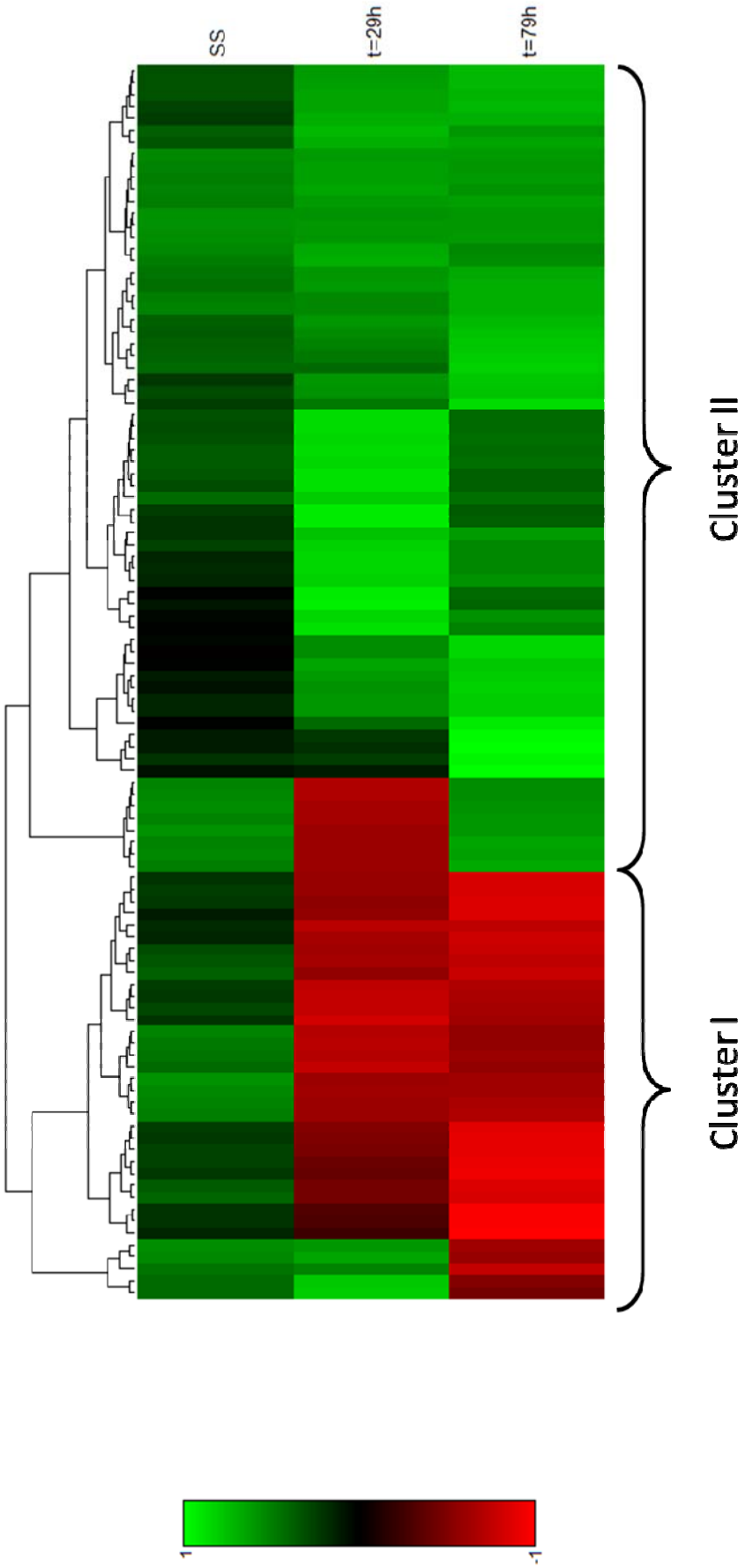


Figure 4.Clustering analysis of transcriptomic changes during long-termadaptation to high NaCl concentration (0.5 M). Time points are ordered chronologically.

3.3. Significantly changed genes specific to a very high NaCl concentration (0.8 M).

Clustering analysis revealed that 48 genes were significantly downregulated, whereas 181 genes were significantly upregulated at the second stationary state (Fig. 5, Clusters I and II, respectively). The complete GO enrichment analysis is included in an additional file. A selection of GO terms is shown in Table 5. Interestingly, besides the common GO terms previously explained, *de novo* pyrimidine nucleotide metabolism, as well as alcohol dehydrogenase activity (adhE), were repressed. Moreover, the expression of several fermentation enzymes, was altered, namely, alcohol dehydrogenase (*adhE*), which was downregulated, and formate (*fdhF and fdoI*) and lactate (*ldhA*) synthesis enzymes genes which were upregulated, suggesting a redistribution in the fermentation pathways. Additionally, fumarate transport (*dctA*) was strongly upregulated.

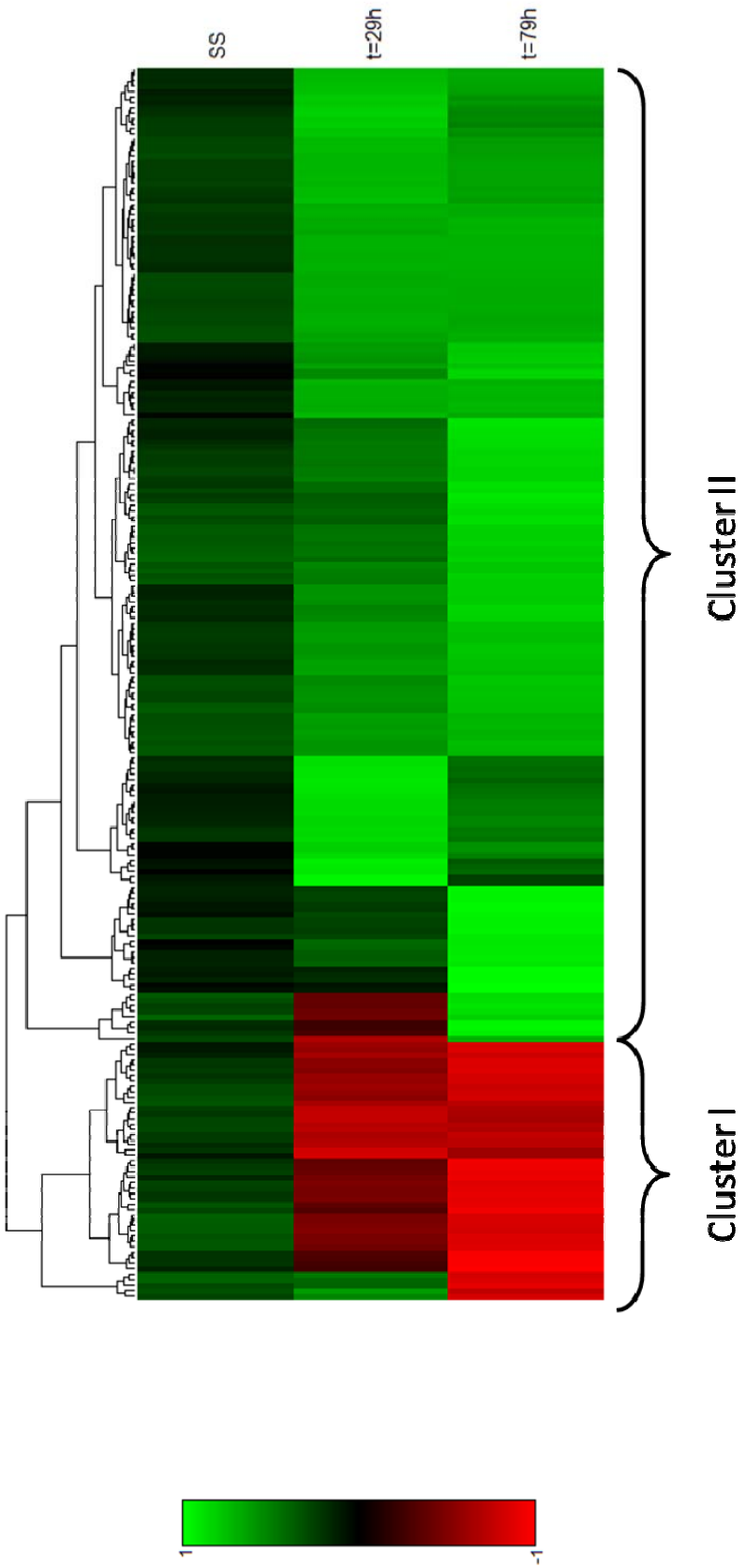


Figure 5.Clustering analysis of transcriptomic changes during long-termadaptation to very high NaCl concentration (0.8 M). Time points are ordered chronologically.

Table 5: Selected Enriched Gene Ontology clusters of *E. coli* in the long-termadaptation to very high NaCl concentration (0.8 M).

GO number	GO name	Count[1]	%[2]	P-value	Gene Ecocyc Ids[3]
Cluster I. Long-term downregulated Genes					
Biological Process					
GO:0009296	flagellum assembly	2	11.74	5.91E-03	*flgK*, ***flhD***
GO:0009173	pyrimidine ribonucleoside monophosphate metabolic process	1	25.00	2.74E-02	*yeiA*
Molecular Function					
GO:0004022	alcohol dehydrogenase (NAD) activity	1	16.67	3.15E-02	*adhE*
Cluster II. Long-term upregulated Genes					
Biological Process					
GO:0009127	purine nucleoside monophosphate biosynthetic process	9	52.94	1.27E-10	*guaB*, ***guaA, purH, purK,purM,*** *purL,purB, purN, purE*
GO:0009408	response to heat	4	14.81	5.67E-03	*hslJ, ldhA, pspA, yhbO*
GO:0015741	fumarate transport	1	100.00	2.72E-02	*dctA*
Molecular Function					
GO:0015419	sulfatetransmembrane-transporting ATPase activity	2	28.57	1.29E-02	*cysA*, ***cysP***
GO:0008863	formate dehydrogenase activity	2	18.18	3.16E-02	*fdhF*, ***fdoI***

[1]Number of found genes for each GO term.

[2]Coverage of the total number of genes for this GO term.

[3]Genes are underlined if they are up/down regulated with a fold-change of more than 10 and bold text indicates a fold change of more than 5.

4. Intra-metabolome comparison of the long-term adaptation to osmotic up-shift at high (0.5 M NaCl) and very high (0.8 M NaCl) salt concentrations.

The intra-metabolome analysis can be found as Appendix A. 2D Clustering was carried out and two clusters of metabolites were found: Cluster I and II. The main changes were related to a decrease of ATP, NADH/NAD and GSH/GSSG ratios and an increment of several amino acids. It is important to highlight that the concentration of NADPH increased by salt presence, particularly at 0.8 M (Tables and figures in Appendix A).

Despite the stress exerted on the environment of cells, their metabolism tended to withstand the robustness of the system. In fact, the acetyl-CoA/CoA ratio remained constant in the three assayed conditions (control, 0.5 and 0.8 M salt concentration).

5. Fermentation profile of the adapted strain to very high salt concentration (0.8 M NaCl).

To check the fermentation profile change due to the osmoadaptative response and to corroborate the presence of an adaptive mechanism, both the wild type and the adapted strain were grown in batch cultures under the three assayed salt concentrations (0.085 (control), 0.5 and 0.8 M NaCl). The metabolic changes were followed through the evolution of the main fermentative metabolites (lactate, ethanol, acetate and succinate) and are summarized in Fig. 6. In both strains, a similar decrease in the acetate and succinate levels was observed. However, lactate and ethanol evolved differently depending on the strain. In fact, the same alteration was shown in the previous continuous experiment. While lactate was not detected in the wild type strain, its concentration increased in the adapted strain with the exposure to salt. On the other hand, the production of ethanol was similar in the wild type regardless of the NaCl concentration, whereas the level of ethanol decreased in the adapted strain as the salt concentration increased.

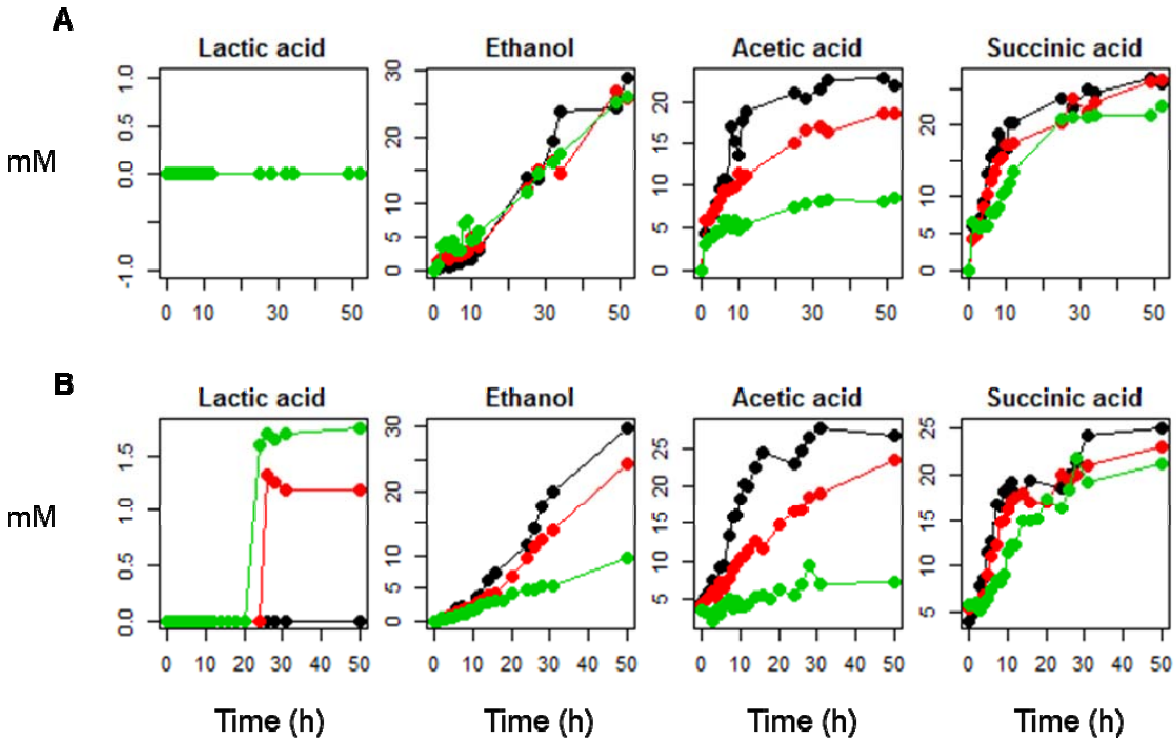


Figure 6. Exometabolite evolution in three different batch cultivations: basal NaCl concentration (0.085 M, black dots), high NaCl concentration (0.5 M, red dots) and very high (0.8 M, green dots). (A) *E. coli* O44K74 (wild type) and (B) *E. coli* O44K74 AS (adapted after 400 h of culture in a chemostat at 0.8 M NaCl).

DISCUSSION

Integration of the different levels assayed (metabolomic, fluxomic and transcriptomic) established the main processes involved in the adaptative response to high salt stress in anaerobic chemostat cultures using a complex medium. Several works have reported the results of the effect of short-term salt stress. The scarce information on the global osmotic response under anaerobic conditions indicated that the number of upregulated genes is lower in anaerobic than in aerobic conditions, emphasizing the overexpression of several porins such as OmpC, transport proteins such as ProV and a scavenger of H_2O_2 [12]. Moreover, even fewer works describe this adaptation using glycerol as C-source, despite its increasing use in anaerobic cultivations to produce fermentation metabolites such as lactate, ethanol or H_2 [25]. Further, glycerol is a byproduct from the bioethanol and biodiesel industries, and its increased production has led to drastically reduced prices [24]. However, the glycerol residue produced in these industries has a high NaCl content; for example, the glycerol residue from palm kernel oil was seen to contained 64.3 % ash, whose principal component was NaCl [26]. Although the ash content can be reduced, it still represents 7% to 20%, depending on the purification conditions [53], and so, NaCl will be present in high concentrations when either crude or refined glycerol residue is used.

To gain a concentration-dependent view of the anaerobic osmoadaptation response, two salt concentrations (0.5 and 0.8 M) were selected since they were shown to result in different survival [9]. We found that some strategies in the long-term salt adaptation were similar for both NaCl concentrations used, even though they were more acute at 0.8 M, namely (i) increased glycerol specific consumption rate [9], (ii) increased relative flux through succinate reduction from fumarate, supported by the increase in the relative flux through fumarate reductase and the higher expression of the transport of fumarate gen (*dctA*), (iii) decreased the relative flux through PDH and increased flux through PYRFL/Formate dHase, as shown in Fig. 2 and in the transcriptomic analysis since the formate dehydrogenase activity

(*fdoI)* was also upregulated, (iv) reduced relative flux towards acetate, supported by the metabolic flux analysis, (v) increased extracellular amino acid uptake as can be seen in Fig. 1 and also supported by the transcriptional analysis, (vi) downregulation of several genes related to motility and locomotion (*cheZ*, *fliS* and *tap*) as previously reported [33] and (vii) increased *de novo* synthesis of purine nucleotides, supported by several upregulated genes of this pathway (Tables 4 to 6) and probably driven by the reduction in the ATP concentration (Appendix A). In general, it seemed that strains developed different strategies to minimize ATP consumption and increase its production. For example, biological processes such as locomotion, which entails the consumption of energy, disappeared while amino acids were taken from the medium instead of being synthesized *de novo*. In order to increase ATP production, the glycerol consumption rate as well as the oxidation of fumarate to succinate increased. This has been shown to act as a mechanism of energy generation similar to that of oxidative phosphorylation mediated by a proton motive force in accordance with Mitchell's chemiosmotic model [54] and similarly with PYRFL/Formate dHase [55]. Surprisingly, the acetate flux proportion was reduced compared with the control NaCl condition and remained constant in both NaCl concentrations, suggesting potential saturation of this reaction and supported by the higher acetyl-CoA and Acetyl phosphate levels (Appendix A).

From an observation of the above cellular strategies, this is the first time that the global amino acid depletion in the medium has been reported in *E. coli*. In complex media, *E. coli* accumulates proline selectively in the face of high osmolarity and no other osmoprotectant such as L(-)-carnitine or betaine is supplemented. If one of these is supplemented, proline accumulatation ceases [3]. Surprisingly, our results showed that amino acid uptake occurred from the beginning of the osmotic stress at both concentrations (0.5 and 0.8 M NaCl). All measured amino acids decreased from the beginning of the salt upshift and this response was maintained throughout the stress despite the fact that biomass also decreased. Although most studies have focused on the role of proline and betaines in stress conditions [3, 5, 56, 57], the presence of other amino acids in the medium seems to exert a positive effect on the

adaptation process. In this regard, several works have reported the capacity of cells to use the resources of the environment in order to economize energetic costs [58-60]. Therefore, the depletion of amino acids in the medium might not be due to their use as osmoprotectors [38], but a way of saving energy and redirecting cofactor redox and coenzymes to other pathways since this would avoid their *de novo* synthesis and they would be available for the turnover of proteins and enzyme machinery if necessary [61]. Moreover, a recent publication of our group pointed out the necessary consumption of peptone [23] and, furthermore, peptone enhanced the growth of *E. coli* even more than glycine-betaine, which is the most powerful compatible solute in bacteria [38]. The transcriptional results agreed with this, since four genes (*pheP*, *proW*, *proV* and *proX*) with the GO term "amino acid transport" were significantly upregulated. Moreover, this was a concentration-dependent effect as was observed in the transcriptional analysis (Table 3), in the uptake dynamics (Fig. 1) and in the intracellular content (Appendix A). Besides, the expression of some of these genes was even higher than 10-fold the control levels at 0.8M NaCl (Table 5). Additionally, genes related to sulphur transport as well as sulphate assimilation were strongly overexpressed at both concentrations (even more at 0.8M NaCl) and intracellular L-cysteine was also depleted (Appendix A), which could suggest that the extracellular level of this amino acid was not sufficient to satisfy its demand at high salt concentrations. This could be important since L-cysteine is an essential amino acid for GSH synthesis [62].

In addition to the above mentioned results, different strategies dependent on salt concentration were found. First, the fermentation pattern was drastically altered: relative ethanol synthesis increased at 0.5 M NaCl, whereas it decreased at 0.8 M, in contrast to lactate, whose ratio was maintained constant at 0.5 M, but increased about 2-fold at 0.8 M (Fig. 2). Transcriptomic analysis supported these results since *adhE* expression was found to be downregulated whereas *ldhA* was upregulated at 0.8 M, reflecting the fluxomics outcome (Fig. 2). Furthermore, the change in the fermentation pattern was also conserved in the adapted strain, as confirmed in batch cultures (Fig. 6). Therefore, these alterations could be considered as an adaptation

mechanism produced as a consequence of long-term adaptation. Subsequently, this mechanism could be available when cells are exposed to the same stress environment. Secondly, the level of redox cofactors was altered depending on NaCl concentration as seen by the shift in the form of the redox cofactor. Thus, at 0.8M NaCl, NADH was depleted, whereas an increase higher than two orders of magnitude was observed in the level of NADPH and NADP, even though their ratio was reduced 2-fold. Thirdly, differences in the level of GSH and GSSG were found, confirming alternative processes to long-term osmoadaptation that depend on the salt concentration. Thus, the level of GSH was similar in control and 0.5 M conditions, while it was not detected at 0.8 M. However, the level of GSSG showed the opposite behaviour it was not observed in control conditions, whereas it presented very low concentration at 0.5 M, and increased at 0.8 M. These findings suggest that a complex process of redox adaptation was being carried out in order to ensure cell survival at very high salt concentrations. GSH could probably be involved, since *E. coli* mutants deficient in their synthesis or regeneration were unable to grow in a medium of high osmolarity [63].

The reorganization of fluxes would be focused on obtaining an adaptive response whose tendency would help to maintain cellular survival and optimize every pathway to avoid unnecessary processes or energy waste. Although the response at 0.8 M NaCl reflected a higher impact of this concentration on the metabolism than 0.5 M, the modifications undertaken allowed cellular integrity to stabilize and the severe conditions of the environment to be overcome. The system could therefore be considered robust and stable since the availability of the main metabolic coenzymes was not limited as the acetylCoA/CoA ratio was relatively constant in the three assayed conditions (control, 0.5 and 0.8 M) (Appendix A).

Overall, our findings are relevant concerning the use of crude glycerol as C-source since a high NaCl concentration seems to be unavoidable without expensive purification. Moreover, it is important that the amino acid depletion of the medium is taken into account when the supplemented medium has a high or very high NaCl

level and an osmoprotectant is present. The shift in the fermentation pattern due to the NaCl concentration could be important for several fermentation products such as lactate, ethanol or H_2 since their relative fluxes have been demonstrated to be drastically altered. Moreover, we observed an increase in succinate reduction reaction from fumarate, which could also be important at biotechnological level, since it has been reported that fumarate can be produced using rice brand and converted into succinic acid using a bacterial fermentation process with glycerol as C-source [29].

CONCLUSIONS

The integration of metabolomic, fluxomic and transcriptomic analyses provides a relevant approach to studying the response to the long-term anaerobic salt exposure. The main strategies observed at the three –omic levels focused on maintaining the availability of energy. Further, the response to the oxidation state and the redistribution of metabolic fluxes pointed to a behavior that depended on salt concentration. Moreover, this work revealed adaptation mechanisms to very high salt concentrations which were conserved by the adapted strain, highlighting their importance. In addition, the information as a whole could be used to enhance biotechnological processes in which crude glycerol is used. Finally, further studies should be carried out to establish the relevance of the switch of redox cofactors observed and the effect of the oxidation state in the stressing conditions.

APPENDIX A

Material and methods. Endometabolome analysis.

Internal metabolic analysis was carried out in two steps: (i) quenching to stop enzymatic activities as quickly as possible, and (ii) metabolite extraction. Quenching was performed by harvesting cells and introducing them into the quenching solution, 60% (vol/vol) methanol/water supplemented with 0.85% ammonium bicarbonate (AMBIC), kept at -48ºC. AMBIC was added since it seemed to reduce osmotic shock and leaking in bacterial [64] as well as eukaryotic cultivations [65], moreover it is compatible with LC/MS analysis. However, controversial results have been found [66]. Afterwards, cells were pelleted by centrifugation at 3,000 x g for 5 min at -12ºC. The contact time with methanol solution was kept as short as possible and the temperature was always kept below -10°C, since both factors have been demonstrated to be critical [67]. The supernatant was removed by aspiration and kept for subsequent analysis to check and quantify leaking [68], samples were kept at -86ºC until extraction.

Metabolite extraction was based on the work of Lazzarino and collaborators [69] with some modifications. Firstly, cultivation samples from the previous step or standard mixtures were re-suspended in 2 mL of extraction solution (acetonitrile + 10 mM KH_2PO_4 (3:1 v/v) at pH 7.4) and then incubated in a wheel for 30 minutes at 4ºC. This homogenate was then centrifuged at 15,000 x g for 20 min at 4ºC. Secondly, the supernatant was separated and added to 4 mL of chloroform and centrifuged again at 15,000 x g for 5 min. This yielded a biphasic system, from which the aqueous phase was harvested. This process was carried out twice more. The extraction procedure was finished by filtering through a sterile 0.2 μm filter before being analysed. It must be remarked that lyophilising or freezing both during and after the extraction procedure was avoided. Therefore, samples were prepared when the analysis platform was ready to avoid potential metabolic degradation since

lyophilisation has been proven to alter the composition of metabolic mixtures [43, 70].

Previous to the quantification process, unequivocal identification of the metabolites was enabled by using the retention time and relative intensities of the diagnostic ions of a pool of samples. Mass spectra of single and pure standards were recorded and compared with the mass spectra of a pool of samples at the corresponding retention time. At least three diagnostic ions (preferably including the molecular ion) should be found, and their relative intensities should correspond to that of the sample, see recent EU regulation for details [71]. If the concentration of the metabolites in the sample was not sufficient to generate a clear spectrum, and a metabolite could not be unequivocally identified, pure calibration standards were spiked and the mass spectrum was recorded again. Due to the limitations of a single quadrupole regarding in the case of isobaric compounds, which cannot be unequivocally identified, their chromatographic separation was confirmed.

Measurements for quantification were conducted using single ion monitoring (SIM). LC-MS experiments were performed on a 1200 series HPLC instrument (Agilent Technologies; California, USA) coupled to an Agilent 6120 single quadrupole mass spectrometer with orthogonal ESI source. The apparatus can be used in positive or negative ionization mode in either SCAN or SIM mode (Agilent Technologies). The mass spectrometer was operated in the positive ESI mode, using the SIM mode for the m/z of each compound. The ion spray voltage was set at 4000 V. Nitrogen with a flux of 12 l/min was used as the sheath gas (35 psi) and the auxiliary gas. The ion transfer capillary was heated to 300ºC. The fragmentation voltage was set at 100 V.

The separation was carried out as previously described [72] on an injection volume of 10 µl using a ZIC-HILIC stationary phase, 150 mm x 4.6 mm internal diameter, and 5 µm particle size, provided with a guard column, 20 x 2.1 mm, 5 µm (Merck SeQuant, Marl, Germany) at a temperature of 25ºC. For metabolite elution, a

gradient method was used with a flow rate of 0.5 ml/min. Mobile phases were 20 mM ammonium acetate (adjusted to pH 7.5 with NH_4OH) in H_2O (solvent A) and 20 mM ammonium acetate in AcN (solvent B). Gradient elution was performed, starting with 0% A and increasing to 80% A over 30 minutes, then back to starting conditions (80-0% A) for 1 minute followed by a re-equilibration period (0% A) of 14 minutes (total run time, 45 minutes). Data were acquired by a PC using the Agilent Chemstation software package provided by the HPLC manufacturer. Afterwards, EasyLCMS [73] was used for automated quantification. Standard and sample areas were normalized using N-acetyl-glutamine since it gives similar results to isotope-labelled standards for several metabolic groups including nucleoside bases, nucleosides, nucleotides, amino acids, redox carriers (NAD^+, $NADP^+$, …), and vitamins, among others [74].

Quality control.

The quality of the results was assessed by: (i) checking the extraction method with standard mixtures, (ii) internal standard (IS), and (iii) quality control samples (QC). The extraction method was validated by comparing the concentration of standard mixtures with and without the extraction process. Recoveries were higher than 85% in all of the analysed metabolites (results not shown). N-acetyl-L-glutamine (m/z 189) was added as IS [74], reaching a final concentration of 50 μM in each analysed sample, and the analysis was monitored by controlling that the internal standard area and retention time were always within an acceptable range. An acceptable coefficient of variation was set at 20% for the peak area and 2% for retention time.

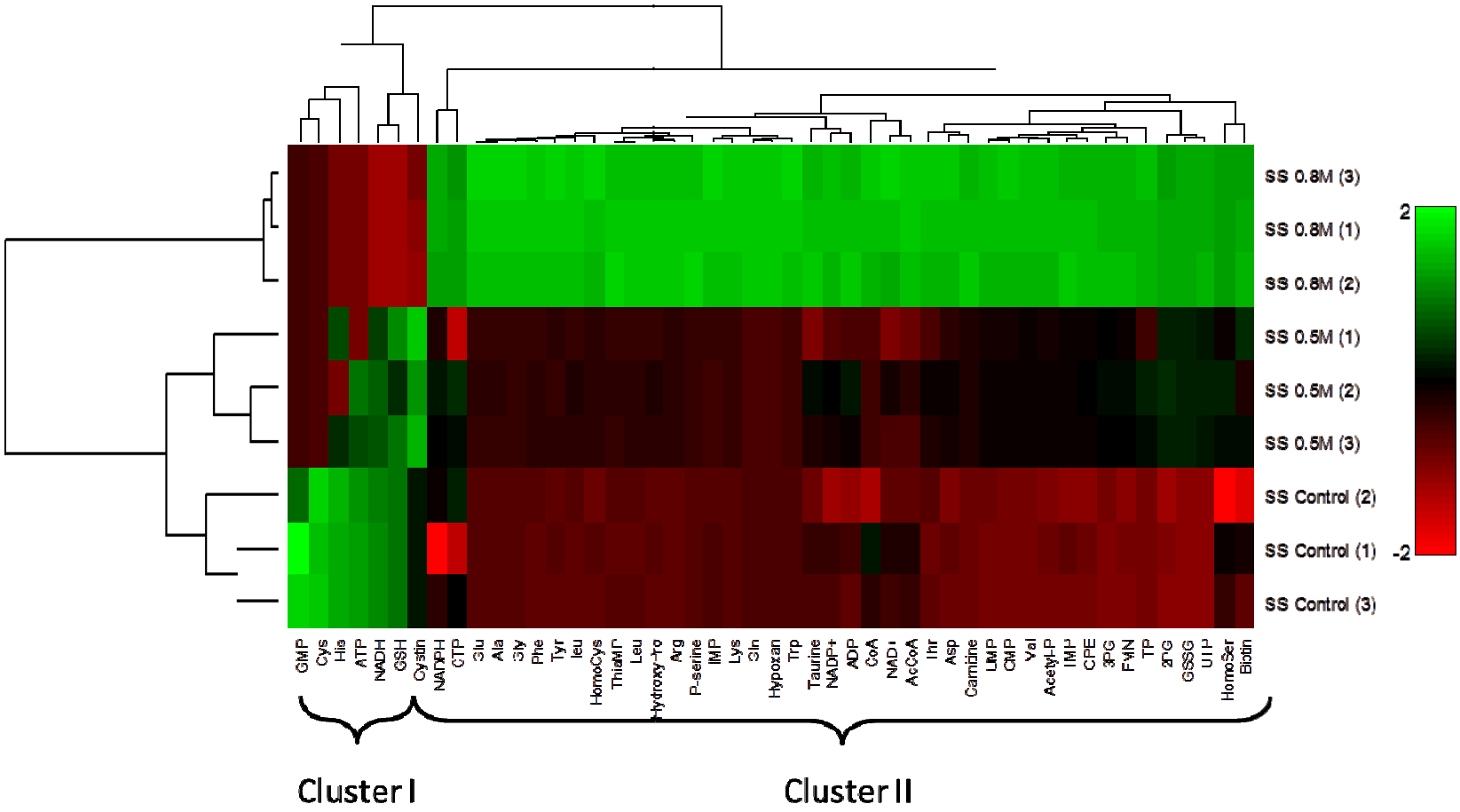
With respect to quality control samples, two types of QCs were incorporated in the analysis: (i) a pool of samples and (ii) a pool of standards. QC analysis was performed in all of the analysed metabolites in the standard pool samples, and in all of those in which concentrations were over the quantification limits in the sample pools. This was carried out by comparing the corrected areas. For the standard pool,

the theoretical corrected area was calculated for the measured concentration. Regarding the pool of samples, the corrected areas were compared among all of the samples. An acceptable coefficient of variation was set at 20% for the peak area and 2% for retention time. QC samples were included in the analysis of the whole set of 20 biological samples. Additionally, samples were randomly introduced into the analysis.

Results.

Intra-metabolome comparison of the long-term adaptation to osmotic up-shift at high (0.5 M NaCl) and very high (0.8 M NaCl) salt concentrations.

To validate the transcriptional response described above, 70 intracellular metabolites were measured. Fig. 1 depicts the metabolites whose concentration changed to a statistically significance extent (ANOVA $p<0.05$) after reaching a new steady state in the presence of 0.5 or 0.8 M NaCl. When 2D clustering was carried out, two clusters were found: metabolites whose concentration fell with increased NaCl level (Cluster I), and those whose concentration increased (Cluster II). As regard the former, it is important to highlight that the concentration of NADH, GSH, His, sulphur-containing amino acids (Cys and Cystin) and ATP were almost depleted in 0.8 M NaCl, and, as a consequence, the NADH/NAD and GSH/GSSG ratios were reduced. As regard Cluster II, the remaining aminoacids increased in concentration at 0.5 M, some of them (Val, Gly and Asp) over 2-fold. Although the same tendency was present at 0.8 M NaCl, the increase in the amino acid concentration was at least 10-fold and Val even showed a 100-fold increase. Moreover, the concentration of NADPH increased with increasing NaCl concentration, particularly at 0.8 M when the concentration was 100 times higher than in control conditions.


SS 0.8M (3)
SS 0.8M (1)
SS 0.8M (2)
SS 0.5M (1)
SS 0.5M (2)
SS 0.5M (3)
SS Control (2)
SS Control (1)
SS Control (3)
2
-2
GMP
Cys
His
ATP
NADH
GSH
Cystin
NADPH
CTP
Glu
Ala
Gly
Phe
Tyr
Ileu
HomoCys
ThiaMP
Leu
Hydroxy-Pro
Arg
P-serine
IMP
Lys
Gln
Hypoxan
Trp
Taurine
NADP+
ADP
CoA
NAD+
AcCoA
Thr
Asp
Carnitine
UMP
CMP
Val
Acetyl-P
IMP
CPE
3PG
FMN
TP
2PG
GSSG
UTP
HomoSer
Biotin
Cluster I
Cluster II

APPENDIX B

List of abbreviations.

Ala	L-Alanine
Arg	L-Arginine
Asn	L-Asparagine
Asp	L-Aspartic acid
Cys	L-Cysteine
Glu	L-Glutamic acid
His	L-Histidine
Iso	L-Isoleucine
Leu	L-Leucine
Lys	L-Lysine
Met	L-Methionine
Pro	L-Proline
Thr	L-Threonine
Tyr	L-Tyrosine
Val	L-Valine
GSH	L-Glutathione
GSSG	L-Glutathione oxidised form
cheZ	cytosolic phosphatase which functions in the chemotaxis signal transduction complex
dctA	proton motive force-dependent C_4-dicarboxylate transporter
fdoI	formate dehydrogenase
fliS	substrate-specific chaperone of the flagellar export system
OmpC (*ompC*)	outer membrane porin OmpC
pheP	phenylalanine APC transporter
ProU (*proU*)	*proU* operon: *proVWX*
ProV (*proV*)	ATP-binding component of transport system for glycine, betaine and proline

proVWX	high-affinity transport system for the osmoprotectant glycine betaines
tap	chemoreceptor
CR	crotonobetaine
CRCoA	crotonobetainyl-CoA
LC	L(-)-carnitine
LCCoA	L(-)-carnitinyl-CoA
GBB	γ-butyrobetaine
GBBCoA	γ-butyrobetainyl-CoA
AcCoA	acetyl-CoA
cit	citrate
etoh	ethanol
form	formate
fum	fumarate
gly	glycerol
glycp	glycerol phosphate
icit	isocitrate
lact	lactate
mal	malate
oac	oxaloacetate
pep	phosphoenolpyruvate
pyr	pyruvate
suc	succinate
ACO	aconitase
AdhE (*adhE*)	alcohol deydhrogenase
CIS	citrate synthase
FDH	fumarate dehydrogenase
FUMR	fumarate reductase
GLYK	glycerol kinase
ICL	isocitrate lyase

LDH (*ldhA*)	lactate dehydrogenase
MDH	malate dehydrogenase
MS	malate synthase
PDH	pyruvate dehydrogenase
PEPCX	PEP-carboxylase
PK	pyruvate kinase
PTA (*pta*)	phosphotransacetylase
PYRFL	pyruvate formate lyase
TCA	tricarboxylic acids cycle

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DISCUSSION

Strategies for optimising biotransformation process from crotonobetaine to L(-)-carnitine by *Escherichia coli* are applied in this work. Several engineered strains were obtained using different molecular biology techniques. These engineered strains were capable of carrying out the biotransformation of crotonobetaine into L(-)-carnitine with, to the best of our knowledge, the highest yield reported to date. Moreover, one of the constraints impairing the carnitine metabolism was successfully overcome, that is, the production of L(-)-carnitine under aerobic conditions. In addition, many questions related to the response of *E. coli* to salt stress have been unveiled, increasing our background of knowledge of the osmoadaptation process. Moreover, this new perspective offers more information about the metabolic processes and important targets related to cell adaptation to hostile environments, which is important when implementing scale-up processes with complex media with a salt content.

As regard L(-)-carnitine production, the optimization of the biotransformation process followed different strategies, which evolved according to the techniques applied. The first aim was to improve the bioprocess at macroscopic level, namely: the biocatalytic environment (i.e. reactor type), the physicochemical operation conditions (i.e. temperature, inducer, presence of oxygen) and cell biocatalytic operation states (growing and resting cells, cell permeabilization or cell immobilization) [1-8]. In this work, aims were focused on obtaining recombinant strains, for which knowledge about the carnitine metabolism and techniques of molecular biology and metabolic engineering were essential. By combining all of these factors, L(-)-carnitine production was improved and relevant information about the best way to develop the optimization of the biotechnological process was obtained.

Although the main genes involved in the carnitine metabolism have been described, no studies have been developed to check the influence of individual proteins on the biotransformation of crotonobetaine to L(-)-carnitine. In light of the good results attained using recombinant strains [9], *caiT*, *caiB* and *caiC* genes, which

codify the transporter, the transferase and the ligase, respectively, of the carnitine metabolism, were selected to be cloned and overexpressed separately in different strains.

In this work, the *caiC* gene was selected to be cloned and overexpressed in *E. coli* (Chapter 1). As expected, the engineered strain, overexpressing *caiC* gene, showed a high yield and productivity. In contrast, *caiB* overexpression led to lower production values. Together, these results emphasized the importance of the CaiC enzyme activity in the carnitine metabolism. Moreover, both enzymes, CaiB and CaiC, were essential for the biotransformation of L(-)-carnitine from crotonobetaine. In fact, the biotransformation could not be carried out by a mutant strain with the *caiB* gene knockout and the *caiC* gene overexpressed. Nevertheless, it has to be remarked that in spite of its important role in the carnitine metabolism, the function of CaiC had only been assigned by sequence homology. Therefore, in this work, CaiC was characterized for the first time and was established as a ligase and transferase enzyme. In addition, the activity of CaiC was proved ATP-dependent according to the results obtained in other works related with L(-)-carnitine production, where the dependence between the biotransformation process and the energetic cellular level (ATP) was indicated [10, 11]. Furthermore, after assaying several substrates, a high specificity of CaiC was determined.

In this work, several techniques are used in order to optimize this biotransformation process. Besides metabolic engineering, cofactor engineering was also shown to be a good tool. The carnitine metabolism was seen to requiere intermediaries of coenzyme A, and, therefore, an alteration in the acetyl-CoA/CoA ratio might affect the fluxes involved in L(-)-carnitine production. To modify the level of the cofactors involved and observe their effect on L(-)-carnitine production different approaches were taken. First, panthotenate, which is a precursor of CoA, was added to the culture medium. This provoked an increase in the availabe CoA and, as a consequence, higher productivity in all strains checked. Then, pyruvate and acetate, which are involved in the availability of acetyl-CoA, were added to the

culture medium separately. Both of them showed a similar effect, decreasing L(-)-carnitine production. Thus, both recombinant DNA technology and controlling the cofactors of the enzymes in the metabolic pathways concerned enhanced the biotranformation process.

Moreover, the use of plasmids as expression systems produces several problems in large scale production processes in continuous systems. Previous works have pointed to the loss of plasmids as a result of segregation [12, 13]. Moreover, it is necessary to use antibiotics and to optimize their concentration since a low level can favour the loss of the plamids concerned. In addition, plasmid-bearing cells suffer a decrease of the specific growth rate and an even higher amount of cellular energy is required to maintain the presence of the introduced plasmid DNA in the host cell [13]. All these factors have led to a reduction in the L(-)-cartinine yield. For this reason, new strategies to achieve a recombinant expression in *E. coli* were implemented (Chapter 2).

Genetic strategies are the primary source of bioproces innovation and are continuously evolving. In this work, several such strategies were applied to enhance L(-)-carnitine production and overcome limitations due to the expression system used, as described above, the expression and regulation of carnitine metabolism and the formation of a side-product, which reduces the L(-)-carnitine production yield [1, 14, 15]. The main strategies were based on the directed disruption of chromosomal genes, abolishing undesired metabolic activities, and the replacement of an endogenous promoter by a constitutive promoter following an optimized procedure [16, 17]. In this way, several engineered strains were obtained, which were characterized by different modifications (knockout genes and/or strong overexpression of target genes due to a constitutive promoter). Thus, an enhancement in L(-)-carnitine production was attained by fine-tuning of the whole gene expression system.

The main modifications were related with the carnitine metabolism, which affected the expression of the structural *cai* operon and the expression of the

transcriptional factor regulator (CaiF). Nevertheles, as in a previous work alteration of the central metabolism led to an improvement in L(-)-carnitine production [18], and so, these modifications were selected for the further engineering of *E. coli* strains. The modifications were performed step by step in *E. coli* BW25113 and their effect was studied by following L(-)-carnitine production. The modifications carried out on the carnitine metabolism provided the best results, a 40-50% increase in the yield with respect to the wild type. Moreover, modification of the central metabolism also showed an improvement of 20% in the relative yield with respect to the wild type. According to these results, alteration of the flux through the TCA cycle is as an important modification, since it exerts a positive effect on the enhancement of L(-)-carnitine production.

Another strategy considered was to implement every positive mutation in a single mutant to obtain a double and even a triple mutant hopefully with the whole set of improvements related to L(-)-carnitine production analyzed in the single strains.

The methodology used allowed the process of cell mutations easily and sequentially. Firstly, the *aceK* gene was deleted. Secondly, the replacement of the promoter p*cai* (endogenous promoter) by p37 (constitutive promoter) was carried out. Finally, the *caiA* gene was also deleted. Nevertheless, an alternative strategy had to be followed to avoid the deletion of *caiT*. The standard procedure involved an antibiotic-marker gene flanked by FRT sites, which allowed elimination of the antibiotic-marker gene by site-specific recombination mediated by FLP recombinase. However, the sequences of the promoter p*cai* and *caiA* gene were so close to each other that the *caiA* gene was deleted using loxP sites. The combination of these modifications led to an overproducer strain whose capacity to produce L(-)-carnitine from crotonobetaine was the highest recorded so far, nearly 100% conversion being possible in the conditions assayed.

Besides the remarkable response of the triple mutant to biotransform crotonobetaine into L(-)-carnitine, the repression of biotransformation under aerobic

condition was also successfully overcome. Throughout the optimization process of L(-)-carnitine production, the presence of oxygen has been regarded as a serious drawback. Moreover, anaerobic conditions were essential for the expression of the carnitine metabolism [1, 14, 19-21]. In this work, all strains, in which the endogenous promoter was replaced showed an ability to produce L(-)-carnitine in the presence of oxygen, with yields ranging from 40 to 60%. Thus, use of the artificial promoter led to the overexpression of target genes and eliminated the regulation of the expression of these genes. In the same way, the modification that replaced the endogenous promoter of *caiF* by an artificial promoter had similar positive effects, increasing L(-)-carnitine production and allowing the biotransformation to be carried out under aerobic conditions, impaired in the wild type strain. Nevertheless, regulation of the expression of *caiF* could not be totally eliminated since the response on L(-)-carnitine production was lower than expected.

Based on the results obtained, all engineered strains showed higher L(-)-carnitine production under anaerobic than aerobic conditions, which suggests that the different metabolisms affect L(-)-carnitine production at energy and cofactor availability level. According to the results presented in Chapter 1, two factors influenced L(-)-carnitine production, the availability of coenzyme A and ATP. Moreover, in Chapter 2, the study on the central metabolism led to similar conclusions, emphasizing the role of acetyl-CoA as a required intermediary in a variety of metabolic pathways. Therefore, an increase in the requirement of acetyl-CoA by other metabolic pathways would decrease its availability, which would be deletereus for an efficient carnitine metabolism in the cells.

In summary, the triple mutant strain was a stable and an overproducing strain, which has been engineered by the optimized methods using simple, easy and sophisticated tools. The properties of this strain would allow its use in continuous systems or in environments under any selective pressure without altering its main capabilities. Therefore, generation of the recombinant strains and modification of any biotransformation process can be implemented in a friendly biotechnology process.

Throughout the two preceding chapters, different techniques were used to optimize a biotechnological process in a direct and individual way with successful results. However, scaling-up a production process could lead to negative consequences and alter results since cells may suffer more environmental pressures than expected. Therefore, even though in this work one of the main aims was the optimization of L(-)-carnitine production, revealing the effect of osmotic stress on the cell metabolism was another goal. This is of prime importance when scaling-up a bioprocess using complex and cheap carbon sources with a high salt content, as is the case with glycerol [22]. In this case the process becomes more complicated as the substrate and the product are osmoprotectants [23, 24] and this affects productivity.

L(-)-carnitine is involved in the response to osmotic stress, where it acts as osmoprotector. This property could offer an advantage in a continuous production system under salt stress, even though it might affect the bioprocess yield and productivity. Thus, a global exploration on the metabolic, fluxomic and genetic response was planned to identify significant points in the osmoadaptation process (Chapter 3 and 4). Using suitable techniques many changes in the physiology and metabolism of the microorganisms were detected. Moreover, a system biology approach offered a way to obtain and integrate a large amount of information, preventing some of the information being overlooked by the massive amount of data.

When the osmoadaptation process of the central metabolism in *E. coli* O44K74 was studied, key points were identified which could be exploited to enhance any industrial biotransformation.

In Chapter 3, the cells grown in continuous reactors were maintained at different salt concentrations (moderate, high and very high) in order to identify (or determine) the key points in the long-term effect. In addition, anaerobic growth, complex media and the presence of an osmoprotector such as crotonobetaine and L(-)-carnitine were considered for the adaptation process [25]. The response to salt stress was followed by measuring the biomass content, certain selected key enzyme activities of the central metabolism and the main fermentative metabolites produced

in a continuous reactor at three different salt concentrations (Chapter 3). Moderate salt concentration produced a subtle response, emphasizing the capability of cells to tolerate that level of stress. On the other hand, the higher salt concentrations led to evolution in the metabolic response, depending on the concentration of salt in the media. Moreover, the effect on the biomass growth was indicative of the pressure exerted by the environment. As mentioned above, the strongest responses were obtained from the cells growing within the highest salt concentration. This salt concentration (0.8 M) provoked a considerable decrease in the biomass level. However, in these conditions, higher specific rates of metabolite production and glycerol consumption were observed, pointing to the necessity of energy and the optimization of the available resources. In fact, the ATP cell content showed a noticeable increase when the NaCl content was increased to 0.5 and 0.8 M. Despite the ATP level being restored, the requirement of energy was a constant feature in the cell stress response. Therefore, the increase in the acetate formation rates underlined the importance of the Pta-Ack pathway, which would allow ATP production. Moreover, in the extreme environment the whole metabolism was rearranged to maintain the cell survival state. In fact, the modification of the activity of the key enzymes involved in the central metabolism supported the necessity of increasing biosynthetic precursors, emphasizing glyoxylate and anaplerotic pathways as two important nodes in the stress response.

On the other hand, *E. coli* cells isolated from the reactor at 0.8 M NaCl were used to verify osmoadaptation. Thus, wild type and adapted strains were grown in batch reactors under standard conditions. Although biomass concentrations reached similar values, the growth rate was higher for the adapted strain. Moreover, production rates of several metabolites and the consumption rates of fumarate and glycerol showed the same tendency, revealing changes in the metabolism. Most results suggested that adapted cells showed different degrees of metabolic intensity. Furthermore, genes related to stress response were analyzed, corroborating the results mentioned above.

In Chapter 4, the metabolic changes resulting from the osmotic stress were studied at different cellular levels (metabolic, fluxomic and transcriptomic). Moreover, using system biology, all the results were included in a model to attain an integrated view of the mechanisms used by the cells to adapt to extreme conditions. Furthermore, this work identified key points which could be modified in order to improve any biotechnological process.

The cell metabolism responded by showing both similar and alternative strategies influenced by the salt concentration. In general, the requirement of energy was the main response. Thus, many biological processes where ATP was necessary were down-regulated, most of them related to motility. Surprisingly, another way to reduce energetic costs was revealed in relation to the uptake of amino acids from the medium. In this way, amino acids could be available for the turnover of proteins without using energy. Furthermore, many reactions with the capability to generate ATP were increased, e.g. the reduction of fumarate to succinate or even the reaction catalyzed by PYRFL/Formate dHase, since in both of them a proton gradient could be generated [26, 27]. Moreover, the glycerol consumption rate increased with the salt concentration (Chapter 3 and 4).

A metabolic flux analysis study under the different salt concentrations showed shifts in the distribution of the main fluxes of the main fermentation pathways. Beside the cells' need for energy, this flux redistribution could be associated with the oxidation state of the cell internal environment [28]. Thus, whereas at high salt concentration the main fluxes involved in the consumption of glycerol were predominantly directed towards ethanol and acetate, at a very high salt concentration the flux towards ethanol switched to lactate. Surprisingly, although acetate production was shown as an alternative way to produce ATP (Chapter 3), the acetate flux remained at the same level, pointing to a possible saturation of the process.

The differences noticed within the cell metabolism due to salt concentrations were also registered at transcriptomic level. Thus, at the very high salt concentration

the alcohol dehydrogenase expression was down-regulated, whereas lactate dehydrogenase, formate metabolism as well as fumarate transport were up-regulated. Moreover, the transcriptome analysis provided an expression pattern that depended on the salt concentration in the medium. Thus, a set of genes showed a similar response independently of the salt level. Most of them were related to the proper and efficient use of the available resources. On the other hand, the effect of salt was observed in another set of genes which changed their level of expression, leading to general or specific responses according to the concentration of salt within the reactor.

Finally, the level of alteration of the main redox cofactors confirmed the salt concentration-dependent changes in the cell oxidation state and the adaptation process achieved. Moreover, the constant acetylCoA/CoA ratio meant the new cell metabolic state was stable and robust. In fact, the adaptation acquired in this process remained in the adapted cells, which adopted similar strategies to overcome analogous stress situations.

In summary, in this work, a biotransformation process was enhanced using metabolic and genetic engineering tools. Moreover, the implementation of several techniques led to advances in the strategy of strain improvement. Thus, many restrictions which limit production were successfully overcome. Both the system biology approach and the use of experimental data within the *in silico* model increased our understanding of the cell adaptative response to osmotic stress. Furthermore, many key points have been highlighted, contributing to the background knowledge concerning the related processes. In addition, this information could be used to improve biotechnological processes under the stress conditions found in high-scale industrial bioreactors using complex media containing glycerol.

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CONCLUSIONS

1. The application of recombinant DNA techniques enhanced L(-)-carnitine production by *Escherichia coli* LMG194. Cloning and overexpresion of the *caiC* gene enabled this increment in the biotransformation process. Moreover, *caiC* gene was characterized for the first time as a betaineCoA: ligase and transferase protein coding gene.

Cofactor engineering was shown as an alternative and feasible tool for rearranging metabolic fluxes related to the biotransformation process. Moreover, the increase in the availability of the cofactors had a positive effect on L(-)-carnitine production. In addition, Coenzyme A was necessary for substrate activation in the reactions catalyzed by CaiC.

2. *E. coli* BW25113 was engineered by implementing diverse strategies at chromosomal level (deletion of *aceK* and *caiA* and replacement of the *cai* promoter). Mutations in the genome related with carnitine metabolism led to successful results in L(-)-carnitine biotransformation.

The highest increase in specific L(-)-carnitine production rate and yield (95% of conversion) was obtained with the triple mutant *E. coli* BW25113 Δ*aceK* Δ*caiA* p37*cai*.

Replacement of the endogenous promoters p*caiF* or p*cai* by the constitutive promoters p8 or p37 allowed that L(-)-carnitine to be produced under aerobic conditions, overcoming the major drawback in L(-)-carnitine production.

The triple mutant, in resting conditions, reached almost total conversion from crotonobetaine to L(-)-carnitine in less than two hours with a productivity 2-fold higher than the wild type.

3. *E. coli* O44K74 was adapted to high osmotic stress as a result of long-term exposure to a high salt concentration in continuous reactors. The adapted strain presented a more active metabolism than the wild type under the same conditions.

Moreover, the Icdh/Icl, Pta/Acs and Pck/Ppc ratios were involved in the response to stress and long-term adaptation, emphasizing involvement of the glyoxylate shunt and anaplerotic pathways to provide biosynthetic intermediaries and acetate pathway to obtain energy.

4. The main processes involved in the adaptative response under anaerobic conditions and in a complex medium are reported in this work by implementing metabolomic, fluxomic and transcriptomic analyses.

A common response was revealed regardless of the salt concentration: the energetic state. Energy availability was highly important in the response to stress and the metabolism was focused on decreasing unnecessary ATP-dependent activities, whereas alternative pathways to produce ATP increased.

The cell internal oxidation state was influenced by the salt concentration. Variation of redox cofactors and flux redistribution were taken as an adaptative mechanisms which was shown in both chemostat and batch reactors.

Supplementation of amino acids and osmoprotector in the medium could be considered a good strategy in biotechnological processes using glycerol as an inexpensive C-source and even where high or very high salt concentrations are present.

At very high salt concentration a whole set of metabolomic, fluxomic and transcriptomic responses seems to be involved in maintaining the survival state, exploiting the resources available in the environment.

System biology proved to be an efficient tool to unravel and understand a complicated process in which many experimental data are involved. Moreover, it should contribute to the development of further experiments to increase our background knowledge related to this process.