Universidad de Murcia

Facultad de Química

Departamento de Bioquímica y

Biología Molecular B e Inmunología

PRODUCCIÓN DE L(-)-CARNITINA MEDIANTE CEPAS DE *Escherichia coli*: APLICACIÓN DE LAS INGENIERÍAS METABÓLICA Y GENÉTICA

Vicente Bernal Sánchez

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Murcia, 15 de enero de 2007

D. JOSÉ LUIS IBORRA PASTOR, 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 "Producción de L(-)-carnitina mediante cepas de *Escherichia coli*: aplicación de las ingenierías metabólica y genética", realizada por D. Vicente Bernal Sánchez, bajo mi inmediata dirección y supervisión, en el Departamento de Bioquímica y Biología Molecular B e Inmunología, y que presenta para la obtención del grado de Doctor por la Universidad de Murcia.

En Murcia, a 15 de enero de 2007.

Fdo: Dr. D. José Luis Iborra Pastor.

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 "Producción de L(-)-carnitina mediante cepas de *Escherichia coli*: aplicación de las ingenierías metabólica y genética", realizada por D. Vicente Bernal Sánchez, bajo mi inmediata dirección y supervisión, en el Departamento de Bioquímica y Biología Molecular B e Inmunología, y que presenta para la obtención del grado de Doctor por la Universidad de Murcia.

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Fdo: Dr. D. Manuel Cánovas Díaz

D. JOSÉ CARLOS GARCÍA BORRÓN, Catedrático de Universidad del Área de Bioquímica y Biología Molecular y Director del Departamento de Bioquímica y Biología Molecular B e Inmunología, INFORMA:

Que la Tesis Doctoral titulada "Producción de L(-)-carnitina mediante cepas de *Escherichia coli*: aplicación de las ingenierías metabólica y genética", ha sido realizada por D. Vicente Bernal Sánchez, bajo la inmediata dirección y supervisión de D. José Luis Iborra Pastor y D. Manuel Cánovas Díaz, y que el Departamento ha dado su conformidad para que sea presentada ante la Comisión de Doctorado.

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Fdo: Dr. D. José Carlos García Borrón.

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

Artículos

- Link between primary and secondary metabolism in the biotransformation of trimethylammonium compounds by *Escherichia coli.* 2003. M. Cánovas, **V. Bernal**, T. Torroglosa, J.L. Ramírez, J.L. Iborra. Biotechnol Bioeng 84:686-699.
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- Analysis of *Escherichia coli* cell state by flow cytometry during whole cell catalyzed biotransformation for L-carnitine production. 2007. M. Cánovas, V. García, **V. Bernal**, T. Torroglosa, J.L. Iborra. Process Biochem 42:25-33.
- *Escherichia coli* central and trimethylmmonium metabolism model for L-carnitine production optimisation. 2005. A. Sevilla, J. W. Schmid, K. Mauch, **V. Bernal**, J.L. Iborra, M. Reuss, M. Cánovas. J Biotechnol 118S1:S3.
- Role of the energetic coenzyme pools on the optimization of the L(-)-carnitine production by *Escherichia coli.* 2006. M. Cánovas, A. Sevilla, **V. Bernal**, R. Leal, J.L. Iborra. Metab Eng 8:603-618
- Salt stress affects the central and carnitine metabolisms of *E. coli.* 2007. M. Cánovas, **V. Bernal**, A. Sevilla, J.L. Iborra. Biotechnol Bioeng (in press). DOI: 10.1002/bit.21128
- Plasmid maintenance and physiological adaptations of a genetically engineered *Escherichia coli* strain durnig continuous L-carnitine production. **V. Bernal**, M. González, M. Cánovas, J.L. Iborra. Process Biochem (submitted)
- Fuctional characterization and role of L-carnitine transport in an industrial strains: *Proteus sp.*. **V. Bernal**, M, Olk, M. Cánovas, J.L. Iborra. (in preparation).
- Role of betainyl-coenzyme A ligase (CaiC) in the activation of betaines and the transference of coenzyme A in *Escherichia coli.* **V. Bernal**, P. Arense, V. Blatz, M.A. Mandrand-Berthelot, M. Cánovas, J.L. Iborra. Biotechnol Lett (submitted).
- Cofactor engineering for the improvement of L(-)-carnitine production in *Escherichia coli.* **V. Bernal**, B. Masdemont, P. Arense, M. Cánovas, J.L. Iborra. J Biotechnol (submitted).

Capítulos de libro

- Modelling of biotransformation processes in high-density cell recycle membrane reactors: production of L-carnitine by *E. coli* and *P. mirabilis* strains, a case study. M. Cánovas, **V. Bernal**, A. Sevilla, J.L. Iborra. In: ECCE. 4th European Congress of Chemical Engineering. "Chemical Engineering, a Tool for Progress". Topic 11 Abstracts. 629th Event of the European Federation of Chemical Engineering, pp. 1-3. ISBN: 84-88233-33-7
- Integrating the microkinetics of the L-carnitine and central metabolism in *Escherichia coli* with the macrokinetics of a high-cell density reactor. (2004) M. Cánovas, A. Sevilla, **V. Bernal**, J.L. Iborra. In: Computer Applications in Biotechnology 147-152.
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- Metabolic flux improvement through cofactor engineering during L(-)-carnitine production by *E. coli*. **V. Bernal**, P. Arense, B. Masdemont, A. Sevilla, M. Cánovas, J.L. Iborra. In: "Understanding and Exploiting Systems Biology in Bioprocess and Biomedicine". Eds.: J.L. Iborra Pastor, M. Cánovas Díaz. ISBN. 84-611-1135-4. Depósito Legal: MU-1036-2006.

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- Respuesta dinámica a perturbaciones del metabolismo de *E. coli* O44K74 en la producción de L-carnitina en reactores de alta densidad celular. M. Cánovas, A. Sevilla, **V. Bernal**, R. Leal, P. Holbach, V. del Pino, J.L. Iborra. Poster. BIOTEC 2004. Oviedo, 2004.
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- Redirecting metabolic fluxes through cofactor engineering: role of CoA-esters pool during L(-)-carnitine production by *E. coli*. **V. Bernal**. P. Arense, B. Masdemont, A. Sevilla, M. Cánovas, J.L. Iborra. Oral communication. 6th European Symposium on Biochemical Engineering Science. Salzburg, 2006. Austria.
- Expresiones enzimáticas y niveles de coenzimas energéticos tras perturbaciones metabólicas durante la producción de L-carnitina por *Escherichia coli*. M. Cánovas, A. Sevilla, **V. Bernal**, J.L. Iborra. Oral communication. Biospain-Biotec 2006. Madrid. Spain.
- Uso de células durmientes, permeabilizadas y dañadas en la mejora de biotransformaciones con enterobacterias: la producción de L(-)-canitina. A. Sevilla, C. Bernal, R. Teruel, **V. Bernal**, P. Arense, B. Masdemont, M. Cánovas, J.L. Iborra. Poster. Biospain-Biotec 2006. Madrid. Spain.
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- El transporte de L(-)-carnitina en *Proteus sp.* **V. Bernal**, M. Olk, A. Sevilla, B. Masdemont, P. Arense, C. Bernal, R. Teruel, M. Cánovas, J.L. Iborra. Poster. Biospain-Biotec 2006. Madrid. Spain.
- Conexión del pool de coenzima-A con el metabolismo secundario en *Escherichia coli*: optimización de la producción de L(-)-carnitina mediante ingeniería de cofactor. **V. Bernal**, P. Arense, B. Masdemont, A. Sevilla, C. Bernal, R. Teruel, M. Cánovas, J.L. Iborra. Poster. Biospain-Biotec 2006. Madrid. Spain.
- Análisis de flujos metabólicos del metabolismo central y el de carnitina en *Escherichia coli* para incrementar su biosíntesis. R. Teruel, A. Sevilla, C. Bernal, **V. Bernal**, P. Arense, B. Masdemont, M. Cánovas, J.L. Iborra. Poster. Biospain-Biotec 2006. Madrid. Spain.

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Capítulo 4

El firmante de esta memoria ha disfrutado, durante el periodo 2002-2005, de una beca de la Fundación Séneca (Comunidad Autónoma de la Región de Murcia) para la Formación de Personal investigador (FPI) y, durante el periodo 2005-2006, de una Beca de Investigación de la Fundación CajaMurcia. Este trabajo ha sido subvencionado por los proyectos BIO 2002-04157-C02-01 y PB/10/FS/02 ambos titulados "Optimización de la síntesis de L-carnitina a partir de compuestos de trimetilamonio mediante cepas salvajes y modificadas genéticamente y el uso de ingenierías metabólica y genética" respectivamente, concedidos por la Comisión Interministerial de Ciencia y Tecnología y la Fundación Séneca de la Comunidad Autónoma de la Región de Murcia.

Murcia, a 15 de enero de 2007.

Vicente Bernal Sánchez.

RESUMEN

En la presente Tesis Doctoral se ha llevado a cabo la optimización de la producción de L(-)-carnitina empleando el metabolismo de compuestos de trimetilamonio de *Escherichia coli* desde los puntos de vista del bioproceso y el metabolismo.

Se ha llevado a cabo un análisis sistemático de los principales factores que afectan al proceso de producción de L(-)-carnitina empleando cepas de *Escherichia coli*, estudiándose el efecto que tiene la disponibilidad de aceptores electrónicos (tales como oxígeno molecular o fumarato) tanto en condiciones de operación en discontinuo como en continuo. El análisis empleando citometría de flujo del efecto sobre las células nos permitió determinar las respuestas en los niveles de DNA, RNA y proteínas, y la integración de estos datos con la heterogeneidad de las poblaciones celulares. Esto nos permitió analizar de una manera novedosa el modo en el que la configuración del reactor afecta a la fisiología de las células. Además, se estudió el contenido en DNA de las células de *E. coli* en cultivos continuos, permitiéndonos determinar los factores que determinan la estabilización genética de la cepa inmovilizada. Además, la expresión del metabolismo secundario se coordinó con la de las rutas centrales a través de proteínas reguladoras generales, permitiendo la evolución de los *pools* de cofactores y metabolitos la unión o integración de ambos metabolismos. El análisis metabólico en condiciones de estrés reveló un aumento en la productividad debido a la permeabilización celular y a la activación de las rutas metabólicas de generación de energía y precursores. Se clonó, sobreexpresó y caracterizó parcialmente la proteína CaiC, revelándose como una ligasa de CoA altamente específica. Además, se construyeron cepas de sobreexpresión y deleción de las actividades CaiB (CoA transferasa) y CaiC (CoA ligasa) estudiándose el efecto de estas modificaciones sobre la producción de L(-)-carnitina y subrayando la importancia de la activación de sustrato. Finalmente, la expresión del ciclo de los ácidos tricarboxílicos, el ciclo del glioxilato y el metabolismo de acetato, que está relacionada con la biotransformación, limita la productividad máxima, y se han desarrollado y comprobado experimentalmente nuevas estrategias de mejora de cepas para la producción de L(-)-carnitina.

En conjunto, el metabolismo y la fisiología celular están estrechamente relacionados con el modo de operación del reactor y contribuyen conjuntamente a en la determinación del rendimiento del proceso. Se ha profundizado en el esclarecimiento del papel del estado metabólico celular en la determinación de la producción de L(-)-carnitina, especialmente en los niveles de cofactores (ATP y

acetil-CoA/CoA). Además, se ha mostrado que el modo de operación del biorreactor, que determina el estado fisiológico de *Escherichia coli,* limita en gran medida el rendimiento del proceso. Además, se ha determinado la función de CaiC y, sobre todo, se ha establecido la importancia de considerar conjuntamente los metabolismos central y secundario en la mejora de cepas con fines biotecnológicos.

INTRODUCCIÓN

PERSPECTIVA HISTÓRICA DE LA L(-)-CARNITINA

La L(-)-carnitina (R(-)-3-hidroxi-4-trimetilaminobutirato) es un compuesto ubicuo que se encuentra en tejidos de origen animal y vegetal, así como en numerosos microorganismos. La L(-)-carnitina se encontró por primera vez a principios del s.XX, en extractos musculares, lo que le dio su nombre (derivado del latín ***caro, carnis,*** carne). Este descubrimiento lo llevaron a cabo, casi simultáneamente, Gulewitch y Krimberg en Rusia y Kutscher en Alemania. Durante muchos años, su función y estructura permanecieron desconocido, hasta que en 1927, Tomita y Sendju confirmaron su estructura química y, ya en los años 30, comenzaron las primeras investigaciones acerca de las funciones fisiológicas de L(-)-carnitina, que fueron llevadas a cabo por Strack en la Universidad de Leipzig. Fraenkel y sus colaboradores descubrieron que L(-)-carnitina era un factor esencial de crecimiento para la larva del escarabajo negro (*Tenebrio molitor*), junto con el ácido fólico y otras vitaminas B. Esto motivó que se denominara a L(-)-carnitina vitamina B_T. En 1955, Fritz encontró que la tasa de metabolización mitocondrial de grasas depende de los niveles de L(-)-carnitina, estableciéndose de esta forma, su función principal por vez primera. Durante la segunda mitad del siglo XX continuó la investigación básica en el papel metabólico de L(-)-carnitina, así como en sus aplicaciones. En los años ochenta, L(-)-carnitina comenzó a estar disponible comercialmente, y en 1993 se reconoció como GRAS (Generally Recognized As Safe) como suplemento dietético por un comité independiente de científicos.

FUNCIÓN FISIOLÓGICA DE L(-)-CARNITINA EN HUMANOS

L(-)-carnitina tiene un papel importante en varios puntos del metabolismo intermediario. Son ejemplos: la β-oxidación de ácidos grasos de cadena larga y media en la mitocondria, la oxidación de ácidos grasos en los peroxisomas, el intercambio de grupos acilo y acetilo con CoA en la mitocondria (alterando así las relaciones acil-CoA/CoA y carnitina-CoA/CoA) y la producción de cuerpos cetónicos. Acetil-L(-)-carnitina puede considerarse una segunda forma de grupo acetilo activado, un sistema tamponador de acetilo o un depósito de grupos acetilo (Löster, 2003). Por tanto, L(-)-carnitina se considera una parte integral y esencial del catabolismo de ácidos grasos en la mitocondria, estando íntimamente ligada a acetil-CoA en diversos orgánulos, en los que tiene una función fundamental en el metabolismo energético. La metabolización de los lípidos de reserva permite obtener energía en periodos más

prolongados y en mayor cantidad que los correspondientes carbohidratos de reserva, por lo que L(-)-carnitina se usa, en general, en todas las funciones corporales con una demanda energética elevada. Esta función tiene especial relevancia en el corazón, puesto que, por su superior necesidad energética, depende fundamentalmente de la metabolización de lípidos y ácidos grasos (Löster, 2003). Además, acetil-L(-)-carnitina participa en el metabolismo neuronal como dador de grupos acetilo en la biosíntesis de acetil-colina. Se han establecido muchas otras funciones de L(-)-carnitina. En resumen, se ha probado su implicación en el metabolismo de corazón, hígado, músculo, cerebro y en el metabolismo lipídico, así como se le reconoce un cierto papel en la maduración del esperma, en el sistema inmune y en el tejido conectivo (Löster, 2003).

L(-)-carnitina está presente en cantidades variables en los alimentos de origen animal, mientras que frutas y verduras contienen cantidades muy pequeñas o incluso nulas. El cuerpo humano produce una cantidad pequeña de L(-)-carnitina, encontrándose especialmente concentrado en los músculos cardiaco y esquelético, donde lleva a cabo su función fisiológica. Los adultos almacenan unos 20 g de L(-)-carnitina, fundamentalmente en músculo esquelético, hígado y corazón. Los principales lugares para la síntesis de L(-)-carnitina son hígado y riñón (Löster, 2003) y consiste en una serie de pasos que requieren dos aminoácidos esenciales, lisina y metionina, como sustratos y vitamina C, vitamina B_6, hierro y niacina como cofactores, por lo que una nutrición deficiente puede disminuir su biosíntesis. El cuerpo humano produce diariamente alrededor de 20 mg de L(-)-carnitina, lo que representa un 10% de sus necesidades totales. Una dieta equilibrada puede aportar unos 100-300 mg de L(-)-carnitina por día. Curiosamente, L(-)-carnitina es un componente esencial en la dieta de los niños, puesto que la biosíntesis está muy limitada en los primeros meses de vida.

En resumen, las necesidades del organismo de L(-)-carnitina se pueden suplir tanto por la síntesis endógena como por la alimentación. En un organismo sano este compuesto no se cataboliza, aunque sí se excreta en la orina, principalmente en forma de ésteres. En adultos sanos se almacenan cantidades suficientes de L(-)-carnitina, no siendo precisa una contribución adicional en la dieta, aunque ésta puede ser de importancia en determinadas enfermedades, durante el embarazo o la vejez, así como en el caso de deficiencias específicas (Borum, 1991). L(-)-carnitina puede considerarse, por tanto, un nutriente esencial. Se define su deficiencia como un déficit intracelular de L(-)-carnitina, encontrándose en concentraciones inferiores a 20 µmol/L y concentraciones tisulares de menos del 10-20% de los valores normales. Este déficit

conduce a una acumulación de ésteres de acil-CoA y a la inhibición del transporte de grupos acilo a través de la membrana mitocondrial interna. Las deficiencias de L(-)-carnitina se han subdividido en primarias (debido a defectos básicos del metabolismo de L(-)-carnitina) y secundarias (ocasionadas por otras enfermedades o desórdenes). Las deficiencias primarias de L(-)-carnitina se caracterizan por miopatías, episodios de hipoglucemia hipocetónica, hiperamonemia, fallos en el crecimiento y cardiomiopatías. Las deficiencias secundarias están asociadas a otras anormalidades metabólicas, caracterizadas por síntomas clínicos heterogéneos, con un exceso de lípidos y una baja concentración de carnitina en músculo. En otros casos, la deficiencia puede ser adquirida, como ocurre en la hemodiálisis, cuando se sigue nutrición parenteral, en la terapia con ácido valproico, en pacientes que sufren cirrosis hepática con caquexia, en el síndrome de Reye, en varias enfermedades musculares crónicas, desórdenes endocrinos, síndrome de la inmunodeficiencia adquirida (SIDA), kwashiorkor y en cardiomiopatía ocasionada por difteria.

APLICACIONES DE L(-)-CARNITINA

Se han encontrado numerosas aplicaciones para L(-)-carnitina. En primer lugar, se administra en el tratamiento de las deficiencias específicas tratadas en el apartado anterior. Además, L(-)-carnitina tiene una función importante de soporte del sistema cardiovascular, aumentando la viabilidad del músculo cardiaco, favoreciendo el mantenimiento de un ritmo sano y ayudando al control del peso corporal. Por su importante función en el metabolismo de las grasas, también reporta numerosos beneficios en la mejora del rendimiento deportivo, retrasando la aparición de la fatiga y mejorando la recuperación. Existen datos clínicos que indican su implicación en el mantenimiento de los niveles de colesterol y triglicéridos, mejorando el depósito muscular y el control del peso. En estudios recientes se ha mostrado cómo el suministro oral de L(-)-carnitina mejora la oxidación de ácidos grasos en individuos sanos (Muller et al., 2002) y con exceso de peso (Wutzke y Lorentz, 2004). En combinación con una dieta hipocalórica y ejercicio moderado, la administración de L(-)-carnitina favorece una mayor disminución del peso corporal en individuos obesos, a la vez que regula, además, los niveles de lipoproteínas de baja densidad (LDL) y azúcar en sangre y reduce la presión arterial.

En el cerebro, acetil-L(-)-carnitina tiene funciones de reparación y protección frente al envejecimiento y a la neurodegeneración, favoreciendo el mantenimiento de la función cerebral. Las deficiencias de L(-)-carnitina en la dieta pueden ser

importantes en recién nacidos, vegetarianos y atletas. También se recomienda L(-)-carnitina para en determinados casos de deficiencias en la calidad del esperma, en número, movilidad o forma de los espermatozoides. También se ha descrito un descenso en el contenido tisular de L(-)-carnitina asociado a la edad, debido por un lado al descenso de la demanda energética y al cambio de los hábitos alimentarios, y a un descenso en la síntesis endógena. Para una extensa revisión de la relevancia fisiológica de L(-)-carnitina se puede consultar la monografía de Löster (2003).

PRODUCCIÓN DE L(-)-CARNITINA

MÉTODOS QUÍMICOS Y BIOTECNOLÓGICOS

A raíz de las numerosas aplicaciones encontradas para L(-)-carnitina y de la creciente demanda de este producto, se han encaminado importantes esfuerzos de investigación al desarrollo de métodos para su producción a escala industrial. Existen en la bibliografía numerosos métodos para la producción de L(-)-carnitina, mediante metodologías tan diversas como la síntesis asimétrica (Kitamura et al., 1988; Kolb et al., 1993); la resolución a través de derivados diasteroisoméricos (Cavazza, 1981; Voeffray et al., 1987); técnicas enzimáticas o microbiológicas (Kasai y Sagaguchi, 1992; Hashiguchi et al., 1992; Jung et al., 1993) o el uso de materiales de partida quirales (Takano et al., 1987; Bellamy et al., 1990; Bols et al., 1992). Así, por ejemplo, el método desarrollado por Bellamy y colaboradores consiste en 6 pasos que, empleando como material de partida ácidos (R)- o (S)-málico, respectivamente, obtiene ambos enantiómeros específicamente. Más recientemente, investigadores del Departamento de Investigación Química de Sigma-Tau describieron una síntesis enantioselectiva de L(-)-carnitina usando como material de partida el compuesto aquiral glicerol, empleando un auxiliar quiral en su procedimiento (Marzi et al., 2000). En cualquier caso, pocos de estos métodos son prácticos a escala industrial. Las ventajas potenciales de los métodos biotecnológicos, basados tanto en el empleo de enzimas como de microorganismos, han motivado el estudio en profundidad del metabolismo de L(-)-carnitina y sus derivados en microorganismos (Kulla, 1991, Jung et al., 1993, Kleber, 1997, Naidu et al., 2000).

USO DE BACTERIAS PARA LA PRODUCCIÓN DE L(-)-CARNITINA

Los procedimientos biotecnológicos para la producción de L(-)-carnitina tienen ventajas evidentes sobre los procesos químicos: se genera un 50% menos de residuos orgánicos, se emplea un 25% menos de agua y es necesario incinerar un 90% menos

de residuos. Los materiales de partida más empleados para la producción de L(-)-carnitina son los precursores aquirales (fundamentalmente crotonobetaína, γ-butirobetaína y 3-dehidrocarnitina), así como mezclas racémicas (tales como D,L-acil-carnitina, D,L-carnitinamida y D,L-carnitina) (Jung et al., 1993; Naidu et al., 2000). Estas biotransformaciones las llevan a cabo los microorganismos resumidos en la Tabla 1.

Tabla 1. Precursores empleados para la producción de L(-)-carnitina con microorganismos. (Adaptado de Naidu et al., 2000)

	Sustratos	**Cepas**
Precursores aquirales	Crotonobetaína	*Escherichia coli, Proteus mirabilis, Acinetobacter lwoffi, Achromobacter xylosoxydans*
	γ-butirobetaína	HK4, HK13, HK1349, *S. cerevisiae, Penicillium, Rhizopus, Mucor, Actinomuchor, Neurospora, Aspergillus, Achromobacter, Pseudomonas, Nocardia crassa*
	3-dehidrocarnitina	*Agrobacterium, Pseudomonas*
Mezclas racémicas	D,L-carnitinanitrilo	*Corynebacterium sp.*
	D,L-acil-carnitina	*Fusarium oxysporum sp. lini, Corynebacterium, Bacillus, Pseudomonas*
	D,L-carnitinamida	*Pseudomonas sp.,* DSM 6320 *(Agrobacterium o Sphingomonas sp.)*
	D,L-carnitina	*Acinetobacter calcoaceticus* y *Acinetobacter lwoffi* (asimilación del isómero-D), *Pseudomonas sp.* y *Escherichia coli* (racemización del isómero-D)

Desde principios de la década de los 80, muchas empresas en todo el mundo han patentado bioprocesos para la producción de L(-)-carnitina (Seitetsu, Kyowa Hakko, Chou Kaseihih, Toyo Jozo, Ajinomoto, Sigma Tau, Lonza, Nippon Pet Food,

Yakult Honsha, Elf Aquitaine, Sanofi) (Naidu et al., 2000). Como ejemplo, mientras que los bioprocesos desarrollados para la producción comercial de L(-)-carnitina por Sigma-Tau (Italia) están basados en la biotransformación de crotonobetaína por cepas de *Escherichia coli* y *Proteus mirabilis*, los métodos de Lonza (Suiza) emplean γ-butirobetaína como material de partida y un derivado de la cepa HK4. Esta última cepa se aisló de una muestra de suelo, y se ha encontrado que su ruta de metabolización de L(-)-carnitina asemeja a la que se encontraría en una cepa situada entre *Agrobacterium* y *Rhizobium.* La cepa HK4 es capaz de crecer usando L(-)-carnitina como única fuente de carbono y nitrógeno en condiciones aerobias. En la cepa de producción se bloqueó la degradación de L(-)-carnitina mediante mutagénesis, generándose una cepa derivada, HK13, que carecía de actividad L-carnitina deshidrogenasa (Kulla y Lehky, 1985). Esta ruta de γ-butirobetaína a L(-)-carnitina es análoga, pero no idéntica, a la degradación de ácidos grasos (Kulla, 1991). De forma similar, muchas proteínas Cai de *Escherichia coli* presentan homología con las enzimas participantes en la degradación de ácidos grasos, tales como acil-CoA deshidrogenasa y CaiA, acetato-CoA ligasa y CaiC, y enoil-CoA hidratasa y CaiD (Eichler et al., 1994a).

METABOLISMO DE L(-)-CARNITINA EN BACTERIAS

A pesar de que la función de L(-)-carnitina en células eucariotas está bien establecida, en bacterias no está tan clara (Kleber, 1997). Se ha relacionado la existencia de sistemas de transporte de betaínas con diferente grado de especificidad y en microorganismos muy distintos con las propiedades protectoras de esta familia de compuestos (Jung et al., 1990; Verheul et al., 1998) y, en determinadas especies como *Listeria monocitogenes*, se ha relacionado con su capacidad para crecer y sobrevivir en alimentos y llegar a provocar infecciones *in vivo* (Sleator et al., 2003).

Además de la capacidad protectora de la acumulación de betaínas, algunas bacterias también son capaces de metabolizar los compuestos de trimetilamonio en determinadas condiciones. Dependiendo de las especies y de las condiciones de cultivo (fuentes de carbono y nitrógeno, condiciones aerobias o anaerobias), existen distintas rutas implicadas en el catabolismo de L(-)-carnitina (Fig. 1). En presencia de L(-)-carnitina se inducen las enzimas iniciales de varias rutas catabólicas, pero también, al menos parcialmente, en presencia de otros compuestos de trimetilamonio. Distintos géneros bacterianos son capaces de degradar L(-)-carnitina en condiciones aerobias. Algunas especies de *Pseudomonas* (tales como *Pseudomonas aeruginosa*

A7244 y ***Pseudomonas*** sp. AK1) pueden crecer aeróbicamente usando L(-)-carnitina como única fuente de carbono y nitrógeno. En estas especies, la degradación de L(-)-carnitina comienza por la oxidación del grupo hidroxilo con la formación de 3-dehidrocarnitina mediante una L(-)-carnitina deshidrogenasa (Aurich et al., 1967). ***Pseudomonas*** sp. AK1 también puede crecer en γ-butirobetaína, que es un intermedio de la ruta de degradación (Lindstedt et al., 1977). Esta ruta tiene muchas semejanzas con la ruta de biosíntesis de L(-)-carnitina en eucariotas. Además, algunas especies, tales como ***Acinetobacter calcoaceticus*** 69/V no pueden asimilar el nitrógeno del esqueleto de L(-)-carnitina y en la degradación se generan cantidades estequiométricas de trimetilamina (Miura-Fraboni et al., 1982). Esta bacteria es capaz de metabolizar L(-)-carnitina, L-O-acilcarnitinas y/o γ-butirobetaína como única fuente de carbono. También puede metabolizar D(+)-carnitina pero sólo en presencia de L(-)-carnitina como inductor (Miura-Fraboni et al., 1982). La estereoselectividad encontrada en el metabolismo de ***Acinetobacter*** podría ser el resultado de la existencia de sistemas de transporte distintos para los isómeros D- y L-, puesto que la cepa salvaje ***A. calcoaceticus*** ATCC 39647 es capaz de discriminar enantiómeros debido a la permeabilidad diferencial de membrana (Ditullio et al., 1994).

Por otro lado, las Enterobacterias, tales como ***Escherichia coli***, ***Salmonella typhimurium***, ***Proteus vulgaris*** y ***Proteus mirabilis,*** no asimilan el esqueleto de los compuestos de trimetilamonio, pero son capaces de metabolizar carnitina, a través del intermedio crotonobetaína, a γ-butirobetaína (Kleber, 1997). Para ello es necesaria la presencia de fuentes de carbono y nitrógeno adecuadas para el crecimiento anaerobio (y, en ocasiones, también aerobio) y para que la expresión de la maquinaria de biotransformación sea la adecuada. Además, la biotransformación también transcurre en ausencia de nutrientes, como se ha demostrado en los estudios con células durmientes (en inglés, ***resting cells***) (Castellar et al., 1998).

En contraste con la ubicuidad de L(-)-carnitina, el enantiómero D- no está presente en la naturaleza. Sin embargo, éste es un residuo de determinados procedimientos químicos para la producción de L(-)-carnitina basados en la resolución racémica de carnitina o de sus precursores a través de la formación de diastereoisómeros con ácidos ópticamente activos. No obstante, varias bacterias son capaces de cababolizar y/o biotransformar D(+)-carnitina (Kleber, 1997).

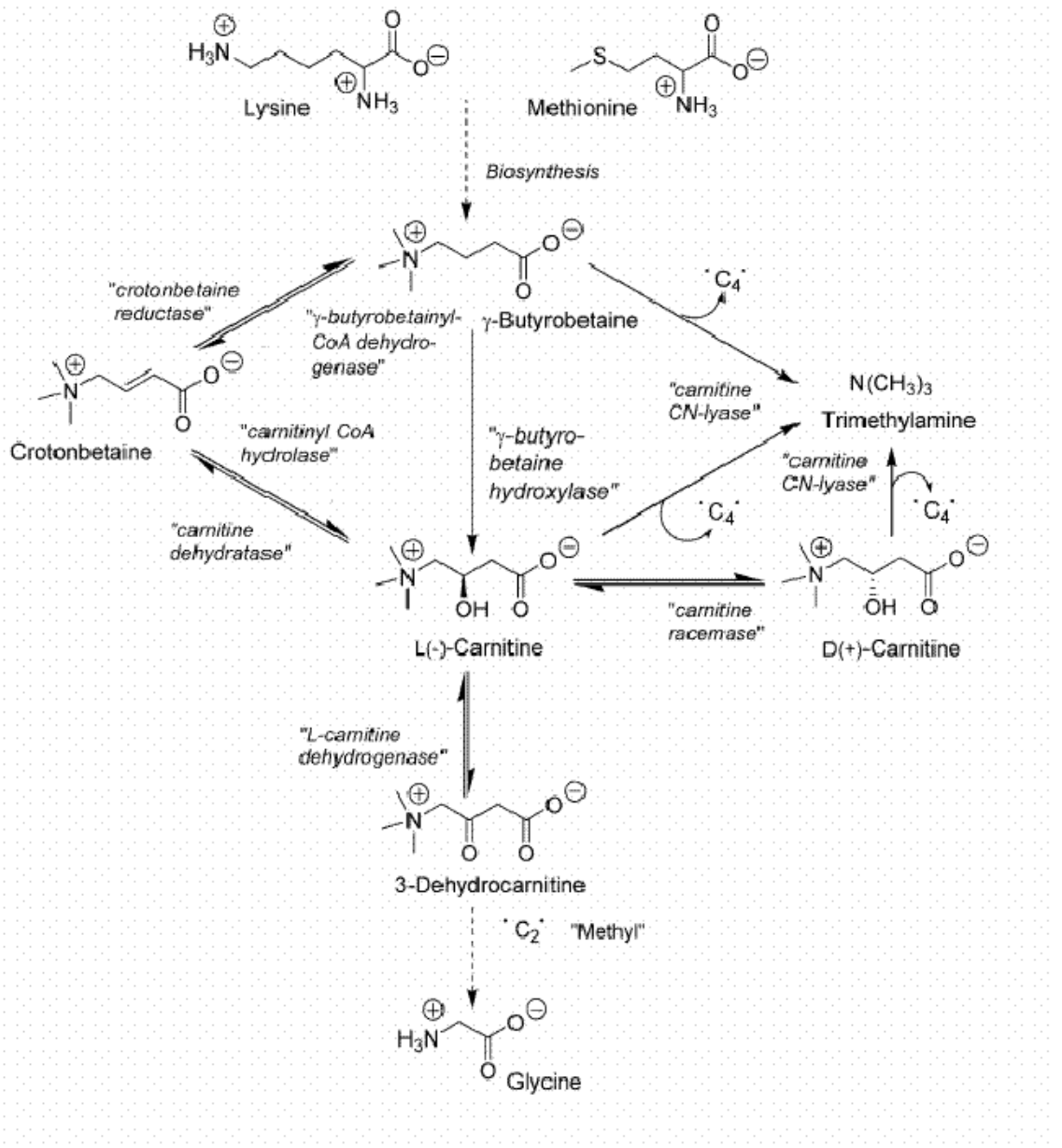

Figura 1. Resumen del metabolismo microbiano de L(-)-carnitina. [Adaptado de: Kamm et al. (2005)].

METABOLISMO DE L(-)-CARNITINA EN ENTEROBACTERIAS: *Escherichia coli* Y *Proteus sp.*

Inicialmente, se propuso un mecanismo en dos pasos para la metabolización de L(-)-carnitina en cepas de *Escherichia coli* y *Proteus sp*. Este mecanismo incluía dos actividades enzimáticas: una L(-)-carnitina deshidratasa (CDH) y una crotonobetaína reductasa (CR) (Eichler et al., 1994b; Roth et al., 1994). Algo después se describió la actividad carnitina racemasa (CRac), que interconvierte los isómeros D- y L- (Jung y Kleber, 1991). Se ha demostrado que este metabolismo está sometido a una gran

regulación en ambas bacterias. Cuando se clonó el operón ***cai*** de ***Escherichia coli*** (Eichler et al., 1994a) se vio que estaba compuesto por seis marcos abiertos de lectura (ORFs) y se asignaron funciones a éstos en base a homologías de secuencia (Tabla 2). Estudios más recientes han permitido determinar que CaiT es un transportador muy específico que lleva a cabo el ***antiporte*** o intercambio de sustratos y productos (Jung et al., 2002, Vinothkumar et al., 2006). Estudios adicionales demostraron que la biotransformación ocurre a nivel de los derivados de CoA de los compuestos de trimetilamonio (Elssner et al., 2000). Además, las actividades CDH y CR descritas inicialmente dependían en realidad de dos proteínas: enoil-CoA hidratasa, CaiD, y crotonobetainil-CoA reductasa, CaiA, respectivamente, actuando de forma conjunta con una transferasa (CaiB) que permite el reciclado del grupo CoA entre productos y sustratos de la biotransformación (Elssner et al., 2001). CaiC (betainil-CoA ligasa) cataliza la síntesis de derivados de compuestos de trimetilamonio, que son sustratos de las actividades CaiD y CaiA (Bernal et al., 2006). La función de CaiE sigue sin haberse determinado, aunque los experimentos iniciales de sobreexpresión sugirieron un posible papel como cofactor para estas dos enzimas (Eichler et al., 1994a). Más recientemente, la clonación y secuenciación del operón ***cai*** de ***Proteus sp.*** mostró que su organización es muy similar, existiendo un elevado nivel de homología entre estas dos cepas (Engemann et al., 2005) (Tabla 3).

El operón ***cai*** se encuentra en el primer minuto del cromosoma de ***Escherichia coli***. Su transcripción se induce durante el crecimiento anaerobio en presencia de L(-)-carnitina, dando lugar a un mRNA policistrónico. En el mecanismo de expresión, es necesaria para la inducción la presencia del activador de operones catabólicos de carbono CRP. Por otro lado, la proteína similar a histonas H-NS y el factor σ^S (RpoS), que regula la activación de genes de fase estacionaria, ejercen un efecto represor sobre el metabolismo de carnitina (Eichler et al., 1994a; Buchet et al., 1998; Buchet et al., 1999). Además, se encontró un gen adicional, denominado ***caiF***, localizado en la región 3' del operón ***cai*** y que se transcribe en dirección opuesta a este desde su propio promotor (Eichler et al., 1996) (Tablas 2 y 3). El producto de este gen es un factor transcripcional que se expresa constitutivamente en anaerobiosis y que, en presencia de carnitina, es capaz de inducir la expresión de los genes ***cai*** (Buchet et al., 1999).

En el extremo 5' del operón ***cai*** se encontró otro operón, formado por cuatro ***ORFs***. Este operón se transcribe desde la misma región promotora/operadora (Eichler et al., 1995) y las proteínas correspondientes muestran homologías de secuencia muy significativas con polipéptidos codificados por el operón *fix*ABCX de ***Azorhizobium***

caulinodans y *Rhizobium meliloti*. A este operón se le ha denominado *fix* y se propuso su implicación en la transferencia de electrones a crotonobetaína (Eichler et al., 1995), lo que se ha confirmado más recientemente (Walt y Kahn, 2002). Estudios de deleción han mostrado que parte de las secuencias *fix* son necesarias para que se exprese el operón *cai* (Buchet et al., 1998).

Tabla 2 Propiedades de los genes *cai* y funciones de los productos correspondientes en *E. coli* O44K74. (Eichler et al., 1994a; Ecocyc-Metacyc: Keseler et al., 2005)

Gen	Tamaño (pb)	Función de la proteína	Referencia
caiT	1515	Proteína de transporte	Jung et al., (2002)
caiA	1143	Crotonobetainil-CoA reductasa	Preusser et al., (1999)
caiB	1218	Betainil-CoA transferasa	Elssner et al., (2001)
caiC	1569	Betainil-CoA ligasa	Bernal et al., (2006)
caiD	894	Crotonobetainil-CoA hidratasa	Elssner et al., (2001)
caiE	591	Desconocida	Eichler et al., (1994a)
caiF	396	Regulador transcripcional	Eichler et al., (1996)

Tabla 3 Propiedades y funciones asignadas de los genes *cai* y de los productos correspondientes en *Proteus sp.* (Engemann et al., 2005)

Gen	Tamaño (pb)	Función de la proteína	Homología con *E. coli* (%)
caiT	1515	Proteína de transporte*	88
caiA	1140	Crotonobetainil-CoA reductasa	92
caiB	1218	Betainil-CoA transferasa	85
caiC	1554	Betainil-CoA ligasa*	69
caiD	783	Crotonobetainil-CoA hidratasa	83
caiE	591	Desconocida	77
caiF	390	Regulador transcripcional*	51

(*) Función postulada en base a similitud de secuencia.

A pesar de todo el conocimiento acumulado, aún se desconoce la función precisa de esta secuencia de reacciones en Enterobacterias. Seim et al. (1982a) postularon que crotonobetaína sirve como aceptor electrónico externo para la respiración anaerobia, de forma similar a lo que ocurre con nitrato, fumarato y el N-óxido de trimetilamina (Haddock y Jones, 1977). Observaciones tales como la estimulación del crecimiento anaerobio de Enterobacterias por crotonobetaína o la supresión de esta reacción por nitrato o glucosa (Seim et al., 1982a, 1982b) apoyan esta hipótesis. La caracterización funcional del sistema de transporte como un intercambiador (Jung et al., 2002) también está de acuerdo con esta idea, explicando además porqué CaiT no está implicado en osmoprotección (Verheul et al., 1998). Sin embargo, la expresión aerobia del metabolismo de carnitina en numerosas Enterobacterias (Elssner et al., 1999, Engemann y Kleber, 2001), incluyendo las cepas de *Proteus*, sugiere una posible pérdida de función debida a mutaciones que afectan a la regulación de la expresión de esta ruta. Además algunos autores han apuntado que el metabolismo de carnitina por la flora del tracto gastrointestinal podría disminuir la disponibilidad de L(-)-carnitina a través de la dieta (Seim et al., 1982b; Kleber, 1997).

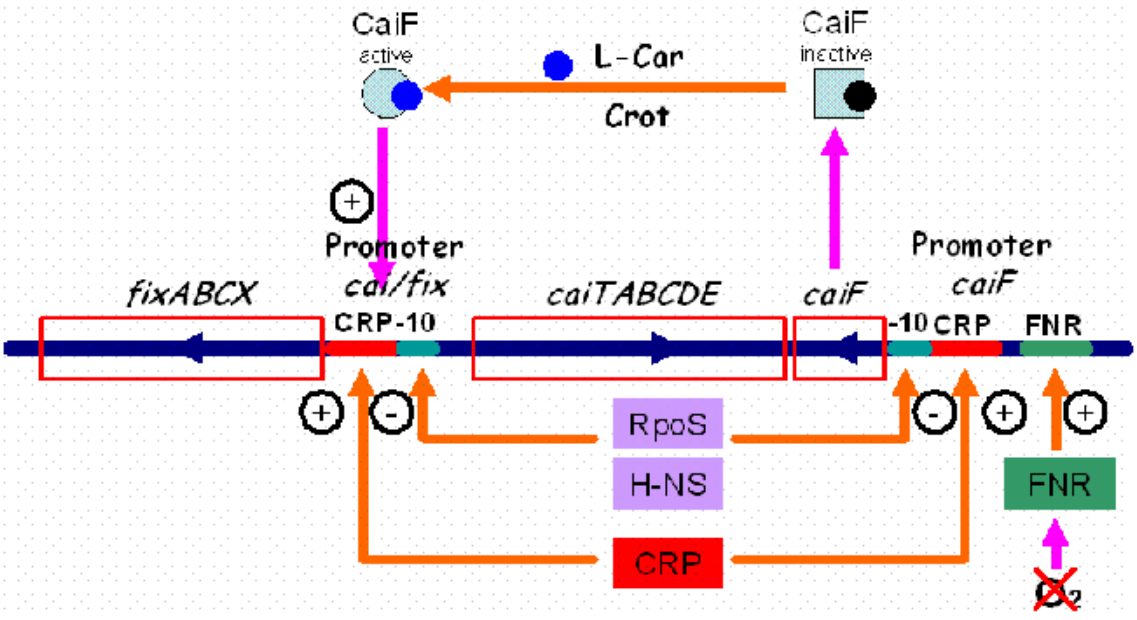

Figura 2. Representación esquemática del mecanismo de regulación de la expresión de los operones *cai* y *fix*. RpoS: subunidad sigma de RNApolimerasa; CRP: proteína receptora de AMPc; H-NS: proteína similar a histonas. FNR: factor transcripcional de anaerobiosis. (Adaptado de: Eichler et al., 1994a).

BIOTRANSFORMACIONES EN LA INDUSTRIA

BIOPRODUCCIÓN DE L(-)-CARNITINA Y LAS INGENIERÍAS METABÓLICA Y DE BIOPROCESOS.

A pesar de que existe una demanda creciente de proteínas para su aplicación terapéutica, el objetivo principal de la industria farmacéutica sigue estando en la obtención de moléculas pequeñas (PM<1000 Da). Además, el mayor conocimiento que se tiene de la distinta actividad biológica de los isómeros ópticos, junto con el creciente aumento en la presión de la reglamentación reguladora ha acelerado el proceso para la fabricación y comercialización, exclusivamente, de compuestos terapéuticos quiralmente puros (Persidis, 1997).

En la naturaleza sólo está presente el isómero L(-)-carnitina. A pesar de que el isómero D(+)-carnitina no es biológicamente activo, se ha demostrado que los sistemas de transporte celulares no son capaces de distinguir entre los dos enantiómeros. De este modo, la administración de la mezcla racémica a humanos tendría el efecto perjudicial de disminuir el contenido celular en L(-)-carnitina. En los últimos 30 años las compañías farmacéuticas han estado produciendo fármacos enantioméricamente puros. La selectividad, así como las condiciones de reacción suaves características de los biocatalizadores los hacen candidatos idóneos para su uso en la producción de compuestos de alto valor añadido. En el caso de reacciones simples, que no requieran un gran número de enzimas o la regeneración de cofactores, se han empleando tanto enzimas puras como células enteras. Cuando las transformaciones requieren pasos multienzimáticos o el reciclado de cofactores, las células completas son más adecuadas (Buckland et al., 2000).

A pesar de los esfuerzos realizados para la optimización de la producción de L(-)-carnitina empleando cepas bacterianas, la mayor parte de los trabajos encontrados en la literatura están dedicados a la mejora del funcionamiento del reactor. Se sabe muy poco acerca de las características que el escenario metabólico debe cumplir para favorecer la producción de L(-)-carnitina. Microbiólogos y biólogos moleculares han descrito en profundidad los determinantes genéticos que controlan la expresión de este metabolismo en *Escherichia* y *Proteus* (Eichler et al., 1994a, 1996; Kleber, 1997; Elssner et al., 2001; Engemann et al., 2001 y 2005). Esto, junto con los estudios de optimización llevados a cabo por ingenieros bioquímicos ha permitido el desarrollo de medios optimizados (Castellar et al., 1998) y determinar el efecto que el estrés tiene sobre el bioproceso (Cánovas et al., 2003b). Se han desarrollado metodologías biosintéticas escalables y aplicables industrialmente (Cánovas et al., 2002; Cánovas et

al., 2003c; Giuliano et al., 2003). Sin embargo, a pesar de que las productividades que se han alcanzado aún están lejos de las que se desearían, el rendimiento obtenido empleando cepas salvajes parece haber alcanzado un máximo y el siguiente paso pasa, probablemente, por la aplicación de nuevas metodologías de mejora para la modificación del metabolismo bacteriano y la modificación genética para la mejora del sistema.

La ingeniería metabólica se definió por primera vez como *"la mejora de las actividades celulares por medio de la manipulación del transporte enzimático y las funciones reguladoras de la célula con el uso de la tecnología del DNA recombinante"* (Bailey, 1991). Esta definición fue extendida por Cameron y Tong (1993) quienes establecieron la ingeniería metabólica como la *"modificación racional del metabolismo intermediario empleando técnicas de DNA recombinante".*

El destacado grado de desarrollo alcanzado por las técnicas de DNA recombinante, que ahora están disponibles para un gran número de organismos, ha aumentado el rango de posibilidades en biocatálisis. Ahora es posible modificar el metabolismo celular eliminando o sobreexpresando rutas completas, lo que ha abierto un campo completamente novedoso en biotecnología: es posible el desarrollo de cepas "a medida" para bioprocesos. Hasta la fecha, muy pocos han sido los trabajos que han pretendido mejorar la producción de L(-)-carnitina empleando cepas modificadas genéticamente (Castellar et al., 2001; Cánovas et al., 2003b). El reto de la ingeniería metabólica es claro, especialmente si se considera que, hasta la fecha, no se han aplicado criterios racionales para la selección de genes diana. Además, la optimización de bioprocesos en los que el metabolismo secundario está implicado no debe obviar la necesidad de establecer el perfil óptimo de expresión del metabolismo primario. Esto es especialmente importante cuando se consideren determinados sustratos o cofactores que son comunes a ambos metabolismos.

Una vez completada la secuenciación de los genomas de diversos organismos, en plena expansión del estudio de proteomas, metabolomas, flujomas y señalomas, es clara la oportunidad que plantea el empleo de la ingeniería metabólica para la potencial mejora de rutas biosintéticas existentes o incluso para el diseño de otras nuevas. La tecnología del DNA recombinante puede aplicarse con fines biotecnológicos, fundamentalmente, para la sobreproducción de un determinado metabolito o algún otro producto celular. La mejora de las rutas metabólicas puede implicar la mejora de las existentes (alterando las redes reguladoras y de expresión) así como la modificación de las rutas por introducción de genes foráneos. Las ventajas

de esta aproximación son claras si se compara con la estrategia clásica de mutagénesis al azar.

Las herramientas empleadas por los ingenieros metabólicos son experimentales, teóricas y computacionales (Mendes y Kell, 1997). Una vez secuenciado el genoma de un gran número de microorganismos, es preciso un mayor conocimiento de los distintos modos de comportamiento o algoritmos de los organismos. En la nueva era post-genómica, el nuevo reto está en la identificación de la función de los genes secuenciados y, sobre todo, en comprender cómo su expresión concertada define el fenotipo celular. Un aspecto novedoso e importante en ingeniería metabólica es el énfasis en la integración de las rutas metabólicas frente a los pasos individuales, considerando el todo resultante de la red de reacciones metabólicas. Esto resulta especialmente importante para comprender y controlar los flujos metabólicos *in vivo* (Buckland et al., 2000) de tal manera que se pueda llevar a cabo un diseño racional de estrategias.

En el campo de los productos naturales, existe un gran espectro de compuestos finales que se pueden obtener como combinación de síntesis química y biosíntesis. Es el caso de los antibióticos, tales como penicilinas, cefalosporinas y macrólidos. El conocimiento de las complejas rutas metabólicas a nivel molecular ha borrado la distinción entre productos naturales y de bioconversiones. Los procedimientos de fermentación microbiana son bien conocidos y son una ruta de confianza y con costes moderados para la síntesis de numerosos compuestos orgánicos (como citrato y glutamato), aunque los rendimientos en metabolitos secundarios son bastante menores. La síntesis de compuestos orgánicos en la industria puede hacer uso de etapas biotecnológicas para la obtención de determinados intermedios, para llevar a cabo bioconversiones o construir moléculas sillares clave.

En la aplicación farmacéutica de la biocatálisis se emplean a menudo reactores de tipo discontinuo-alimentado (*fed-batch*) en tanques agitados, por ser los mejor documentados y ser más sencillo el control del proceso, a la vez que se obtienen rendimientos elevados. Además, en este tipo de reactores es sencillo mantener la necesaria esterilidad de estos procesos. Sin embargo, las numerosas ventajas de los procesos en continuo hacen que su diseño y desarrollo sea un objetivo más que interesante para numerosas aplicaciones a escala industrial. Una de las principales necesidades para la optimización de bioprocesos es el desarrollo de técnicas analíticas adecuadas, especialmente si pueden acortar el tiempo de análisis y permitir un control más preciso del proceso. Además, las técnicas deben permitir determinar e incluso cuantificar el efecto de la configuración del reactor sobre la fisiología

bacteriana, especialmente cuando se emplean cepas modificadas genéticamente. Por tanto, aunque ya se ha avanzado un largo camino, sigue siendo necesario más trabajo para el desarrollo de bioprocesos, especialmente en la interfase entre bioquímica, biología e ingeniería.

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ORGANIZACIÓN DE LA MEMORIA

La optimización de bioprocesos debe abordarse desde un punto de vista multidisciplinar. En el campo de la ingeniería bioquímica y la biotecnología es habitual encontrar grupos formados por microbiólogos, ingenieros, químicos y bioquímicos, puesto que el rendimiento final de un bioproceso depende del conocimiento que se tenga de la fisiología celular, de la optimización del funcionamiento de las redes metabólicas celulares, de la optimización del bioproceso, de la elección de reactores adecuados, del análisis de las variables del proceso y de la modelización.

En esta memoria de Tesis Doctoral hemos elegido como modelo de estudio la producción del fármaco L(-)-carnitina, llevando a cabo una aproximación multiobjetivo. Como ya se ha mencionado previamente, las rutas de biotransformación existentes en Enterobacterias pueden aprovecharse para la producción de L(-)-carnitina. Los datos obtenidos previamente *in vivo* e *in silico* por nuestro grupo de investigación nos han permitido desarrollar metodologías para la optimización de la producción de L(-)-carnitina. La complejidad creciente de los modelos desarrollados ha permitido optimizar las condiciones de operación de bioreactores (Cánovas et al., 2002; Álvarez-Vásquez et al., 2002), un mejor conocimiento de la interfase reactor-microorganismo (Sevilla et al., 2005a) e incluso cuantificar los flujos metabólicos y optimizar la unión entre el metabolismo central y el de carnitina en términos energéticos y de regeneración de cofactores (Sevilla et al., 2005b). Estos trabajos han permitido elegir dianas para la mejora del rendimiento del proceso usando los principios y herramientas de la ingeniería metabólica.

En la primera parte del trabajo se lleva a cabo un análisis sistemático de los principales factores que afectan a la producción de L(-)-carnitina usando cepas de *Escherichia coli* (salvajes y modificadas genéticamente). Se comparan sistemas continuos y discontinuos y se estudia el efecto de las condiciones de operación sobre la estabilidad genética de la cepa.

Además, se hace uso de la citometría de flujo como técnica analítica para determinar cómo el diseño del proceso afecta al microorganismo. Así, se comparan distintas configuraciones de operación (células en crecimiento y durmientes, reactores discontinuos y continuos). También se analiza el efecto de estas variables sobre la fisiología de *Escherichia coli* prestando atención tanto a la viabilidad celular como al contenido en macromoléculas.

La inestabilidad genética de las cepas modificadas genéticamente es la principal desventaja que limita su aplicación en bioprocesos. La estabilidad es especialmente

importante en procesos continuos con células en crecimiento. Así, se analiza la estabilidad de una cepa transformada de *E. coli* en distintas condiciones de operación y el enorme grado de estabilización tras la inmovilización en geles de κ-carragenano. Se hace uso de la citometría de flujo para analizar las diferencias fisiológicas en la cepa en los distintos ambientes de reactor.

El conocimiento en profundidad de la red metabólica implicada en la biotransformación es vital. Así, analizamos la unión entre los metabolismos primario y de compuestos de trimetilamonio de *E. coli* en distintos reactores. Se establece que la unión tiene lugar a través de los niveles de cofactores y que la expresión del ciclo de los ácidos tricarboxílicos, el ciclo del glioxilato y el metabolismo del acetato están relacionados con la biotransformación.

Además, puesto que L(-)-carnitina es un osmoprotector, se estudia el efecto de la presencia de sal en el medio de crecimiento sobre el rendimiento del bioproceso. Se lleva a cabo un análisis del metabolismo tanto en presencia como en ausencia de estrés salino. De hecho, en determinadas condiciones se consigue un aumento en la productividad. Se caracterizaron las restricciones metabólicas relacionadas con esta respuesta en la biotransformación, permitiéndonos detallar aún más la unión entre la biotransformación y las rutas centrales en *Escherichia coli*.

Se lleva a cabo la clonación del cuarto marco abierto de lectura del operón ***cai***, que se había propuesto previamente como carnitina:CoA ligasa (CaiC), y se hace una caracterización preliminar. Se llevan a cabo ensayos de actividad enzimática y se establece con mayor precisión cuál es su papel en la biotransformación en relación con la transferasa de CoA (CaiB).

Finalmente, puesto que la aplicación de técnicas de biología molecular puede resolver las restricciones previamente determinadas en el metabolismo de L(-)-carnitina en *Escherichia coli*, se sobreexpresan actividades seleccionadas pertenecientes al operón ***cai*** y se llevan a cabo estudios de deleción de genes, con objeto de determinar la función de determinadas actividades en las rutas centrales de *E. coli* en la biotransformación.

Por tanto, presentamos el siguiente resumen de los ***objetivos*** de esta Tesis:

1. Determinar los factores principales que afectan a la producción de L(-)-carnitina por *Escherichia coli*, en sistemas continuos y discontinuos, con cepas salvajes y modificadas genéticamente y en presencia o ausencia de aceptores electrónicos tales como fumarato u oxígeno (Capítulo 1).
2. Establecer el efecto de la configuración del reactor en el estado fisiológico de cepas de *Escherichia coli* durante la producción de L(-)-carnitina, en presencia y ausencia de medio de crecimiento (células en crecimiento o células durmientes), en sistemas discontinuos y en reactores continuos de reciclado celular. Además, estudiar estrategias de reutilización celular (Capítulo 2).
3. Establecer los efectos de la configuración de reactor y de la inmovilización celular en el estado fisiológico y la estabilidad genética de una cepa modificada genéticamente de *Escherichia coli* durante la producción de L(-)-carnitina, en cultivos continuos y discontinuos (Capítulo 3).
4. Determinar la unión entre el metabolismo primario (o de carbono) y el metabolismo secundario de carnitina en *Escherichia coli* en condiciones de producción, estudiando las concentraciones de metabolitos primarios y secundarios, así como las actividades enzimáticas, durante el proceso (Capítulo 4).
5. Determinar la unión entre el metabolismo primario (o de carbono) y el metabolismo secundario de carnitina en *Escherichia coli* en presencia de estrés salino, considerando las concentraciones de metabolitos primarios y secundarios, así como las actividades secundarias, durante el proceso y tras pulsos de sal y sustrato (Capítulo 5).
6. Caracterizar la proteína CaiC con objeto de determinar su actividad enzimática y especificidad de sustrato, revelando la relevancia que tiene su expresión *in vivo* en el metabolismo de compuestos de trimetilamonio, usando distintos sustratos y cepas mutantes (Capítulo 6).
7. Clonar y sobreexpresar los genes *caiB* y *caiC* del operón *cai* de *Escherichia coli*, y determinar los factores limitantes en la producción de L(-)-carnitina en cepas de *Escherichia coli*, estudiando el efecto sobre el metabolismo de la modificación de los niveles de derivados de coenzima A de los compuestos de trimetilamonio. Además, analizar la relación de las principales rutas relacionadas con el metabolismo de acetil-CoA (ciclo del glioxilato, metabolismo de acetato y ciclo de Krebs) con la producción de L(-)-carnitina (Capítulo 7).

Capítulo 1

Factors affecting the biotransformation of trimethylammonium compounds into L-carnitine by *Escherichia coli*.

Los contenidos de este capítulo han sido publicados como:

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ABSTRACT

The biotransformation of D(+)-carnitine and crotonobetaine into L(-)-carnitine with wild and transformed *E. coli* strains under batch and continuous operation was optimised. In batch, the best conditions for the transformed strain were 30% oxygen saturation, a temperature of 41ºC and a minimal medium, whereas anaerobic cultures in either complex or minimal media at 37ºC and pH 7.5 were optimal for the wild strain. Studies on the expression of the enzymes involved in trimethylammonium metabolism showed that L(-)-carnitine dehydratase activity was always higher than that of D(+)-carnitine racemase. Experiments with the transformed strain in continuous cell-recycle reactors showed that, despite the higher productivity that could be achieved (0.65-1.2 g/L·h), plasmid-bearing cells were segregated even when a selective medium was used. This fact was also confirmed by studying the evolution of the D(+)-carnitine racemase level. Immobilization of the transformed strain in κ-carrageenan gels allowed continuous operation for L(-)-carnitine production with no plasmid loss. In continuous processes with cell-retention systems, the wild strain showed higher productivity and stability than the transformed strain. Moreover, crotonobetaine was a better substrate for both strains used. Recycling with hollow-fiber cartridges provided the highest biomass level even though the L(-)-carnitine dehydratase/biomass ratio was lower. However, membrane composition and cut-off had less influence on reactor performance as similar levels of productivity were attained. In spite of this, continuous processes attained a L(-)-carnitine production as high as 11.5 g/L·h as a result of the high enzyme induction and biomass levels.

INTRODUCTION

L(-)-carnitine (R(-)-3-hydroxy-4-trimethylaminobutyrate) transports long-chain fatty acids through the inner mitochondrial membrane. Because of this, several clinical applications for L(-)-carnitine have been identified with a consequent increase in its demand on the part of the pharmaceutical and food industries. Most L(-)-carnitine is chemically produced by chiral resolution of the racemic mixture (Jung et al., 1993). The D(+)-carnitine obtained in equimolecular amounts as a waste product could be transformed into L(-)-carnitine by various enzymes (Mönnich et al., 1995; Hanschmann and Kleber, 1997) and microorganisms, such as *Escherichia coli* (Jung and Kleber, 1991), meaning that an otherwise uneconomical waste product can be used as substrate to produce a high value compound (Castellar et al., 2001). For this reason a racemization method for transforming the by-product, D(+)-carnitine and/or the trans-crotonobetaine, arising from D(+)-carnitine chemical dehydration, could be of wide industrial interest.

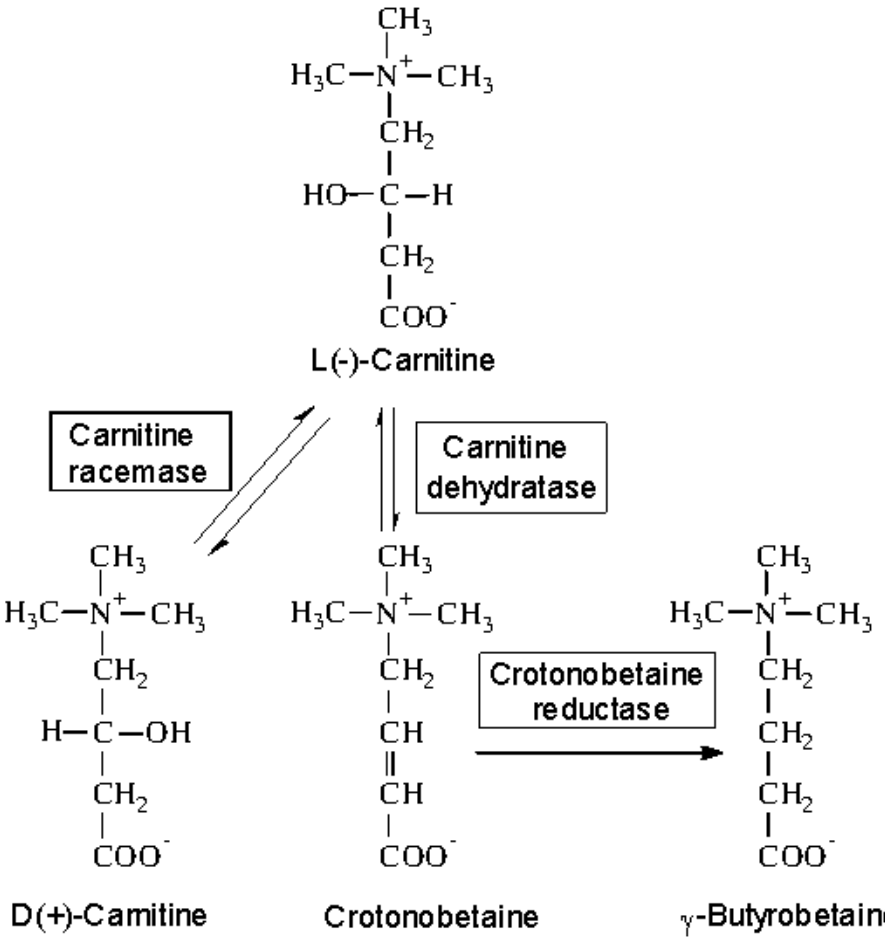

Figure 1. Postulated metabolism of the trimethylammonium compounds in *E. coli* O44K74 (After Kleber, 1997).

Biological procedures for L(-)-carnitine production have been proposed using *Escherichia coli* cells, which are able to metabolize carnitine (through a secondary metabolism) but which do not assimilate the carbon or nitrogen carnitine skeleton. Initial experiments have shown that resting or growing *E. coli* O44K74 cells are able to convert trans-crotonobetaine into L(-)-carnitine (Fig. 1) (Seim and Kleber, 1988; Obón et al., 1997). Furthermore, D(+)-carnitine can be biotransformed under batch resting conditions into L(-)-carnitine (Jung and Kleber, 1991; Castellar et al., 1998 and 2001).

In parallel to the above experiments, genetic studies were conducted to elucidate carnitine metabolism in *E. coli* O44K74. Two divergent structural operons, *caiTABCDE* and *fix*ABCX, are coexpressed during cell anaerobic growth in the presence of L(-)-carnitine or crotonobetaine as inducers. Both operons are modulated positively by general regulators, such as the cyclic AMP receptor protein (CRP) or the transcriptional regulator responsible for anaerobic induction (FNR), and negatively by the DNA-binding protein H-NS, glucose or nitrate (Eichler et al., 1994). In addition, it has been proposed that a positively controlled *caiF* gene acts as a specific transcriptional regulator for carnitine metabolism (Eichler et al., 1996). The transformed strain *E. coli* K38 pT7-5KE32, which carries an insert with *caiD*, *caiE* and *caiF* genes, has been shown to express high levels of carnitine racemase and carnitine dehydratase activities, since CaiD is a bifunctional enzyme and CaiE has been proposed to act as a cofactor necessary for its proper function (Eichler et al., 1994).

The main aim of this study was to characterize the whole cell biotransformation of D(+)-carnitine and crotonobetaine (dehydrated D(+)-carnitine) into L(-)-carnitine by using the D(+)-carnitine racemase and carnitine dehydratase activities from two strains of *E. coli* (wild and transformed, respectively) in batch and continuous reactors under growing conditions, since resting processes have already been studied (Castellar et al., 1998 and 2001). Both aerobic and anaerobic conditions were checked and compared. The effect of variables, such as different oxygen or fumarate concentrations and medium compositions, was evaluated in the batch experiments. During continuous operation, different membrane configurations, compositions and cut-offs for cell retention and recycling were used and processes were run at different dilution rates. Optimum conditions for maximum L(-)-carnitine production were used to perform plasmid stability studies under continuous operation in freely suspended cell, κ-carrageenan entrapped systems and in a cell-recycle reactor so as to evaluate the suitability of the transformed strain. Finally, the

induction and expression of the trimethylammonium compounds metabolism enzymes were followed to determine their effect on biotransformation reaction catalysis and to optimise the reactor set up.

MATERIALS AND METHODS

Strain and plasmids

E. coli O44K74 (DSM 8828) and *E. coli* K38 pT7-5KE32 were used throughout this study. *E. coli* O44K74 contains the complete divergent structural operons, *caiTABCDE* and *fix*ABCX, with carnitine racemase and L(-)-carnitine dehydratase activity. *E. coli* K38 pT7-5KE32 contains the complete *cai*TABCDE and *fix*ABCX operons as well as the plasmids pGP1-2, Kan^R and the pT7-5KE32, a pBR322 derivative, Amp^R, which carries an insert with *caiD*, *caiE* and *caiF* genes from the *E. coli cai* operon. Therefore, this derivative overexpresses carnitine racemase and L(-)-carnitine dehydratase activities (Eichler et al., 1994 and 1997; Chang and Cohen, 1978; Tabor and Richardson, 1985), the non-characterized protein CaiE and the transcriptional activator CaiF. Expression of c*aiF* occurs from its own promotor sequence. Thus the cofactor required for racemase and dehydratase activities is generated due to the expression of the cai operon within its own genome. This cofactor, crotonobetainyl-CoA, has been described as the cofactor required by the biotransformation (Elssner et al., 2001; Cánovas et al., 2003). The genetically modified strain was therefore kanamycin and ampicillin resistant. The strains were stored in glycerol (20%) at –20ºC.

Batch and continuous cultures

The standard minimal medium contained (g/L): 13.6 KH_2PO_4, 2 $(NH_4)_2SO_4$, 0.5 casein hydrolysate, 8.8 glycerol, 1 D,L-carnitine hydrochloride and 7.7 D(+)-carnitine inner salt. The complete medium contained (g/L): 20 bacteriological peptone, 12.6 glycerol, 5 NaCl, 1 D,L-carnitine hydrochloride and 7,7 D(+)-carnitine inner salt. In both cases, the final pH of the medium was adjusted to 7.5 with KOH. Antibiotics were added at the following standard concentrations: 50 µg/mL ampicillin and 25 µg/mL kanamycin, unless otherwise stated in the text. *E. coli* O44K74 was grown in a complex medium with the following composition (g/L): 8.5 crotonobetaine, 5 pancreatic peptone, 5 NaCl, and 50 mM fumarate. The pH was adjusted to 7.5 with KOH. Anaerobic conditions were maintained to induce the enzymes involved in the carnitine metabolism, while D,L-carnitine mixture, D(+)-carnitine or crotonobetaine were used as inducers.

Batch and continuous experiments in aerobic and anaerobic (under nitrogen atmosphere) assays were performed in reactors equipped with temperature, pH, and oxygen probes and pump controllers (Biostat B, Braun, Germany). A 1 L culture vessel with 0.5-0.8 L working volume was used. In continuous as well as in batch

processes the cells were fed on the growing media referred to above (the transformed *E. coli* strain on the minimal media while the wild *E. coli* strain were fed on the complete medium). The media contained crotonobetaine or D(+)-carnitine, depending on experiment (see results section) as the biotransformation substrate. Resting cell assays in batch were performed as follows: after cell batch growth, cells were harvested and resuspended in the biotransformation medium, which contained D(+)-carnitine or crotonobetaine (25-100 mM) in phosphate buffer pH 7.5 and left for 15 and 24 hours for the transformed and wild strain, respectively.

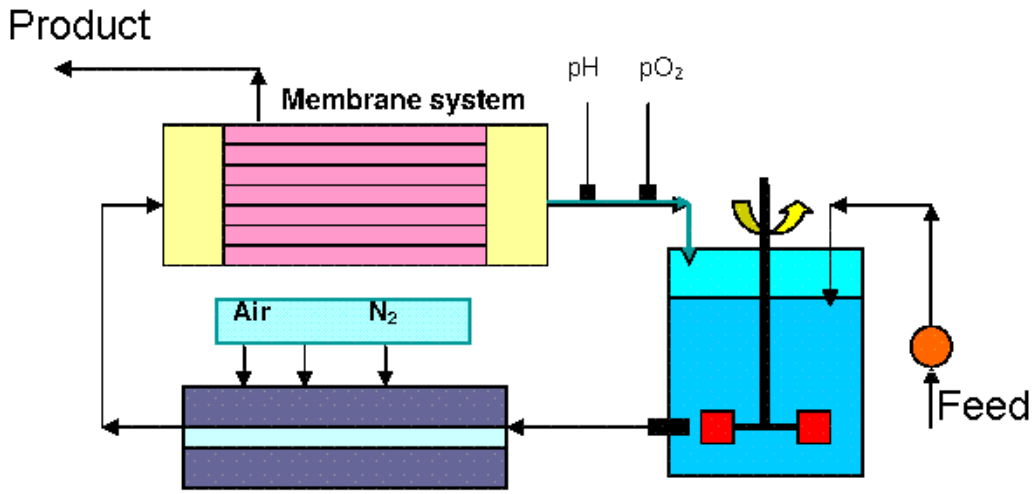

Figure 2. Schematic representation of the experimental set-up for cell recycle cultivation: reactor vessel was connected to a membrane filtration module or hollow fiber by means of a cell recycling pump. Control of the inlet and outlet pumps was that of the Biostat B.

In addition, continuous experiments with cell-recycle devices were also carried out in the reactor system mentioned above adapted to continuous cell-recycle operation by using a cross-flow filtration module (Minitan, Millipore, USA) or a hollow fiber cartridge (Diaflo, Amicon, USA) (Fig. 2). The systems were equipped in a different set of experiments with either four 0.1 μm hydrophilic polyvinylidene difluoride (PVDF) Durapore plates, or with cellulose membranes of 300 kDa nominal cut-off, or with polysulphonated polysulphone membranes of 0.2 μm nominal cut-off of 60 cm^2 area (Millipore, USA), or with ceramic membranes of 30-60 cm^2 area, 0.1 μm nominal cut-off (Amicon, USA). The hollow fiber module was fitted with a membrane system made of polysulphone with a total available surface of 0.03 m^2; the inner diameter of the fiber was 1.1 mm with a membrane nominal cut-off of 0.1

μm. Cells were recycled through the lumen side back to the reactor vessel. Inlet and filtration flows were fixed to keep a constant volume. Continuous operation was started after inoculation. Continuous reactors and high-cell density reactors (as a result of the cell recycle systems used) were run at dilution rates (flow rate/reactor volume ratio) ranging from 0.1 to 2.0 h^{-1}, depending on the experiment and the different membrane systems in use (see the result sections).

For the immobilization of *E. coli* K38 pT7-5KE32 in κ-carrageenan gels, a modification of the method of Takata *et al.* (1987) was used. For this purpose, 6 ml of cells from a culture grown overnight (approximately 14 hours) were mixed with 50 ml of a 2.1% (w/v) κ-carrageenan solution (κ-carrageenan C-1263, type III from Sigma) with continuous stirring and a constant temperature of 40ºC to avoid gelation. The carrageenan solution was previously sterilized by autoclaving. This mixture was then added dropwise to the minimal medium, supplemented with 0.3 M KCl, forming beads with bacteria entrapped inside. Continuous operation of the reactor was performed as previously described, but feeding with a culture medium containing 0.1 M KCl to avoid disruption of the polymer. A filter in the medium outlet prevented beads from leaving the reactor.

κ-Carragenan beads were collected from the reactor. Gel-entrapped cells were released by washing three times with 0.9% NaCl followed by a 10 minutes incubation period at 37ºC. Cells were inmediately pelleted using a benchtop centrifuge and used for further analysis. Biomass concentration was determined by using optical density followed at 600 nm (A_{600}) with a spectrophotometer (Novaspec II, Pharmacia-LKB, Sweden) and correlated with dry cell weight.

Assays

Optical density (OD) was followed at 600 nm with a spectrophotometer (Novaspec II, Pharmacia-LKB, Sweden) as a measure of cell concentration. Viable counts were performed on complete medium-agar plates without carnitine supplementation (20 g/L of bacteriological peptone, 12.6 g/L glycerol and 5 g/L NaCl) in the presence and absence of antibiotics. The plates were incubated at 30ºC for approximately 24 h. Segregational stability was estimated as the ratio between the number of colonies growing on the antibiotic plates (50 μg/mL ampicillin, 25 μg/mL kanamycin) and the number growing on the control plate without antibiotics.

L(-)-carnitine concentration was determined by an enzymatic test, while D,L-carnitine, crotonobetaine and γ-butyrobetaine were determined by HPLC (Obón et

al., 1997) with a Tracer Spherisorb-NH_2 column 3μm, 25 x 0.46 cm, supplied by Teknokroma (Barcelona, Spain). The isocratic mobile phase was acetonitrile/H_3PO_4 0.05 mol/L pH 5.5 (65/35) at a flow rate of 1 mL/min.

Enzyme activity

The L(-)-carnitine dehydratase assay was carried out according to Jung et al., (Jung et al., 1993), and the crotonobetaine reductase assay according to Preusser et al., (Elssner et al., 2001), both started by using crotonobetaine as substrate. D(+)-carnitine racemase activity was determined as described in Jung and Kleber (1991), using D(+)-carnitine as the substrate. Enzyme activity was defined either as the total mmols of substrate consumed per hour (U) or as specific activity, mmol of substrate consumed per hour and mg of protein (mU/mg).

RESULTS AND DISCUSSION

Batch experiments

Influence of electron acceptors: oxygen and fumarate

Resting cell experiments have previously shown the influence that electron acceptors, such as oxygen and fumarate, have on the D(+)-carnitine racemization process carried out by wild *E. coli* strains (Fig 1) (Jung and Kleber, 1991; Castellar et al., 1998). Therefore, their effects on cell growth during D(+)-carnitine racemization and crotonobetaine hydration were studied. Table 1 shows that higher levels of biomass concentrations for *E. coli* K38 pT7-5KE32 were achieved with increased oxygen concentration. Anaerobic growth rendered γ-butyrobetaine as a by-product of the carnitine metabolism, although its levels could be minimized in aerobiosis since oxygen acts as an electron acceptor for cells. In fact, during anaerobiosis, crotonobetaine acts as an electron acceptor and is transformed into γ-butyrobetaine (Fig. 1); moreover, the enzyme crotonobetaine reductase has been shown to be inhibited by oxygen in cell-free extracts (Kleber, 1997). Furthermore, in aerobiosis *E. coli* K38 pT7-5KE32 presented a trimethylammonium metabolism in which crotonobetaine reductase enzyme was not detected (0.5 mU/mg protein). Thus, 15-30% oxygen saturation was fixed as the optimum concentration since L(-)-carnitine was obtained with no concomitant γ-butyrobetaine production (Table 1). With respect to *E. coli* O44K74, batch studies in aerobiosis showed that the metabolism of trimethylammonium was hardly expressed, unlike in anaerobiosis, since low levels of L(-)-carnitine dehydratase and D(+)-carnitine racemase were detected after growth in the presence of crotonobetaine or D(+)-carnitine (4.8± 1.2 mU/mg protein and 2.2± 2.1 mU/mg protein, respectively).

When the effect of fumarate on the biotransformation system was checked, the growth of the strains, as well as the values of the biotransformation parameters, showed that the process was improved by the addition of fumarate. Thus, only the kinetic parameters, L(-)-carnitine yield and process conversion for growing *E. coli* O44K74 using crotonobetaine as the substrate, are presented in Table 2. Parameters were calculated considering different experiments at different glycerol concentrations as the carbon and energy source and fumarate as the electron acceptor (Kleber, 1997), and conventional mathematical techniques (Bailey and Ollis, 1986). The use of resting cells of *E. coli* for D(+)-carnitine biotransformation has also been shown to improve when fumarate is added to the resting process performed under aerobiosis (Castellar et al., 1998). However, the role of fumarate

on the trimethylammonium metabolism of *E. coli* growing under anaerobiosis is not totally understood. Fumarate might affect the crotonobetaine reductase enzyme, inhibiting its activity, as has been reported previously in cell-free extracts (Seim and Kleber, 1988; Obón et al., 1997; Preusser et al., 1999). Furthermore, the effect of fumarate on growth might also be at the level of energy production or the anaplerotic pathways expression.

Table 1. Effect of different oxygen concentrations on L(-)-carnitine production from D(+)-carnitine in batch growth of *E. coli* K38 pT7-5KE32. D(+)-carnitine concentration was 30 mM. Results were obtained after an experimentation time of 35 hours. Medium as in Materials and Methods.

	Oxygen (%)			
	0	15	30	60
Biomass (A_{600})	1.2	3.5	4.5	4.0
L(-)-carnitine (Mm)	3.1	13.1	14.2	5.9
Butyrobetaine (mM)	26.1	5.0	0	0

Table 2. Kinetic parameters for anaerobic growth of *E. coli* O44K74 in batch experiments using crotonobetaine as the substrate. 50 mM fumarate was added to the complex medium (Materials and Methods).

Complex media	**μ_{max} (h^{-1})**	**$qcar_{max}$**	**$qcrot_{max}$**	**Yield carn. (%)**	**Conv (%)**
Control	0.224	0.008	0.189	6.9	40.0
With fumarate	0.431	0.343	0.471	43.0	48.5

Moreover, for both strains, experiments demonstrated that crotonobetaine was the best substrate for L(-)-carnitine production from both crotonobetaine and D(+)-carnitine (Fig. 3B). This effect may be due to enzyme activity expression or even to the fact that membrane transporters for the substrate and product have been said to be the same (Jung et al., 2002), meaning that different transport affinities might occur.

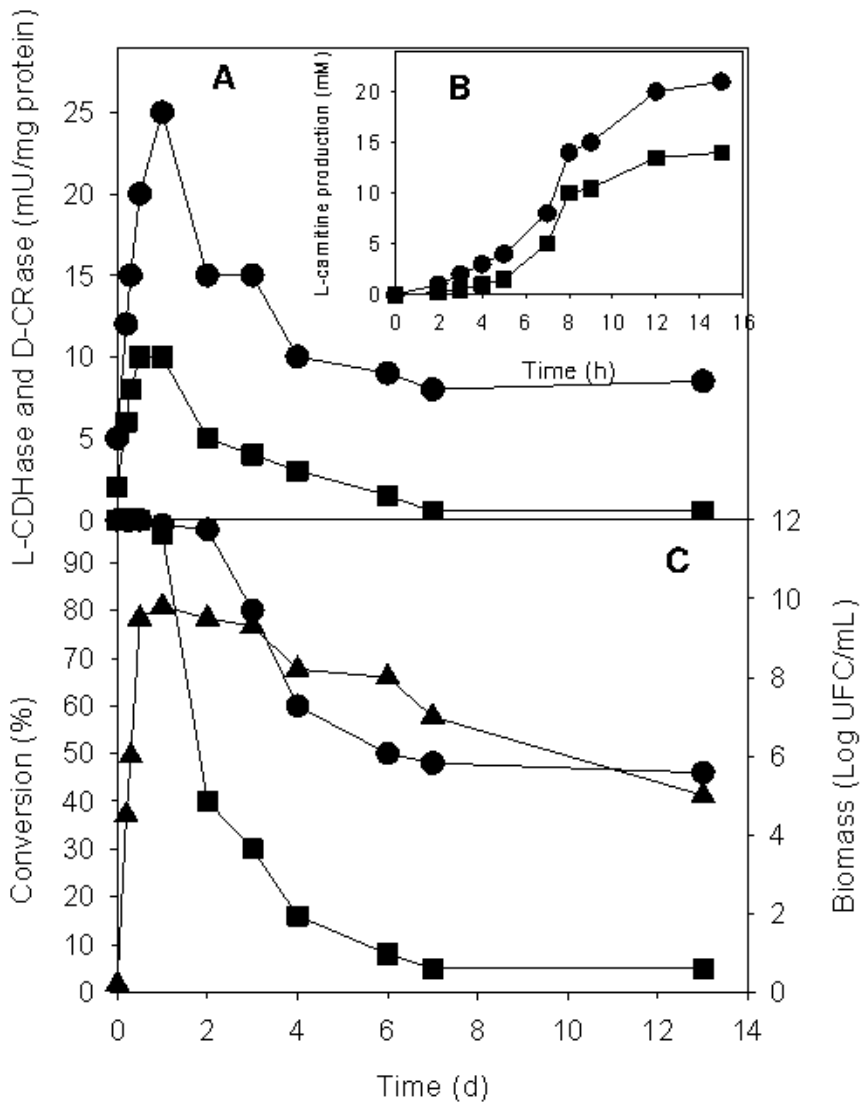

Figure 3. A) L(-)-carnitine dehydratase (●) and D(+)-carnitine racemase (■) expression during *E. coli* O44K74 growth. B) L(-)-carnitine production from crotonobetaine (●) and D(+)-carnitine (■). C) Biomass evolution (▲) and L(-)-carnitine yield with respect to initial levels using crotonobetaine (●) and D(+)-carnitine (■) as the substrates during batch culture. Experiments were made at 37 ºC using complex medium supplemented with 50 mM fumarate under anaerobiosis.

Enzyme expression during growth and biotransformation

Additional batch experiments were carried out, checking the maximum enzyme activity for both strains. In Figure 3A, the evolution of the L(-)-carnitine dehydratase and D(+)-carnitine racemase enzymes for *E. coli* O44K74 is shown. According to the results, maximum enzyme activity was attained after 24 hours of

cell growth, coinciding with the end of the exponential growth phase (Fig. 3C). It was always observed that the level of D(+)-carnitine racemase was half that for L(-)-carnitine dehydratase, whereas the L(-)-carnitine yield at the same substrate concentration was lower for D(+)-carnitine than crotonobetaine (28% for D(+)-carnitine versus 45% for crotonobetaine). Besides this, it was obvious that enzyme activity was lower in the case of D(+)-carnitine racemase, and nearly no activity was detected after six days, while biotransformation with respect to initial levels was close to zero (Fig. 3C).

L(-)-carnitine production as a result of the L(-)-carnitine dehydratase/D(+)-carnitine racemase activity in E. coli.

In previous work (Castellar et al., 2001), L(-)-carnitine production using resting cells of the recombinant strain *Escherichia coli* pT7-5KE32 was studied and optimised with crotonobetaine and D(+)-carnitine as substrates. High biocatalytic activity was obtained after growing the cells under anaerobic conditions at 37-41ºC and with crotonobetaine or L(-)-carnitine as inducer. The best biotransformation conditions were: resting cells grown under aerobiosis (15-30% oxygen saturation, 41ºC and the minimal medium for proper induction of the enzymatic system), with 4 g L^{-1} biomass and 100 mM substrate concentration. In this work and using the same reactor conditions, an L(-)-carnitine productivity of 0.15 g/L·h with a conversion of 32% were achieved with growing cells. In addition, no plasmid loss was observed during batch experiments. In this strain, the batch experiments showed that overexpressed protein (CaiD) presents a racemase activity (Kleber, 1997) capable of transforming D(+)-carnitine into L(-)-carnitine, while γ-butyrobetaine production (Table 1) seems to be coupled to the inherent anaerobic metabolism of the non-transformed *E. coli* K38. This was confirmed by experimental results obtained for the anaerobic growth of non-transformed *E. coli* K38, which rendered low levels of γ-butyrobetaine (12 mM) as final product from D(+)-carnitine (data not shown). Thus, enzyme expression (see Strains and plasmids, Materials and Methods) would be as follows: expression of protein CaiF enhances the expression of the chromosome-coded *E. coli* K38 carnitine metabolism, while the protein CaiE stimulates the inherent crotonobetaine reductase activity, the result being the formation of γ-butyrobetaine, as in the wild strain (Fig. 1), due to the non reversible step. Besides this, under aerobiosis, overexpressed CaiD might show L(-)-carnitine racemase activity coupled to the L(-)-carnitine dehydratase activity. This hypothesis, with two enzymes working in opposite directions but with net equilibrium towards the

hydration side, would explain the ability of *E. coli* K38 pT7-5KE32 to transform D(+)-carnitine into L(-)-carnitine (Fig. 4) and this into crotonobetaine (as in Fig 1); in addition, since the process was aerobic, no γ-butyrobetaine was detected (see Table 1). Therefore, the low levels of D(+)-carnitine racemization obtained could be related to the activity of the dehydration reaction to produce crotonobetaine. The mechanism of D(+)-carnitine racemization is still unknown, although the existence of a protein with different enzyme activities can be postulated. This would resemble the recently described crotonobetaine reductase system, which consists of a multimeric enzyme, codified by ***caiAB***, with two different activities: crotonobetaine reductase and an L-carnitine-CoA transferase (Preusser et al., 1999). Furthermore, the cofactor for carnitine dehydratase and crotonobetaine reductase has been shown to be identical (Elssner et al., 2000). Taken together, these facts would explain the levels of crotonobetaine detected during the batch and continuous studies of D(+)-carnitine racemisation (Fig. 4).

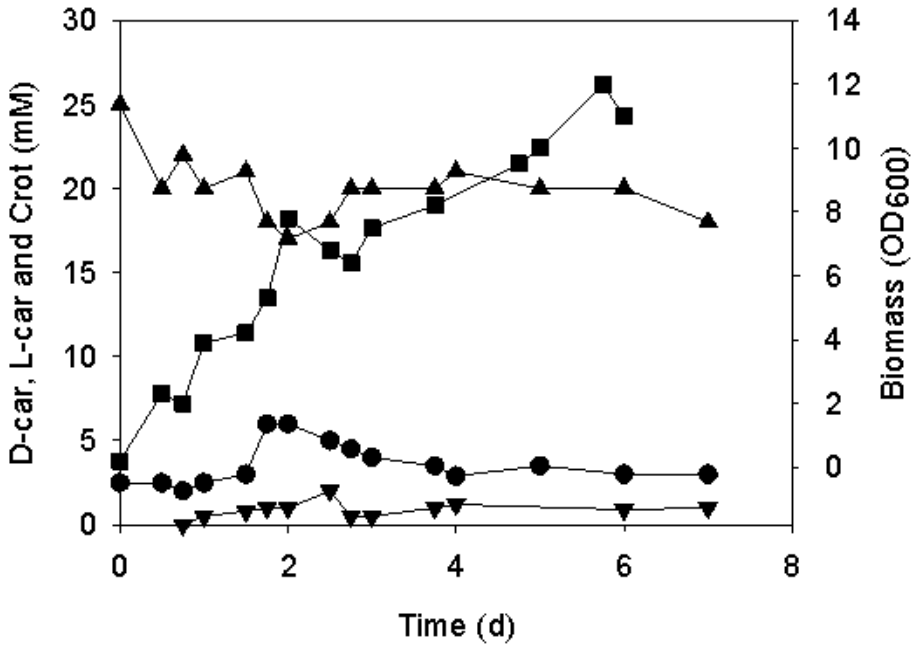

Figure 4. Profiles of optical cell density (■), L(-)-carnitine production (●), D(+)-carnitine level (▲) and crotonobetaine production (▼), determined during continuous cell-recycle culture of *E. coli* K38 pT7-5KE32. Cell culture was performed using minimal medium with a glycerol concentration of 1.3 g/L, 25 mM D(+)-carnitine, a 30% oxygen saturation and 41ºC. The antibiotic concentration used was 50 µg/mL ampicillin and 25 µg/mL kanamycin. Dilution rate was set at 0.9 h^{-1}.

Continuous biotransformation experiments: Cell-recycle reactor with cross-flow modules and hollow fibers cartridges

The choice of appropriate biocatalysts, reaction media and reactor configuration is of vital importance for L(-)-carnitine formation (Lee and Chang, 1989; Obón et al., 1999). In previous studies the biotransformation of crotonobetaine into L(-)-carnitine by a wild *E. coli* strain within packed bed and cross flow filtration continuous reactors was studied and high productivities were attained (Obón et al., 1997 and 1999). However, the success and economic viability of the biotechnological production of this fine chemical depends not only on the choice of biocatalyst but also on the chemical and physical reaction environment. For a good performance of biotransformations catalyzed by transformed strains in continuous systems, there must exist some well-balanced reactor conditions, in which copy number, transcription, and translation efficiency are optimally balanced to maximize productivity. Furthermore, bioreactor development represents the focal point for interaction between the basic scientist and the process designer (Lidén, 2002). Experiments need to be addressed to understanding the cellular physiology in process conditions, using the same cells in the reactor described and subjecting them to production conditions in order to ascertain their dynamic responses. The present study deals with the use of membrane continuous reactors to establish and optimize the process of crotonobetaine and D(+)-carnitine biotransformation into L(-)-carnitine with cells of *E. coli* as biocatalyst. Different membrane configurations were checked as a means of retaining the catalyst within the system and obtaining a clean aqueous product. Culture techniques, such as cell-recycling using hollow fibers and cross-flow filtration modules were used, to improve the retention of the biomass compartmentalized in the reaction vessel and thus determine the productivity and stability of the process. Commercial non-biological membranes made of different materials were used in this work.

Transformed E. coli strain

A continuous process with the transformed strain was started with the antibiotic concentrations used (50 µg/mL ampicillin and 25 µg/mL kanamycin, unless otherwise stated in the text). The systems were equipped with four 0.1 µm PVDF Durapore plates. Figure 5, shows the reduction in L(-)-carnitine yield which occurred after 48 hours, coinciding with an increase in the biomass content of the reactor. This fact could be due to possible loss of the plasmids as a result of a segregation process followed by faster cell growth. Thus, it was decided to study plasmid stability during continuous cultivation and to monitor changes in carnitine

concentration and CaiD with time. Experiments using different antibiotic concentrations were then carried out to check whether these results might be due to instability of the strain. Figure 5A shows that, with a low antibiotic concentration, plasmid segregation started after 2 days of continuous operation. In the following days, cells without plasmids colonized the reactor, and so the residual bacterial CaiD was still able to produce a lower level of L(-)-carnitine. Thus, the evolution of L(-)-carnitine runs in parallel to the presence of plasmid-bearing cells and the rest of cells in the reactor. When a higher antibiotic concentration was used (Figure 5B) plasmid segregation started after 5 days, after which a sharp decline in plasmid-bearing cells as well as in the activity of the racemase was observed. It is interesting to note that plasmid segregational instability occurred while the cells were apparently not growing. The overexpression of plasmid-coded proteins could cause a significant reduction in the specific growth rate of plasmid-bearing cells, while the specific growth rate of plasmid-free cells would remain high. Furthermore, when operating in continuous cell recycle reactors, substrate limitations may become more accused, because of the high cell density values obtained.

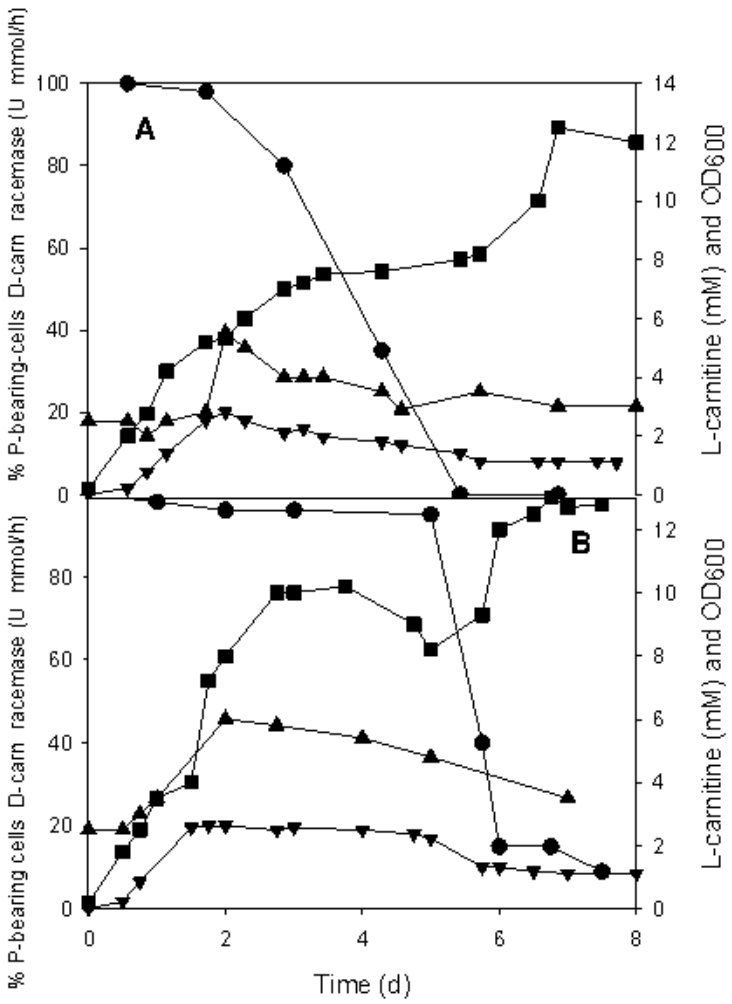

Figure 5. (*Pag. 52*) Profiles of optical cell density (■), % plasmid-bearing cells (●), L(-)-carnitine concentration (▲) and D(+)-carnitine racemase (▼) determined during continuous cell-recycle culture of ***E. coli*** K38 pT7-5KE32. Cell culture was performed using minimal medium with a glycerol concentration of 1.3 g/L, 25 mM D(+)-carnitine, a 30% oxygen saturation, 41ºC, and at 0.2 h^{-1} dilution rate. Two different antibiotic concentrations were tested: (A) 50 µg/mL ampicillin and 25 µg/mL kanamycin, (B) 200 µg/mL ampicillin and 100 µg/mL kanamycin.

Thus, at the end of the run, ***E. coli*** K38 only contained the complete divergent structural operons, ***caiTABCDE*** and ***fix***ABCX, thus maintaining residual L(-)-carnitine production from the function of CaiD, CaiE and CaiF of the trimethylammonium metabolism (Eichler et al., 1994 and 1996). Compared with those from batch experiments, productivity was higher (1.25 g/L·h) and L(-)-carnitine conversion slightly lower (28%). The low conversion values could be due to: a) the energy required for the carnitine racemization process, b) the formation of inclusion bodies (Schein, 1989), or c) the rearrangement of plasmid DNA sequences affecting cloned genes (Peretti et al., 1989). Thus, the recombinant microorganism was found to be segregationally unstable under the conditions tested. Nevertheless, since medium composition and reactor configuration might strongly affect cell stability, new studies were performed in order to overcome this difficulty.

The instability of the strain was not dependent on the reactor configuration, since experiments performed in a chemostat operating at a dilution rate of 0.15 h^{-1} (far below the maximum specific growth rate of the bacteria, 0.4 h^{-1}) showed that the population of plasmid-bearing cells decreased after 4 days and virtually disappeared from the reactor after 6 days of operation (data not shown). Thus, it was shown that the two-plasmid based expression system was highly unstable and inadequate for continuous culture. By plating samples from the reactors in solid media with different antibiotics, it was shown that the first plasmid to disappear was the ampicillin resistant and high-copy, pT7-5, while the pGP1-2, which is kanamycin resistant and low-copy, was apparently more stable. It has been previously stated that the stability of plasmids is dependent on both their copy number and resistance mechanisms. In order to stabilize the two-plasmid expression system, it was decided to restrict the strain growth rate by entrapping the cells in either Ca-alginate or κ-carrageenan gels. Immobilization of ***E.coli*** K38 pT7-5KE32 in carrageenan gels succeeded in genetically stabilizing the strain, allowing for the continuous operation of the reactor with immobilized cells at rates much higher than the specific growth rate with no

plasmid loss. Not only was high stability observed, but also a high increase in productivity compared with the free cell chemostat reactor. After 140 hours of continuous operation at a dilution rate of 0.2 h^{-1}, the strain remained stable and both plasmids were still present. An increase in the dilution rate to 0.5 h^{-1} and 1.0 h^{-1} did not influence the stability of the strain, but increased the productivity, its value being to an extent proportional to the dilution rate employed (Fig. 6).

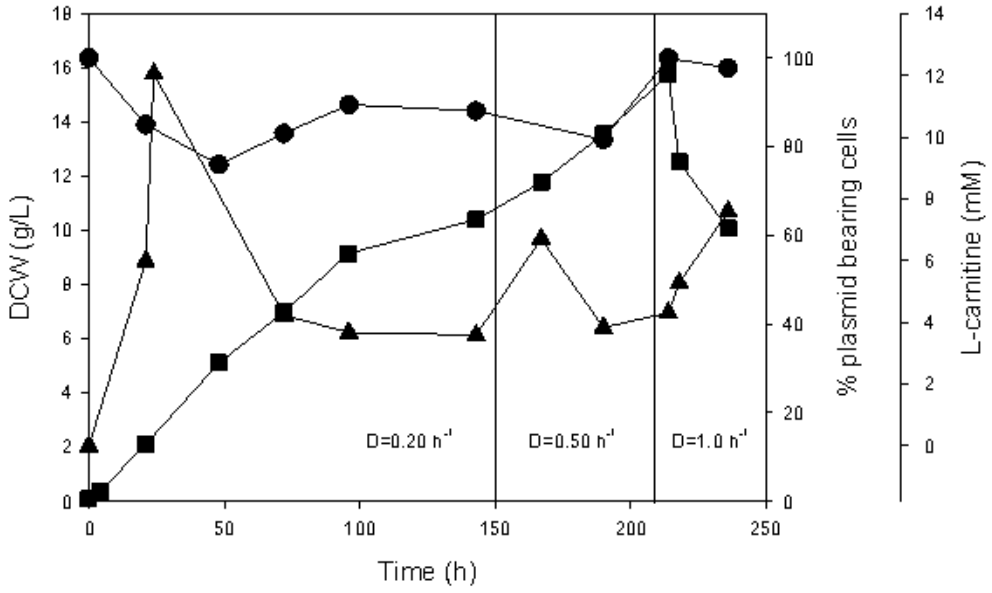

Figure 6. Profiles of optical cell density (■), % plasmid-bearing cells (●) and L(-)-carnitine concentration (▲) determined during continuous culture of ***E. coli*** K38 pT7-5KE32 immobilized in carrageenan gels. Cell culture was performed using minimal medium with 100 mM crotonobetaine, 30% oxygen saturation, 37 ºC, and at three different dilution rates.

In Table 3, biomass levels, and L(-)-carnitine production are shown for both experiments at every working dilution rate. In the chemostat system, the experiments were run at dilution rate values always below the maximum specific growth rate (0.35 h^{-1}), while in the immobilized cells system, the dilution rates ranged from 0.2 to 1.0 h^{-1}. As can be seen, although the dilution rates increased and therefore the residence time decreased, the biomass content of the reactor increased as a result of higher glycerol content, which acts as the carbon and energy source for cell growth availability (Bailey and Ollis, 1986). Productions ranging from 0.20 to 0.71 g/L·h were attained depending on the working dilution rate, while the chemostat with freely suspended cells showed a lower production of

0.07 g/L·h. Furthermore, higher productivities could be obtained by increasing the amount of immobilized biomass inside the reactor, thus modifying the beads to culture medium ratio, since in our work a low ratio was used (1 bead volume/5 reactor working volumes) as only plasmid stability studies were the aim. Further experiments should be performed to optimize this point.

Table 3. Effect of immobilization of E. coli K38 pT7-5KE32 in carrageenan gels. Biomass levels (g/L) and L(-)-carnitine productivities (g/L·h) with free cell reactors (chemostat) and immobilized cells reactors, at different dilution rates. Crotonobetaine was used as substrate at a concentration of 100 mM and oxygen was set at 15-20%. Medium as in Materials and Methods.

	Dilution rate (h^{-1})	**Maximum productivity ($g \cdot L^{-1} \cdot h^{-1}$)**	**Maximum biomass ($g_{DCW} \cdot L^{-1}$)**
Chemostat (free cell reactor)	0.15	0.07	3.2
Carrageenan gel immobilized cell continuous reactor	0.20	0.20	9.2
	0.50	0.56	13.6
	1.00	0.71	15.7

Wild E. coli strain

Escherichia coli O44K74 cells were cultured anaerobically at 37ºC. In order to increase the biocatalyst concentration within the reactor, cells were retained by means of a polysulfone microfiltration membrane of 0.1 μm cut-off and subjected to recycling. The membranes were assembled in a flat-sheet module. Table 4 shows the biomass levels, L(-)-carnitine production, crotonobetaine conversion and the productivity achieved within the system. L(-)-carnitine yield was considered as the percentage of L(-)-carnitine produced from the crotonobetaine supplied (50 mM from 0.2 to 1.5 h^{-1} and 100 mM at 2 h^{-1}). Productivity was determined after reaching the steady state, unless specified otherwise. It was concluded that the continuous production of L(-)-carnitine from crotonobetaine by *E. coli* O44K74 cells in a cell-recycle reactor can reach values as high as 6.5 g/L·h, with conversions into L(-)-carnitine of 35-46 %. When the system was in steady operation at a dilution rate of 1 h^{-1}, and using a crotonobetaine concentration of 50 mM in the feed medium, the concentration was switched to 100 mM and the dilution rate increased to 2 h^{-1}, in

order to observe the evolution of this strain of *E. coli* in new conditions. In Fig 7, the evolution of *E. coli* O44K74 at different dilution rates is observed. Biomass increased to a new steady value and biotransformation almost reached the same L(-)-carnitine yield as in the previous steady operation, though with nearly double the productivity (11.5 g/L·h L(-)-carnitine). Hoeks et al., (Hoeks et al., 1996) reported that L(-)-carnitine productivity during the biotransformation of γ-butyrobetaine was 5 g/L·h. In previous studies of crotonobetaine biotransformation into L(-)-carnitine in cell-recycle systems, productivities as high as 6 g/L·h at dilution rates of 2.0 h^{-1} were reported (Obón et al., 1999). The results obtained in this work represent an important improvement, principally due to the optimization of the cell physiology in process conditions. Thus, the wild strain demonstrated that it was suitable for process operation and even capable of being subjected to dynamic stress, which may be useful for controlling and optimizing strategies (Lidén, 2002). Moreover, it seems that there existed a maximum of L(-)-carnitine dehydratase activity (Fig. 7), since the increase in the biomass level was not matched by any increase in this enzyme activity when the dilution rate was increased. Further, a certain amount of cell debris within the reactor was apparent, which could be the result of the high dilution rate and be partly responsible for the increased optical density. However, although the L(-)-carnitine dehydratase level did not increase, substrate biotransformation did, as productivity was double the previous value, which means the catalysis capability of the cells had been improved. This fact is important and might imply cellular control of the biotransformation reaction catalysis, which, to our knowledge, has not previously been detected in trimethylammonium metabolism studies in *E. coli*.

In order to optimise the continuous production of L(-)-carnitine, the suitability of using a cross-flow filtration module with flat-sheets of different membrane compositions and cut-offs was checked. Thus, systems equipped with three different membrane types were studied: cellulose membranes of 300 KDa nominal cut-off, polysulphonated polysulphone membranes of 0.2 µm nominal cut-off, and ceramic membranes of 0.1 µm nominal cut-off. The continuous reactor system was identical to that previously explained (Materials and Methods). In this case, the medium was added at a 1.5-1.8 h^{-1} dilution rate, and pH and temperature were set at 7.5 and 37ºC, respectively. Table 5 presents the results of reactor performance obtained in the experimentation. No important differences were observed and it was concluded that membrane composition and membrane cut-off had little influence on reactor performance since similar productivities were obtained.

Table 4. *E. coli* O44K74 biomass levels, L(-)-carnitine production, crotonobetaine conversion, and productivity within the continuous membrane cell recycle reactor. Crotonobetaine concentration was 50 mM from 0.2 to 1.5 h^{-1} and 100 mM at 2 h^{-1}. Medium as in materials and Methods.

	Dilution rate (h^{-1})				
	0.2	0.5	1.0	1.5	2.0
Biomass (g dry weight/L)	2.5	6.8	9.4	18.1	27.0
L-carnitine production (%)	38	40	42	38	30
Crotonobetaine conversion (%)	40	44	46	42	35
Productivity (g/Lreactor.h)	0.45	1.75	3.5	5.5	11.5

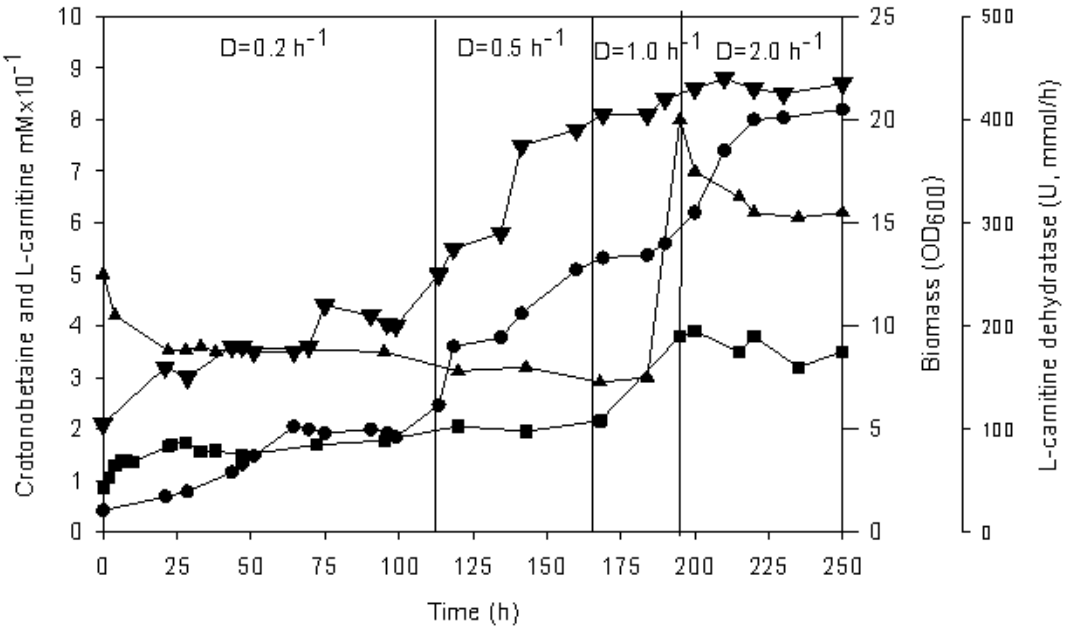

Figure 7. Profiles of optical cell density (●), L(-)-carnitine dehydratase, (▼) and L(-)-carnitine (■) and crotonobetaine (▲) concentrations determined during continuous cell-recycle culture of ***E. coli*** O44K74. Cell culture was performed in complex medium with 50 mM crotonobetaine from 0.2 to 1 h^{-1} and 100 mM at 2 h^{-1} dilution rate, anaerobiosis and at 37 ºC.

Finally, in order to compare operational stability for different membrane configurations, a new reactor set-up was used, based on cell recycling by means of a hollow-fiber cartridge module with fibers made of polysulphone, with a total surface area available of 0.03 m^2. Throughout the set of experiments, the dilution rate was kept constant at 1.8 h^{-1}. The cells were fed, as previously mentioned, with a biotransformation medium containing 50 mM crotonobetaine. Under these conditions, the results presented in Table 5 were obtained. Good stability and easy cleaning up compared with the cross-flow systems were evident. However, the main finding was the L(-)-carnitine dehydratase/biomass ratio obtained, since it showed a lower value than that obtained with the other systems. This was probably a result of cell debris accumulating, possibly due to the increased dilution rate, which could involve a decrease in the active L(-)-carnitine dehydratase with respect to biomass dry weight (Table 5). This was also seen in experiments run at a 2 h^{-1} dilution rate (Fig. 7). However, this system presented a slightly higher productivity, which also meant that a slightly higher level of enzyme was present. Moreover, the stability of the system was remarkably robust throughout the experimental run.

Table 5. L(-)-carnitine production, L(-)-carnitine dehydratase/biomass ratio, maximum productivity, biomass concentration and dilution rate achieved within the continuous reactor operation with ***E. coli*** O44K74 retained by means of different membrane systems. Crotonobetaine concentration in the studies was 50 mM. Medium as in Materials and Methods.

	Dilution rate (h^{-1})	**CDHase/ biomass ratio (U/g_{DCW})**	**L-Car production (%)**	**Max. productivity (g/L·h)**	**Max. biomas (g_{DCW}/L)**
Cellulose (300 KDa)	1.5	18.18	42	5.29	16.5
Polysulphonated polysulphone (0.2 μm)	1.8	15.62	38	5.74	19.2
Ceramic membranes	1.6	16.21	41	5.09	18.5
Hollow fiber system	1.8	12.95	45	6.80	25.1

CONCLUSIONS

A procedure for the production of L(-)-carnitine from crotonobetaine or D(+)-carnitine, characterized by the direct separation of the immobilized microorganisms and the reaction mixture, and using *E. coli* recycling with commercial cross-flow filtration or hollow-fiber modules, allowed us to obtain a clean product. This procedure provided a two-fold increase in L(-)-carnitine productivity (11.5 g/L·h) with respect to other reported systems, and enabled a stable continuous process to be run, since perturbations did not affect steady operation. The physiology of *E. coli* in production conditions is now better understood and different reactor configurations for process optimization are also possible. More importantly, the metabolism of the trimethylammonium compounds of the biocatalyst was found to exercise a certain degree of cell control on the biotransformation reaction catalysis, which suggests the possibility of applying metabolic engineering studies to improve the connection between primary and secondary metabolism and the transport of the biotransformation substrate.

ACKNOWLEDGEMENTS

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Capítulo 2

Analysis of *Escherichia coli* cell state by flow cytometry during whole cell catalyzed biotransformation for L-carnitine production.

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ABSTRACT

Flow cytometry was used to monitor *Escherichia coli* cellular state during the biotransformation of crotonobetaine into L(-)-carnitine using growing and resting cells in batch and high-cell recycle continuous membrane reactors. The cell physiological state and the DNA, RNA and protein cell content were analyzed during the bioprocess. The cell growth cycle was followed by reference to cellular DNA concentration and the entry in the stationary phase resulted in an increase in intracellular protein. The biochemical activity of resting cells was assessed for the first time at the molecular level, protein synthesis being observed despite the absence of nutrients. Freely suspended growing, both in batch and continuous cultures, and, more importantly, resting *E. coli* cells were seen to be made up of subpopulations differing in reproductive ability, metabolic activity and membrane integrity. In the case of growing cells, biotransformation was mostly performed by fully viable cells (68-75%) , while in a resting cell system, also dead cells (1-5%) and cells with doubtful viability (60-70%) appeared to be involved in the process; in later stages, a population made up of phantom cells, containing little or no cellular DNA, was detected. In cell-recycle continuous reactors, the recording of DNA (40-60 fg), RNA (50-120 fg) and protein (100-220 fg) levels per unit of cell, and the evolution of cell population heterogeneity (three different populations of cells) threw light on the stress conditions imposed by high cell densities. The use of FCM allowed to follow the recovery of cell catalytic activity for resting biotransformation batch processes, thus showing its potential for the optimization of bioprocesses.

INTRODUCTION

The optimization of bioprocesses for the production of high value compounds using microbial cells depends on cell physiology and biochemistry since both determine the performance of the developed production systems. The choice of adequate reactor configuration is a key step in the optimization of bioprocesses, since multiple factors are involved in the complicated relationships at the bioreactor-microorganism interface. Biotransformations can be performed in an industrial context using both growing and resting cells as biocatalyst. In resting cell processes, cell growth and biotransformation occur in two successive phases: once grown, the cells are collected and placed in the buffered biotransformation substrate, thus simplifying and reducing the cost of the purification (Gokhale et el., 1996; Wagner et al., 1996). On the other hand, cell immobilization has been used for increasing biocatalyst concentration and improving stability. Recently, an increase in the study and development of membrane bioreactors has been assessed (Giorno and Drioli, 2000). Membrane barrier at the reactor outlet retain cells, while allowing small molecules to pass through. Knowledge on cell physiology is crucial for biotransformations involving resting cells, since the process running time and the best biochemical and physiological conditions affect the whole process if optimization is the aim. However, no information has been available until now on the cell physiology and molecular variables (DNA, RNA and protein per cell unit) of resting cells performing biotransformation processes for use during modelling, control and process optimization. Further, in membrane bioreactors, the cells may be exposed to highly stressing conditions, which will inevitably affect their physiologic and metabolic states (Cánovas et al., 2003) since the high cell density achieved can affect nutrient fluxes, due to transport gradients and stress conditions for cells, finally leading to different cell states.

Within this landscape, proper reactor design and operational conditions are fundamental, while easy to implement monitoring techniques are necessary to control process performance in the steady-state and under perturbation (Cooney, 1983; Hewitt et al., 1999a). It is therefore crucial to monitor intracellular compounds level and to control the cell physiology throughout the bioprocess by means of rapid and easily applicable techniques. Such ability will help to characterize the cell metabolism and physiology in the production-directed environment of the reactor (basic research) so as to fully understand and optimize the processes involved (applied research).

Flow cytometry (FCM) is a powerful technique for the rapid characterization of cell populations which, in recent years, has begun to be used to study prokaryotic cells in different environments (Akerlund et al., 1995; Akermann et al., 1995; García-Ochoa et al., 1998). Cells can be detected on the basis of their light scattering properties in the forward (FALS) and the right (RALS) angle direction and, more importantly, staining with fluorescent molecules allows to probe the intracellular environment even with living cells. FCM has evolved as a leading tool due to its growing role in bioprocess development and monitoring (Hewitt et al., 1998, 1999b, 2000; Looser et al., 2005).

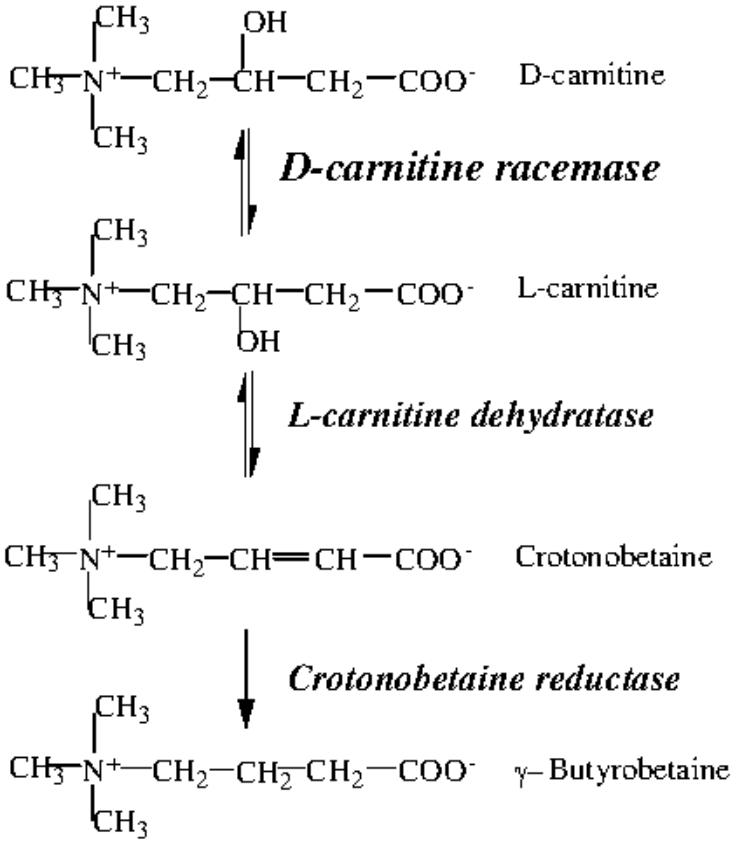


Figure 1. Biotransformation of trimethylammonium compounds in *E. coli* cells.

L(-)-carnitine, which is an essential cofactor for mitochondrial transport of lipids in higher animals, has many clinical applications (Seim et al., 2001). Its chemical production leads to a racemic mixture, which needs to be resolved to obtain the pure biologically active L-isomer. D(+)-carnitine is a waste product, which can be

chemically dehydrated to crotonobetaine and estereospecifically biotransformed into L(-)-carnitine by *Escherichia coli* (Kleber, 1997; Castellar et al., 1998; Obón et al., 1999). *E. coli* is able to catalyze both crotonobetaine hydration and D(+)-carnitine racemization into L(-)-carnitine (Kleber 1997), while crotonobetaine reduction into γ-butyrobetaine side-reaction (Figure 1) can be prevented by enzyme inhibition (Cánovas et al., 2002).

The aim of this work is to report on how FCM can be used to determine the cell physiological state during the biotransformation of crotonobetaine into L(-)-carnitine by *E. coli* cells, in batch reactors with both growing and resting cells and in continuous high-cell density membrane reactors so as to develop strategies for bioprocess optimization. Therefore, changes in cell viability and the evolution of different cell populations within the reactors throughout the experimentation process have also been studied. In this way, *E. coli* cells were characterized in the bioreactor environment during the biotransformation process in stress situations, including resting media, where only a biotransformation substrate and a buffer are present and high-cell density membrane reactors with highly viscous media, where toxic metabolites are produced and nutrient shortages are common during the process.

MATERIALS AND METHODS

Growth of the bacteria

Escherichia coli O44K74 (DSM 8828) was grown under the different conditions stated in the text. The cultures were inoculated with a 3% (v v^{-1}) of a pre-culture stored at -20ºC in 20% (v v^{-1}) glycerol and grown at 37ºC. The complex (CM) and minimal (MM) media employed have been previously described (Obón et al., 1999; Cánovas et al., 2002). Glycerol was used as carbon source while crotonobetaine was the substrate for the biotransformation; the pH of the media was adjusted to 7.5 with 1 M KOH prior to autoclaving.

Biotransformation experiments

Growing cells. The carnitine metabolism (Fig. 1) is principally related to growth under anaerobic conditions (Castellar et al., 1998; Obón et al., 1999). Batch biotransformation experiments in anaerobic assays (under nitrogen atmosphere) were performed with either the CM or MM medium using crotonobetaine (50-100 mM) as the substrate. The reactors were equipped with temperature and pH controllers (Biostat B, Braun, Germany). A 1 L culture vessel with 0.5-0.8 L working volume was used.

Resting cells. Assays were performed as follows: cells were batch grown, and at the end of the exponential growth phase were harvested by centrifugation, washed and resuspended in the biotransformation medium, which contained crotonobetaine as the substrate (50-100 mM) in phosphate buffer 67 mM, pH 7.5 and transferred to the reactor vessel under sterile conditions.

Continuous high-cell density membrane reactor. The same reactor vessel used for batch cultures was adapted to continuous cell-recycle operation by incorporating the membrane cross-flow filtration module (Minitan, Millipore, USA) (Cánovas et al., 2002) (Fig. 2). The system was equipped with four hydrophilic polyvinylidene difluoride (PVDF) Durapore plates of 0.1 µm pore size and a total area of 60 cm^2 (Millipore, USA), which retained the biomass in the system, while clear cell-free product was purged in the filtrate. The pump control was that of Braun Biostat B (Germany); the medium inlet and filtration flow rates were controlled to keep a constant working volume.

Assays

Biomass and biotransformation variables. The optical density of the sample was followed at 600 nm (A_{600}) with a spectrophotometer (Novaspec II, Pharmacia-

LKB, Sweden) and correlated with dry cell weight. L(-)-carnitine concentration was determined by an enzymatic test, as previously described (Cánovas et al., 2002), while crotonobetaine was determined by HPLC, using a Tracer Spherisorb-NH_2 column (3 μm, 25 cm x 0.46 cm) supplied by Teknokroma (Barcelona, Spain). The isocratic mobile phase was acetonitrile/H_3PO_4 0.05 mol l^{-1} pH 5.5 (65/35) at a flow rate of 1 ml min^{-1}.

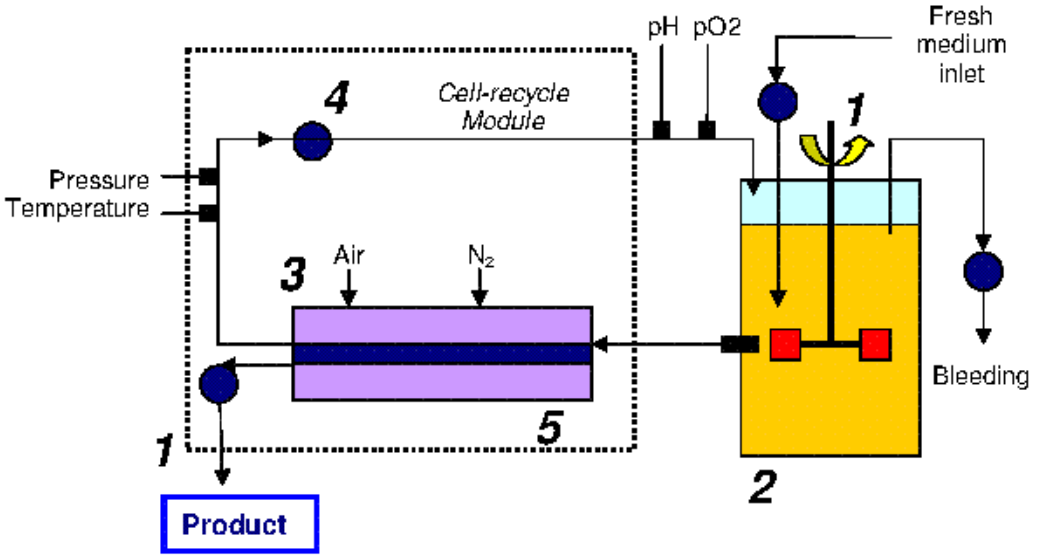


Figure 2. Experimental set up for cell-recycle experiments. 1. Inlet and outlet pumps. 2. Reactor vessel. 3. Air/N_2 supply (aerobic or anaerobic system). 4. Recycling pump. 5. Membrane filtration or hollow fiber module. The controller of the system was that of the Braun Biostat B. For batch experiments, experimental set up was the same without the cell recycle module.

Flow Cytometry. The reactor samples were run in a Becton Dickinson FASort model (San José, USA) equipped with an argon laser for excitation at 488 nm and 15 mW and equipped with filters at 530, 585 and 650 nm. Samples were analyzed in the linear photomultiplier gains mode, a total of 10,000 cells being analyzed for each sample at a rate of 800-2,000 cell s^{-1}. The FCM probes fluorescein isothyocyanate (FITC), ethydium bromide (EB), propidium iodide (PI) were purchased from Sigma-Aldrich, while bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$ or BOX) was purchased from Molecular Probes Inc. EB was used for DNA and RNA analysis (after cell treatment with RNase and DNase) and FITC for protein analysis on fixed

cells. PI and BOX were used for viability studies on living cells. Stained cells were diluted in PBS buffer pH 7.2 (g l^{-1}: NaCl 8.00, KCl 0.20, Na_2HPO_4 0.91 and KH_2PO_4 8) previously sterilized by 0.22 µm filter. FALS and RALS values, allowed cell debris discrimination and a total of 10,000 events were used for statistical data analysis.

Calibration for DNA, RNA and proteins. For the quantification of the intracellular content of nucleic acids and proteins, FITC and EB staining was employed. The optimized staining protocol consisted on the fixation of cells with 3% (v v^{-1}) glutaraldehyde for 30 min at 4ºC. The fixed cells were then stained with either 30 µg ml^{-1} FITC in 0.1 M Tris/HCl buffer, pH 7.4, 0.1 M NaCl, 1 mM EDTA for 5 h or 100 µg.ml^{-1} EB in 0.1 M Tris/HCl buffer, pH 7.4, 0.1 M NaCl, for 15 min at room temperature. To avoid non-specific fluorescence signal (background signal) the samples were washed with buffer at least twice. EB stained cells were subsequently treated with either RNase or DNase as follows: cells were suspended in 50 mM Tris/ClH, pH 7.4 buffer in 5 mM $MgCl_2$ with 1 mg ml^{-1} RNase and 0.1 M Tris/ClH, pH 7.4 buffer in 0.1 M NaCl with 5 mM $MgCl_2$ with 1 mg ml^{-1} DNase, respectively. Both treatments were performed in a volume of 50 µl and cells were incubated at 37°C for 1 h in the dark. Calibration curves for DNA, RNA and protein were obtained using a fluorimeter (Floustar-BGM, Offenburg, Alemania). EB was excited at 485 mm and recorded at 590 nm, while FITC was excited at 485 nm and recorded at 520-535 nm. The cytometer outputs were correlated against the fluorimeter (see García-Ochoa et al., 1998) employing six sets of independent experiments with *E. coli* O44K74. The calibration curves obtained for DNA, RNA and protein per cell unit were: y= ax + b, DNA (a=14.9, b=484.9; r^2=0.958), RNA (a=6.9, b=423.3; r^2=0.969) and protein (a=5.1, b=649.7; r^2=0.949), where *x* is the cytometer mean fluorescence intensity in relative units and *y* is the content in DNA, RNA or protein in fg-$cell^{-1}$-10^2. Samples were analyzed in triplicates.

Viability studies. To determine cell viability by FCM, double staining was performed according to Hewitt et al. (1998, 1999a). Heat stressed cells treated at 60°C for 5 min and exponentially growing cells were used as positive and negative controls respectively (Hewitt et al., 1998). The green fluorescence channel for BOX-stained cells (X axis) was plotted versus the red fluorescence channel for PI/BOX stained cells (Y axis). Dot plot representations are in log scale, while data refer to the channel mean fluorescence, with a resolution of 1,024 channels. The WinList programme, supplied by the flow cytometer manufacturer was used for data representation.

RESULTS

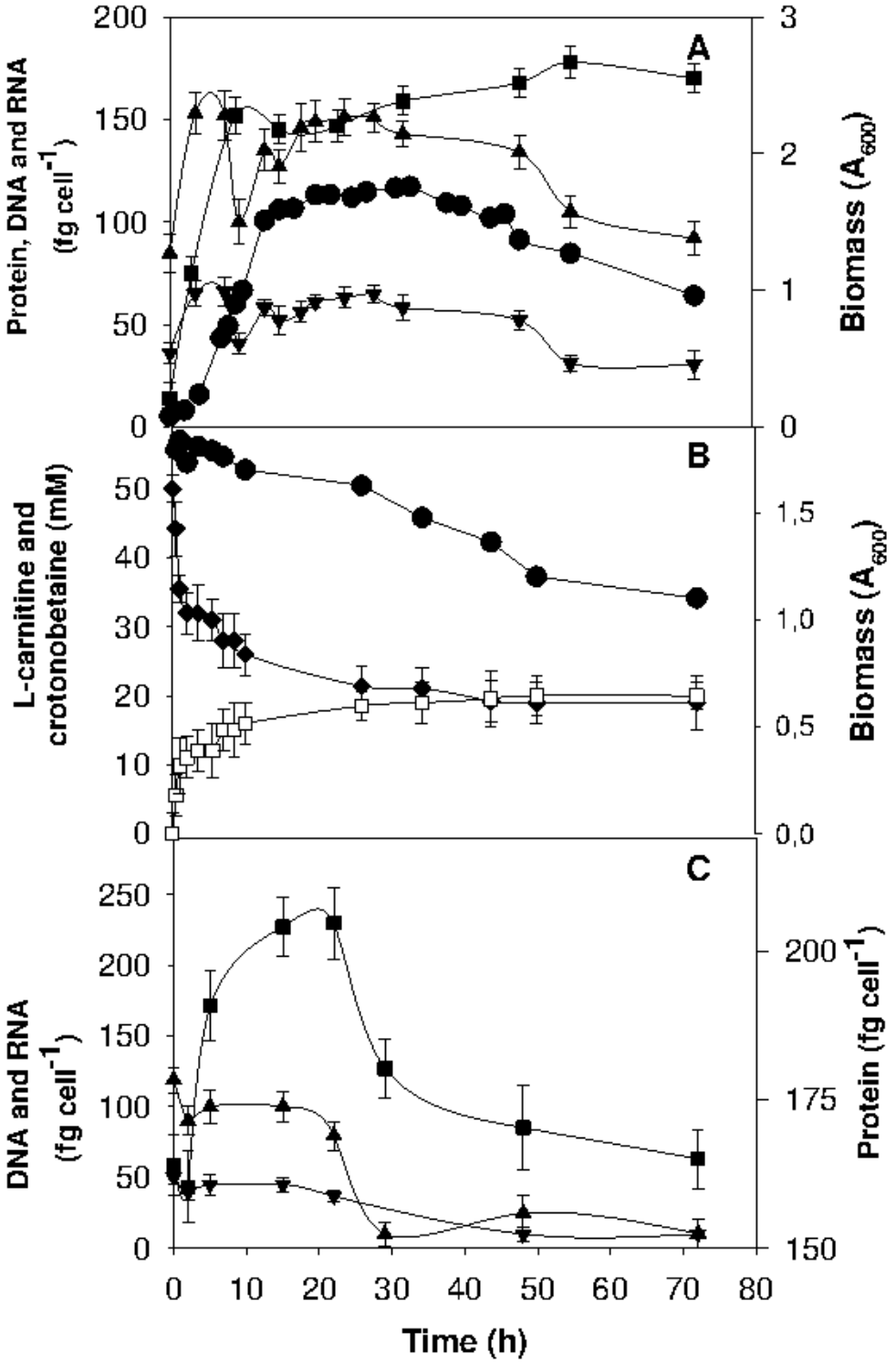
Evolution of DNA, RNA and protein per cell unit

The macromolecular content of *E. coli* cells was followed in order to assess the effect of different growth conditions and reactor configurations. Glutaraldehyde-fixed cells were stained following optimized protocols and samples were run in the flow cytometer (see Material and Methods section).

Batch biotransformation: growing and resting cells

In batch reactors with growing *E. coli* O44K74 in biotransformation medium, protein concentrations increased up to values of 150 fg cell^{-1} after 9 h of growth on the CM and anaerobiosis (Fig. 3A). The slight increase in protein content between 30 and 70 h did not coincide with the measured decrease in A_{600} (start of death phase) nor with the decrease in RNA cell^{-1}, this latter reflecting the lower cell biochemical activity as a result of cell death or deterioration. Regarding DNA cell^{-1} and RNA cell^{-1}, both showed a maximum at the beginning of the exponential growth phase (3-8 h). Levels decreased after 13 h, and still further from 45 h onwards, mirroring the biomass decay. During the early exponential growth phase, the DNA cell^{-1} was nearly double that observed at the beginning of the experiment. Further, levels fell again, due to cell division, increasing afterwards, at 13-30 h, when DNA replication occurred, though both this and the cell division were far slower since cells had entered the stationary phase. A similar behaviour was observed for the RNA cell^{-1} during cell population growth.

Figure 3. (*Pag. 76*) A) (■) Protein cell^{-1}, (▼) DNA cell^{-1}, (▲) RNA cell^{-1} and (●) biomass evolution for *E. coli* O44K74 growing cells in batch reactors under anaerobic conditions with CM at 37ºC and pH 7.5 during the biotransformation of 50 mM crotonobetaine. B) (□) L(-)-carnitine, (◆) crotonobetaine and (●) biomass and C) (■) Protein cell^{-1}, (▼) DNA cell^{-1} and (▲) RNA cell^{-1} evolution during the biotransformation of crotonobetaine into L(-)-carnitine using resting cells of *E. coli* O44K74. Assays using resting cells were performed with an A_{600} of 2, 50 mM crotonobetaine at 37ºC and pH 7.5 in batch reactors.



For the resting cells experiments, previously grown cells were harvested and fed onto a biotransformation medium (Materials and Methods), and L(-)-carnitine production, crotonobetaine consumption and cell evolution were followed at different times of the process (Fig. 3C). While performing the biotransformation, the protein, DNA and RNA per unit of cell were followed with time (Fig. 3B). At zero time, the

cells, from a 15 h culture, were at the end of the exponential phase, replicating their genetic content (Fig. 3B), thus being nearly diploid, while the protein content approached the maximum level. The most noticeable feature was that the protein level of resting cells reached 200 fg cell ^{1} after 20 h and then fell until the end of the experiments. The maximum peak of protein coincided with steady levels of RNA and DNA (60 and 110 fg cell ^{1} between zero and 25 h, respectively). From this moment onwards there was a decrease in both RNA and DNA, parallel to the decrease in A_{600}, reaching levels below the technique's sensitivity limit. The recorded A_{600} values probably included large amounts of dead and broken cells since L(-)-carnitine reached steady values from 15 h onwards (Fig. 3C). At the end of the process a considerable number of cells should probably be regarded as phantom cells as no nucleic acids could be detected.

Biotransformation in continuous high-cell density membrane reactors.

Reactors were also run with a membrane retention system (Fig. 2), and the evolution of DNA, RNA and protein per cell unit in the bacterial populations was followed in order to assess: a) the effect of high-cell density on bacterial biochemistry and physiology, and b) the effect of stress due to viscosity and nutrient shortages imposed by the reactor environment on the bioprocess itself. In Figure 4A, the evolution of total biomass and the consumption of crotonobetaine to produce L(-)-carnitine (Fig. 1) are shown, while Figure 4B depicts the DNA, RNA and protein cell ^{1} with time throughout the experimental runs. DNA levels were of the order of those found in batch reactors, although three different peaks for molecular variables were observed. The time spacing these peaks increased as cell density reached higher values, meaning that replication and cell division were slower during the early steady state. Furthermore, DNA values were in accordance with the presence of more than one copy of the chromosome, so that cells seemed to be retained in a state prior to cell division. RNA and protein levels were lower than maximum values seen in batch systems, showing quite similar behaviour to that of DNA. A maximum in the content of all these three macromolecular components was observed to coincide with the entrance into steady state, probably as a result of stress responses and the beginning of nutrient shortages.

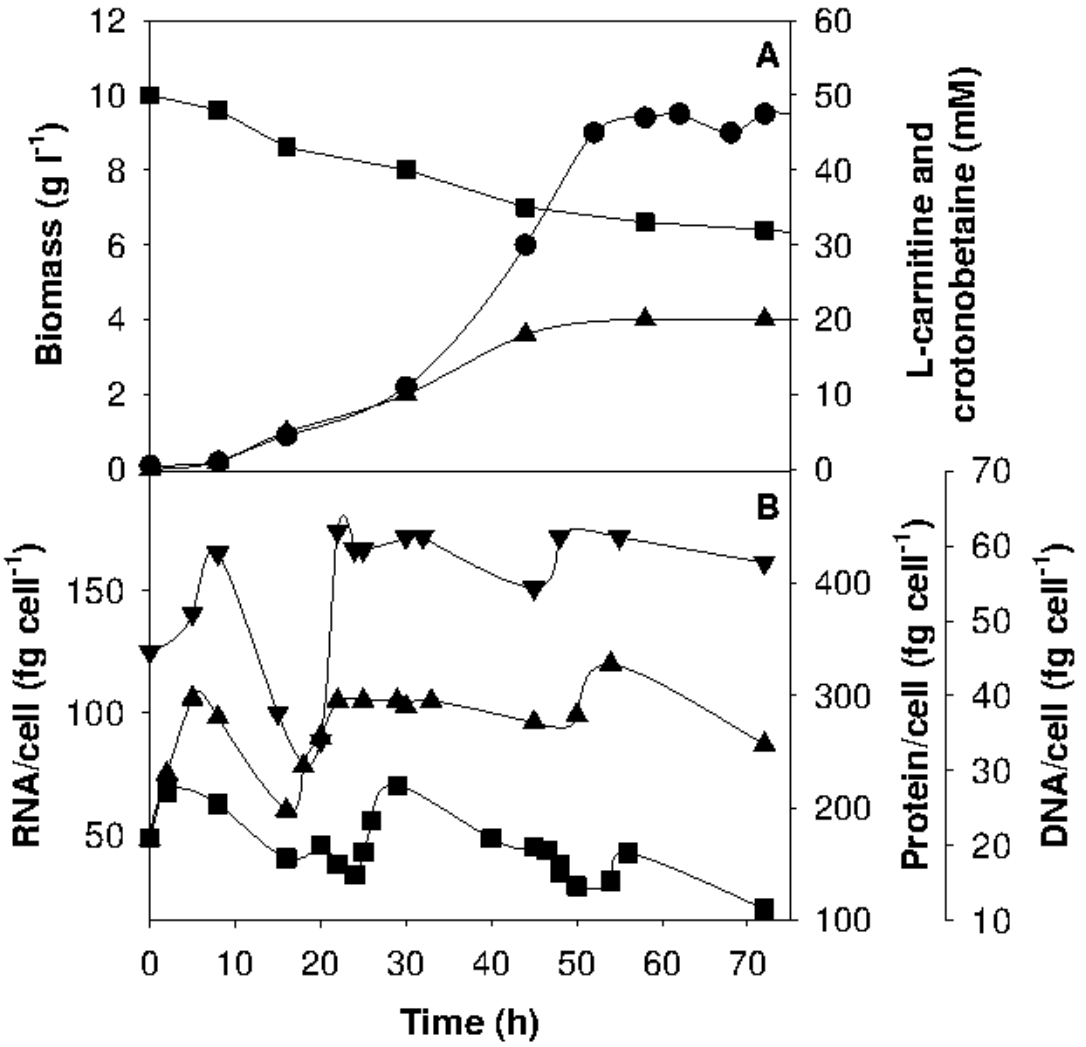


Figure 4. A) (▲) L(-)-carnitine, (■) crotonobetaine and (●) biomass and B) (■) Protein cell^{-1}, (▲) RNA cell^{-1} and (▼) DNA cell^{-1} evolution for growing *E. coli* O44K74 cells during the biotransformation of crotonobetaine into L(-)-carnitine in a high-density cell recycle continuous membrane reactor. Cells were cultured under anaerobic conditions with CM at 37ºC, pH 7.5 and 50 mM crotonobetaine.

Bacterial populations within the reactor

Changes in the different cell populations were followed during the biotransformation using the optimized multistaining technique to relate physiological cell state with reactor performance (Materials and Methods section) in different reactor scenarios.

Batch biotransformation: growing and resting cells

A progressive change in the physiological state of *E. coli* was observed within the three main sub-populations of cells described by Hewitt et al. (Hewitt et al., 1998, 1999a). In the dot plots presented, the lower left square represents non-stained cells, the lower right rectangle the BOX-stained cells and the upper right square the simultaneously PI/BOX-stained cells. Briefly, BOX-stained cells were depolarized cells, while PI/BOX-stained cells were permeabilized and dead cells; non-stained cells were healthy viable cells (Hewitt et al., 1998, 1999a). Cell debris was identified on the basis of the FALS and RALS values. After 15 h of batch growth only a small portion of cells was BOX-stained (1%), simultaneously PI/BOX-stained cells (dead cells) rose to 1.8%, the rest being non-stained fully viable reproductive cells (Hewitt et al., 1999a; Nebe von Caron et al., 2000). After 30 h, the percentage of depolarised cells increased to 2.5%, while the dead cells represented 4.2%. At 48 h, cells stained with BOX reached 5.0% and those stained with PI and BOX 2.1%. This coincided with the fall in A_{600} and DNA cell^{-1} detected by FCM, indicating that the diminution in biomass was a consequence of cells disappearing through degradation and death (Fig. 3A).

For the resting cells experiments, cells grown in CM under anaerobiosis and expressing the metabolism of trimethylammonium compounds were collected by centrifugation and placed in the biotransformation medium (see Materials and Methods). Cell viability was followed at different stages of the bioprocess (Fig. 5) and the cell populations were seen to decline faster in these conditions. At zero time, and during the first hours of operation viability was almost 100% (Fig. 5A). After 15 h and especially after 24 h (Fig. 5B), 20% of the cell population was BOX-stained, while the percentage of PI/BOX-stained cells (dead cells) was 5.9%. Moreover, after 48 h (Fig. 5C) 68% of the population was stained with BOX, while dead cells represented less than 1% of the total. This low percentage was clearly not only due to cell disappearance (as a result of cell lysis), but also to the decrease in the nucleic acid content (Fig. 3C), so that cells could not be stained by PI. At the end of the experiments (72 h), 68.4% of the population was BOX-stained while only

1.3% was PI-stained (Fig. 5D). Dead cells could be underdetermined, since at this point, hardly any DNA was detected and RNA traces were observed (Fig 3C), most of the population therefore consisting of phantom cells.

In this context, cell-reuse experiments were carried out based on two different schemes. Cells directly harvested from the biotransformation medium and resuspended in fresh resting biotransformation medium could be used three times, although there was a sharp decrease in the final L(-)-carnitine yield, thus their biocatalytic capability dramatically decreased. However, if following each 16 hours biotransformation cycle cells were reenergized by incubation in fresh culture medium for 8 hours, no decrease in final productivity was detected (Fig. 7).

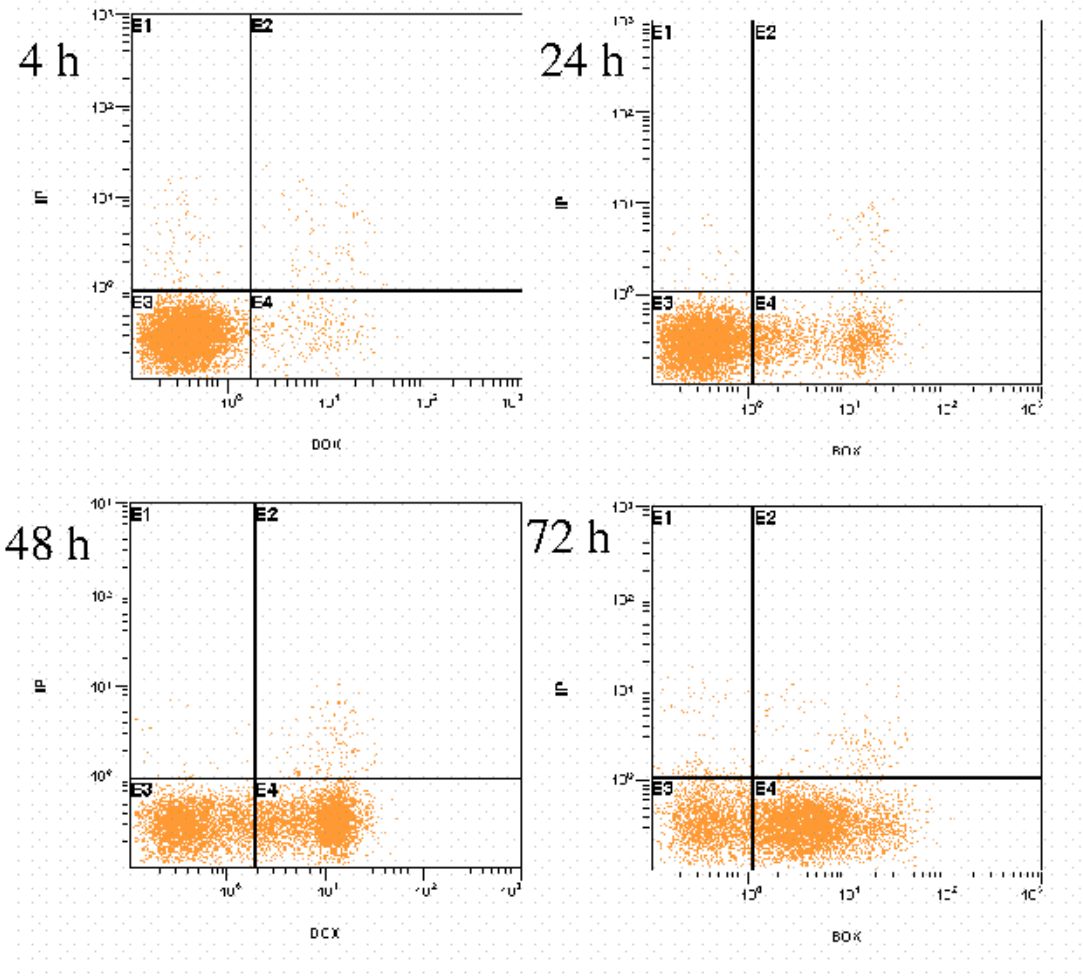


Figure 5. Viability of *E. coli* O44K74 **resting cells** performing the biotransformation of crotonobetaine into L(-)-carnitine. Green fluorescence of cells (FL1, axis X) due to BOX is plotted versus red fluorescence (FL3, axis Y) due to PI. Samples were taken at A) 4, B) 24, C) 48 and D) 72 h. Simultaneous PI/BOX staining was performed as explained in the corresponding Materials and Methods section.

Biotransformation in continuous high-cell density membrane reactors.

When studying the different cell populations of the reactor during the biotransformation process at high cell densities (10-40 g DCW l^{-1}), the cell population evolved parallel with the biomass profile. Furthermore, a steep decrease in viability was observed when steady state biomass values were reached at which time a 14% of the population showed depolarized cell membranes and 15% of the cells were dead (Fig. 6). Such a high accumulation of permeabilised and dead cells was not observed with any of the other systems, probably reflecting the fact that the death rate was much higher than that of disappearance in this system.

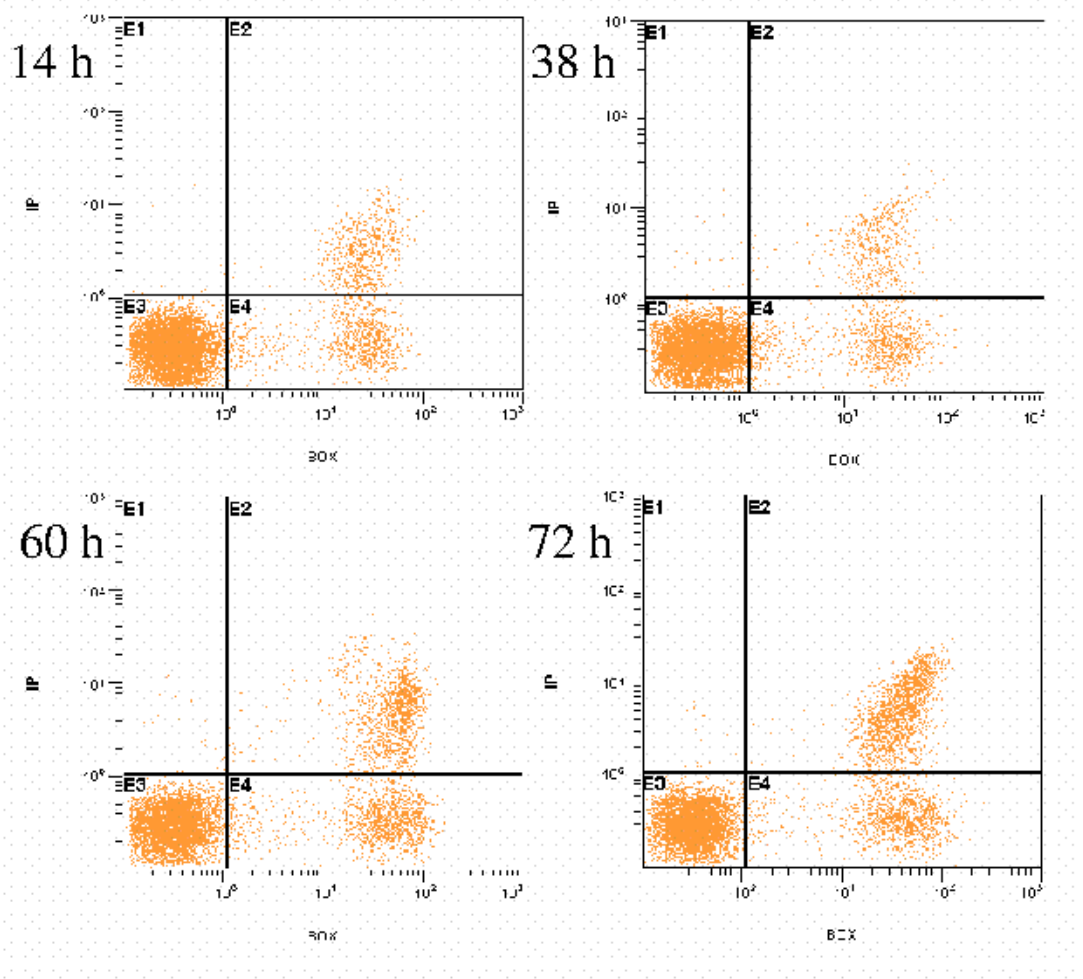


Figure 6. Viability of *E. coli* O44K74 **growing cells** performing the biotransformation of crotonobetaine into L(-)-carnitine in a high-density cell recycle continuous reactor, operated at a dilution rate of 0.15 h^{-1}. Green fluorescence of cells (FL1, axis X) due to BOX is plotted versus red fluorescence (FL3, axis Y) due to PI. Samples were taken from the reactor at A) 14, B) 38, C) 60, and D) 72 h. Simultaneous PI/BOX staining was performed as explained in the corresponding Materials and Methods section.

To determine whether changes in the dilution rate affected the composition of the cell population, multisteady-state reactor experiments were run. In Table 1 the retained biomass and the amount of depolarized and dead cells observed at each dilution rate are shown. There was no increase in depolarized cells which remained almost constant and even decreased from 15 to 8% of the total cell population. Dead cells became more apparent as the experiment progressed, increasing from initial values of 5% to final 16-18% as a result of accumulation.

Table 1. Viability of growing *E. coli* O44K74 cells performing the biotransformation of crotonobetaine into L(-)-carnitine within a high-density cell recycle reactor under anaerobic conditions. Samples were withdrawn throughout the experiment; the results shown correspond to steady values in the stationary phase. The study was performed using BOX and PI, as explained in the corresponding Materials and Methods section.

Dilution rate (h^{-1})	**0.15**	**0.3**	**0.6**
Sampling time (h)	80	140	220
Dry Weight (g l^{-1})	9.4 (0.7)	15.8(0.2)	34.6(3.3)
Non stained cells (%)	71.1(2.1)	69.0(0.6)	74.2(1.31)
BOX stained cells (%)	13.7 (1.5)	12.0(0.99)	8.1(1.2)
PI/BOX stained cells (%)	14.6(0.5)	18.4(1.8)	16.4(0.99)

Standard deviation is presented in parentheses.

DISCUSSION

An important finding was that at the end of the early stationary phase of batch cell growth biotransformation, the quantified DNA cell $^{-1}$ was very high due to the slow-down in the cell division rate caused by nutrient limitation (Fig. 3A). When studying *E. coli* growth using FCM, without DNA quantification Akerlund et al. (1995) observed that cells tended to increase their DNA content as a result of substrate limitation. Further, by running the cells to substrate limitation, it has been seen that cells complete the DNA replication cycle, resulting in distinct multiple genome copies (Akermann et al., 1995; Hewitt et al., 2000). Similarly, in this work a high DNA cell $^{-1}$ level was also found at the end of the start up of the high-cell density membrane reactor (early steady state, Fig. 4B), which could also be due to the slow down in the cell division rate, caused by nutrient limitation or the metabolically stressing environment imposed by high-cell density.

In addition, when using resting cells, the RNA and DNA contents per cell throughout the process pointed to a decreasing trend. Using starved *E. coli* cells, Poter et al. (1995) suggested that less than one entire chromosome was present per cell, indicating that the starvation stress would mediate changes in the DNA structure, such as the degree of supercoiling and more intimate contact with protecting proteins. This would indicate that in our work DNA would be less available for staining as time was progressing. Moreover, in the first 24 h of the bioprocess, the protein cell $^{-1}$ content in resting cells increased with respect to the basal content (value at zero time of the biotransformation), probably due to the synthesis of trimethylammonium compound metabolism enzymes which are involved in the biotransformation (Kleber, 1997; Cánovas et al., 2003), as well as to the response to the absence of nutrients. The subsequent decrease in protein level ran parallel to that of RNA (Fig. 3C). At this point, the registered fluorescence of EB-stained cells was below the technique's detection limit. Biomass decreased (Fig. 3B) as a result of cell decay, but at a much lower rate than intracellular nucleic acids. Therefore, the higher protein levels or even its nearly steady value compared with the initial state, and the decreasing trend shown by DNA and RNA cell $^{-1}$ may also have been due to the production of cell stress proteins and even to the triggering of death. These observations coincide with the findings obtained by other authors concerning the production of stress proteins in *E. coli* (Poter et al., 1995) and *S. aureus* (Diaper and Edwards, 1994) placed in sterile water and measured by biochemical methods.

During operation of the continuous cell-recycle membrane reactor, the variation in DNA and RNA cell $^{-1}$ reflected cell divisions within the reactor. The

protein cell^{-1} levels compared to those in batch biotransformation indicated that the membrane reactor contained a cell population with very high biochemical and metabolic activity throughout the reactor start up, while the decrease in the levels after the steady state had been reached implies a certain slow down as a result of nutrient limitation. Furthermore, the build up of dead or permeabilised cells would also explain the overall decrease in the "average value" of these variables. The evolution of RNA cell^{-1} and protein cell^{-1} followed similar trends, whereas the DNA cell^{-1} showed the different doublings of cell population within the system (Fig. 4B). The increase in the time spacing peaks pointed to the gradually slower replication rate and/or cell division rate in the early steady state, reflecting the cell behaviour also observed in batch systems when approaching the stationary phase.

Although quantification was the aim, the absolute values obtained for DNA and RNA must be carefully considered, since it has previously been stated that DNA and RNA staining can be affected not only by the interaction with proteins but also by their tridimensional folding (Poter et al., 1995) which could lead to an underestimation of the actual concentrations. However, the results here presented are an approach to cell behaviour under the tested conditions and mainly represent useful information for bioprocess control and optimization.

On the basis of cell viability, the cell populations within a growing cell process have been classified into viable, viable but non-culturable (those that do not form colonies in a plate) and dead cells (Poter et al., 1995; Davey and Kell, 1996; Nebe von Caron et al., 1998). Moreover, depending on the cellular metabolic activity and physiological state, cells can have a depolarized and/or permeabilized membrane and cell wall (Hewitt et al., 1998; Nebe von Caron et al., 2000). These different states can be distinguished on the basis of differential degrees of permeability to fluorescent molecules. Briefly, DNA-intercalating dyes, such as PI and EB bind nucleic acids, although the former can only cross the cell membrane if the cell is permeabilized (uncertain viability or dead cell), while the latter can cross the cell membrane although it is pumped out in reproductive viable cells (Hewitt et al., 1999b; Looser et al., 2005). In addition, bis-(1,3-dibutylbarbituric acid) trimethine oxonol (BOX or $DiBAC_4(3)$) is a lipophilic compound, insensitive to the existence of efflux pump systems (Davey and Kell, 1996) which acts as a slow-response potentiometric probe. Therefore, in this work the use of these probes demonstrated that the amount of damaged (mainly depolarized cells) or dead cells was higher with resting cell systems (Fig. 5 and 6), despite the fact that biotransformation levels were high in these conditions (Fig. 3B). As cells become stressed, the metabolic

membrane pumps are gradually inactivated as a result of energy depletion while transmembrane potential is eventually dissipated, finally resulting in cell permeabilisation and death. However, after 72 h, part of the cell population was still viable and reproductive (or vital) despite the substantial degree of stress resulting from the lack of nutrient and the accumulation of toxic by-products (Fig. 5D). At the end of the experiments with resting cells, very low levels of DNA were detected and only traces of RNA could be observed, most of the population consisting of phantom cells (Figs. 3C and 5D). There is no information on continuously operated reactors in the literature, since most research efforts have been devoted to batch and fed-batch systems. In these systems, the build up of over-flow metabolites, which might be toxic for the cells, has been proposed as the most likely reason for the loss in viability. The amount of damaged cells detected did not exceed 15% (Hewitt et al., 1999a) using *E. coli*, 10% (Lu Chau et al., 2001) with *S. cerevisiae* and 35 % with *P. pastoris* (Hohenblum et al., 2003). In our case, since the medium was continuously purged from the fermentation vessel, the accumulation of toxics was lower and the loss in viability was presumably mainly the result of nutrient limitation. In a cell-recycle membrane reactor also the build up of bacterial debris greatly increases viscosity which might affect cells. Further, the increase in dilution rates did not involve an increase in depolarized cells levels (Table 1), supporting that during operation cells were not exposed to further stress. The percentages of depolarised and dead cells indicated that cells were weakened and eventually died, while 65-75% remained healthy and viable. Other authors have previously shown that starvation does not lead to cell death (Looser et al., 2005). Further, in our experiments, low percentages of depolarized cells were detected. Moreover, preliminary experiments showed that when cells were kept in resting medium (phosphate buffer) in the absence of substrate, the cell decay rate was much higher and populations evolved much faster towards depolarization and death (data not shown).

Methods based on measuring biomass are intrinsically erroneous, since heterogeneity in the growth and evolution of bacterial populations is the norm (López-Amorós et al., 1995; Nebe von Caron et al., 1998; Hewitt et al., 2000; Looser et al., 2005). From the biotransformation point of view, the sequential steps from polarised to depolarised membrane and then to permeabilised cell have a detrimental effect on the bioprocess performance. The results presented further support the idea that cell depolarisation indicates a decline in cell functionality, because of energy depletion, but does not imply cell death. In our laboratories,

optimization studies with resting cells showed that cells could be fully reactivated for biotransformation if following a biotransformation cycle (16 h), they were reenergized in fresh culture medium for another 8 h (Fig. 7). The catalytic properties of reused cells were practically the same as those of fresh cells, suggesting that the loss in viability associated to this initial period of the experimentation can be readily recovered and no irreversible genetic damage occurs. These results indicate that the biotransformation process run time could be lengthened by controlling cell state and pulsing fresh medium when needed. This is why this technique gives useful information for process optimization. Further, in general, damaged cells catalytical capacity is lower than that of fully viable ones and FCM might also help in the screening of media and conditions for increasing cell stability, and in identifying agents to improve cell structural stability and viability and/or the internal biotransforming enzyme environment, such as polyethyleneglycol or the energetic substrates fumarate and citrate (Castellar et al., 1998). This is important since the state of each cell contributes to the overall rate and efficiency of the metabolic activity of the cell population as a whole, and the quantification of heterogeneity during biotransformation constitutes an important achievement for process control and optimization.

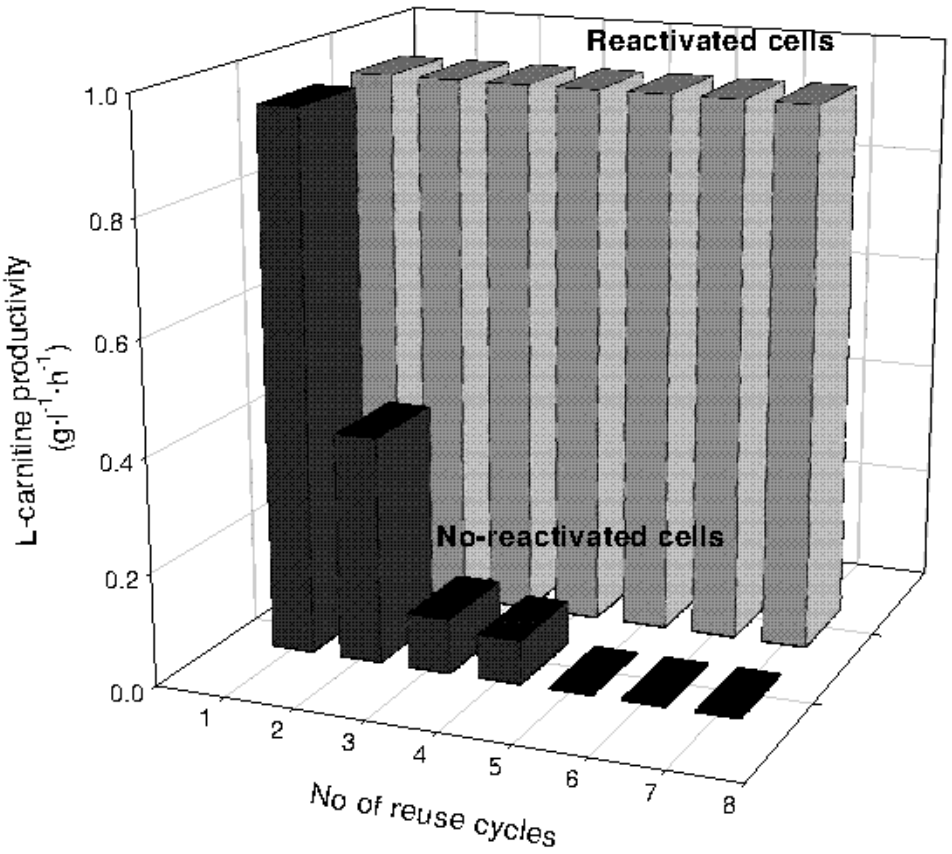


Figure 7. (*Pag. 87*) Reuse of resting cells in successive biotransformation cycles. L(-)-carnitine productivity versus the number of reuse cycles of no-reactivated cells (control) and re-energized cells. Re-energization consisted of 8 h incubation in fresh culture medium following the 16 h biotransformation cycle.

CONCLUSIONS

E. coli physiological stability was checked during the production of L(-)-carnitine within three different reactor systems. Taken together, the data indicate that the end of the biotransformation is linked to the build-up of damaged cells in the reactor. However, with resting cells, a considerable number of damaged cells appeared during the first 24 h, paralleling the biotransformation, and much higher yields in L(-)-carnitine than growing cell systems were assessed even though cell decline was faster and the occurrence of damaged cells higher, probably as a result of cell permeabilization. As regards the cell-reuse experiments, it can be concluded that the integrity of the cellular membrane potential must be maintained for a sufficient biocatalytic activity, together with a proper intracellular energy and coenzyme pools. Classical optical density and dry weight methods for the measurement of biomass content, or even cell counts, such as total or CFUs, are not accurate and do not provide *on site* information about the bioprocess or the biochemistry, molecular biology and physiology of the cells. Further stabilization studies should be also carried out to increase the stability of cells for process optimization. Finally, FCM is an important and valuable instrument in bioprocess control for the study of cell molecular processes (DNA, RNA and protein production), the cell proteomic machinery, the starting point of cell death, the real biotransforming state of a cell biocatalyst in bioproduction, the processes governing cell population evolution at the reaction site and the factors that must be controlled for process optimization.

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LIST OF ABBREVIATIONS

BOX: DiBAC4(3) or bis-(1,3-dibutylbarbituric acid) trimethine oxonol

DW: dry weight

EB: ethidium bromide

FALS: forward angle light scatter (also, FSC forward scatter)

FCM: flow cytometry

FITC: fluorescein isothyocyanate

MM: minimal medium

PI: propidium iodide

RALS: right angle light scatter (also, SSC side scatter)

Tris: tris(hydroxymethyl)-aminomethane

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Capítulo 3

Plasmid maintenance and physiological adaptations of a genetically engineered *Escherichia coli* strain during continuous L(-)-carnitine production.

Los contenidos de este capítulo han sido enviados para su publicación como:

Bernal V, González M, Cánovas M, Iborra JL. Plasmid maintenance and physiological adaptations of a genetically engineered *Escherichia coli* strain during continuous L(-)-carnitine production. Process Biochem. (submitted).

ABSTRACT

Cell immobilization is a general strategy to avoid plasmid loss, which is one of the major drawbacks in the application of genetically engineered cells in bioprocesses. Flow cytometry was applied for the first time to assess the physiological effects of plasmid maintenance in freely-suspended and immobilized genetically engineered cells. The genetic stability of the strain could be assessed, regardless of cell growth on selective media based methods, from the intracellular DNA levels measured. DNA, RNA and protein levels were analyzed in batch and continuous reactors and physiological adaptations resulting from immobilization were observed. Immobilized cells showed increased protein, whereas the RNA concentration was seen to be tightly controlled by the cellular machinery, remaining almost constant during continuous processes. Continuous operation was followed by a decrease in the DNA content, even in immobilized cells. The observed changes in macromolecule synthesis rates were related to genetic stabilization of the strain, since lessened metabolic burden.

INTRODUCTION

The genetic engineering of microorganisms has become a fundamental tool in biochemical engineering science. In applied biocatalysis genetic engineering has improved strain performance and permitted the development of new bioprocesses, greatly increasing yields and allowing the synthesis of new products not obtainable through chemical synthesis.

Though techniques for chromosomal gene insertion, deletion and recombination are available, plasmids are still the most frequently used vehicles for gene expression. However, the application of transformed cells in bioreactors has often been hampered by the loss of plasmid-coded genetic material, especially in continuous processes. Genetic instability of transformed strains arises from segregational effects (due to failure in plasmid distribution during successive cell divisions) or structural instability (implying sequence modification). Segregational instability has been related to multiple factors, such as plasmid size, plasmid copy number, plasmid-coded antibiotic resistance gene, gene-expression levels, cell genotype, cell growth rate in continuous processes and nutrient limitation (Walls and Gainer, 1988; González-Vara et al., 2003). Thus, environmental factors, such as temperature, pH and nutrient limitations, can enormously affect cell physiology and the cell response to segregational stability (Kumar et al., 1991; Chaves et al., 1999). Further, when the growth rate is lower, plasmid stability is higher (Caulcott et al., 1987; Jones and Keasling, 1998). Process performance is also affected by other factors, such as metabolic burden (effected by plasmid maintenance and protein overexpression), cell density and varying substrate availability, as well as variations in the micro-environment of non-ideal bioreactors (Looser et al., 2005). With the aim of genetically engineering bacteria in a stable way, transformation systems have been improved. However, cell immobilization is still the most widely used technique to overcome biocatalyst instability (Nasri et al., 1987).

Since the early 1980s, its multiple advantages have rapidly extended the use of cell immobilization. Among these advantages, the genetic stabilization of transformed cells, the lower risks of contamination, higher specificity of biotransformation environments and easier downstream processing may be mentioned. Improved resistance to environmental stresses is a characteristic of immobilized cells (Doleyres et al., 2004). In many cases, immobilization decreases division rates, minimizing the differences in the growth rate between transformed and non-transformed cells, allowing the occurrence of plasmid replication and lowering segregational effects.

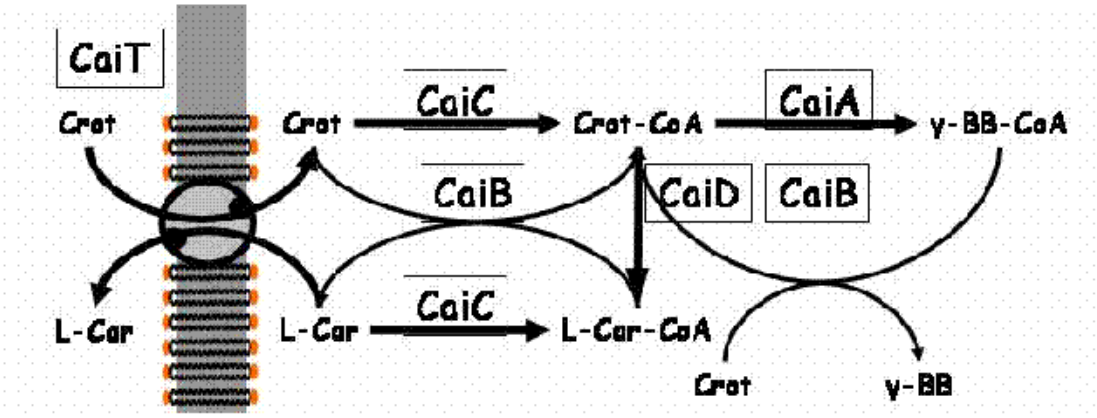

Figure 1. Anaerobic carnitine metabolism in *E. coli* (after Elssner et al., 2001). Crot, crotonobetaine; L-Car, L(-)-carnitine; γ-BB, γ-butyrobetaine; Crot-CoA, crotonobetainyl-CoA; L-Car-CoA, L(-)-carnitinyl-CoA; γ-BB-CoA, γ-butyrobetainyl-CoA; CaiT, carnitine:crotonobetaine:γ-butyrobetaine antiporter; CaiA, cronotonobetainyl-CoA reductase; CaiB, betainyl:CoA transferase; CaiC, betainyl-CoA ligase (putative); CaiD, enoyl-CoA hydratase.

However, the effect of immobilization on cell growth, biocatalytical efficiency and microbial physiology can still not be predicted and the physiological behaviour in the immobilized cell state remains paradoxically uncharacterized. Very little is known on the effects at the molecular level and even when considering macroscopic variables, contradictory responses have been observed. Physiological analysis of the effect of immobilization on cells is a current research topic which is showing that immobilized bacteria are physiologically different from the freely suspended organism (Junter and Jouenne, 2004).

To obtain a deeper knowledge of the physiological state of cells, flow cytometry is a technique capable of providing potentially unlimited information, independently of cell culturability (Davey and Kell, 1996; Hewitt et al., 1999; Nebe von Caron et al., 2000). By adequately selecting fluorophores, nearly *in vivo* intracellular conditions can be probed. Cell populations in the bioreactor have been shown to depend on the environment imposed by operational conditions (Cánovas et al., 2007). In addition, the study of intracellular compounds has shown their applicability to mathematical modelling of growth and biotransformation processes (García-Ochoa et al., 1998; Cánovas et al., 2007).

In this study, the production of the nutraceutical and pharmaceutical L(-)-carnitine by a recombinant *E. coli* strain was taken as model system. The divergent

cai operon is involved in L(-)-carnitine metabolism in ***Escherichia coli*** (Eichler et al., 1994). Functions for the proteins in ***cai*** operon have been described elsewhere (Elssner et al., 2001; Cánovas et al., 2003). L(-)-carnitine biotransformation occurs at the CoA level (Eichler et al., 1996) by the joint action of two proteins (Fig 1): CaiB (carnitine:crotonobetaine CoA-transferase) and CaiD (enoyl-CoA hydratase). The side reaction catalyzed by CaiA (crotonobetainyl-CoA:reductase) leads to the formation of γ-butyrobetaine, although the addition of fumarate to the growth medium can prevent this reaction (Cánovas et al., 2003). Further, ***caiF*** is a specific transcriptional activator for ***cai/fix*** operons and is expressed from its own promoter/operator region (Eichler et al., 1996). The ***E. coli*** K38 pGP1-2 pT7-5KE32 strain used in this work has previously been shown to be capable of producing high yields of L(-)-carnitine (Castellar et al., 2001; Cánovas et al., 2003 and 2005).

Our aim was to determine the physiological changes that take place in a transformed ***E. coli*** strain in the face of different cultivation strategies. Plasmid stability and L(-)-carnitine production were analyzed, while the effect of plasmid maintenance and gene expression on cell physiology was followed using flow cytometry. Therefore, the performance of batch and continuous systems with freely-suspended cells and that of continuous systems with immobilized cells were compared. A deeper insight into the effects of continuous feeding and immobilization on cell physiology for process monitoring and design was pursued.

MATERIALS AND METHODS

Strain, plasmids and media.

E. coli K38 pT7-5KE32 was used throughout this study. This strain contains the complete structural *cai* operon and two plasmids: pGP1-2 (Kan^R) and pT7-5KE32 (Amp^R) (Eichler et al., 1994), which carries *caiD*, *caiE* and *caiF* from *E. coli*. Therefore, this derivative overexpresses carnitine racemase and L(-)-carnitine dehydratase activities (CaiB:CaiD) and the transcriptional activator CaiF (Eichler et al., 1996; Elssner et al., 2001). The expression of *caiF* arises from its own promoter sequence. The rest of the genes were expressed from chromosomal *cai* operon. The strains were stored in glycerol (20%) at −20 ºC.

Biotransformation experiments

Bacterial Batch Cultivation.

Batch biotransformations with *E. coli* K38 pT7-5KE32 were performed under the different conditions stated in the text. The media employed have been previously described (Cánovas et al., 2003). Glycerol was used as the carbon source for sustaining growth. Both the complex (CM) and the minimal (MM) media were supplemented with 100 mM crotonobetaine as inducer and substrate for the biotransformation. The pH of the media was adjusted to 7.5 with 1 M KOH prior to autoclaving. With the transformed strain, ampicillin and kanamycin were employed at 100 and 50 µg/mL working concentrations, respectively. Cultures were inoculated with a 3% ($v \cdot v^{-1}$) of pre-cultures stored at -20ºC in 20% ($v \cdot v^{-1}$) glycerol and grown at 37ºC unless otherwise stated. A 0.5 L culture vessel with 0.2 to 0.3 L working volume was used. Reactors were equipped with temperature, pH, oxygen and pump controllers (Biostat B, Braun, Melsungen, Germany). Oxygen saturation was kept below 30%.

Chemostat reactor.

Continuous biotransformation experiments were carried out in the same reactor vessel used for batch cultures using Biostat B pumps for medium feeding and purge. No cell retention was performed and flow rates were fixed in order to keep the working volume constant. Continuous operation was started after inoculation. Experiments were run at a dilution rate of 0.15 h^{-1} to avoid cell wash.

Continuous reactor with immobilized cells.

Cell immobilization in κ-carrageenan gels was performed using a modification of the method described by Chibata et al. (1987). For this purpose, 6 mL of cells

from an over-night grown culture were mixed with 50 mL of a 2.1% (w/v) κ-carrageenan solution (κ-carrageenan C-1263, type III from Sigma, St. Louis, MO, USA) with continuous stirring and a constant temperature of 40ºC to avoid gelation. The carrageenan solution was previously sterilized by autoclaving. This mixture was then added drop-wise to the reactor using cultivation media supplemented with 0.3 M KCl in order to stabilize the gel beads.

Continuous reactors with immobilized cells were run at dilution rates (flow rate/reactor volume ratio) ranging from 0.15 to 1.0 h^{-1}. The culture medium was supplemented with 0.1 M KCl to avoid disruption of the polymer. A filter in the medium outlet prevented beads from leaving the reactor.

For cell de-immobilization, beads were collected from the reactor and gel-entrapped cells were released by washing three times with 0.9% NaCl followed by a 10 min incubation at 37ºC. Cells were immediately pelleted using a benchtop centrifuge and used for further analysis.

Analytical assays

Biomass and biotransformation variables.

The absorbance of the sample was followed at 600 nm with a spectrophotometer (Novaspec II, Pharmacia-LKB, Uppsala, Sweden) and correlated with dry cell weight. L(-)-carnitine and crotonobetaine concentrations were determined by an enzymatic test and HPLC respectively, as already described (Cánovas et al., 2003).

Flow Cytometry.

Reactor samples were run in a Becton Dickinson FASort model cytometer (San José, CA, USA), equipped with an argon laser with excitation at 488 nm at 15 mW and FL1 (530 nm), FL2 (585 nm) and FL3 (650 nm) filters. Samples were analysed with photomultiplier gains linear, a total of 10,000 cells being analysed in each sample at a rate of 800-2000 cell s^{-1}.

1. *Calibration for intracellular DNA, RNA and protein.* Ethidium bromide (EB) was used for DNA and RNA analyses after RNase and DNase cell treatment, while fluorescein isothiocyanate (FITC) was used in the case of protein. The staining and calibration procedures used have already been described (Cánovas et al., 2007).

2. Viability studies. Double fluorescent staining was performed, using 5 $\mu g \cdot ml^{-1}$ propidium iodide (PI, 1 $mg \cdot ml^{-1}$ in water) and 10 $\mu g \cdot ml^{-1}$ bis-(1,3-dibutylbarbituric acid) trimethine oxonol (BOX, 2 $mg \cdot ml^{-1}$ in dimethylsulfoxide) (Cánovas et al., 2007).

Staining was performed at room temperature for 10 min in the darkness and samples were run in an Epics XL analyser cytometer from Beckman Coulter (Fullerton, CA, USA), equipped with an argon laser of 15 mW for the excitation of the fluorophores at a wavelengh of 488 nm. Fluorescence detection was carried out using filters of 525 nm (for BOX) and 675 nm (for PI), and spectral overlap was corrected by using a numerical compensation. Positive (heat treated cells) and negative controls (either non-stained cells or cells stained with only one of the fluorophores and exponentially growing *E. coli* cells) were undertaken to avoid false positives. The BOX and PI fluorescence compensation was according to Hewitt et al. (1999, 2000) using heat treated cells (60^{o}C for 5 min).

Scanning electron microscopy.

Beads with immobilized bacteria were washed twice in 67 mM phosphate buffer (pH 7.4) and fixed for two hours in 2.5% glutaraldehyde. After washing, samples were continuously dehydrated in ethanol-water mixtures with increasing ethanol concentrations. Samples were dried by critical point after substitution of ethanol by liquid carbon dioxide. Samples were observed at 15 KV in a JEOL 6100 (Tokyo, Japan) scanning electron microscope.

RESULTS

Continuous L(-)-carnitine production with a genetically engineered strain plasmid stabilization

L(-)-Carnitine production with the transformed Escherichia coli K38 strain: aerobic expression of carnitine metabolism.

Preliminary batch experiments were performed in order to determine the optimum cultivation conditions for L(-)-carnitine production with *E. coli* pT7-5KE32. Crotonobetaine (dehydrated carnitine) was employed as substrate and growth media were supplemented with 2 g/L fumarate to be used as alternative electron acceptor and inhibitor of crotonobetaine reductase activity (Cánovas et al., 2003). Aeration was controlled to keep oxygen saturation below 30%, since higher productivity has been assessed (Cánovas et al., 2005). The effect of medium composition and cultivation temperature were also analyzed (results not shown). The optimal conditions selected included the use of MM and 37ºC (see Materials and Methods section). Batch L(-)-carnitine biotransformation assays were performed under the optimal conditions, using 100 mM crotonobetaine as the substrate. The transformed *E. coli* K38 was compared with the isogenic wild-type strain. Yield and specific productivities for both strains are outlined in Table 1. Under the tested conditions, L(-)-carnitine production was three-fold higher with the transformed strain. The combined effect of fumarate and oxygen allowed the inhibition of crotonobetaine reductase, thus improving the final yield of L(-)-carnitine. Note that the optimal expression of T7 RNA polymerase from the temperature-dependent phage λ promoter should occur at higher temperature. However, leaky expression of plasmid-coded *caiD* and *caiE* from the T7 promoter and, especially, expression of *caiF* from its own promoter region, led to the overexpression of carnitine metabolism at 37ºC under aerobic conditions.

Continuous L(-)-carnitine production with freely suspended cells: genetic stability of the strain.

Continuous processes are desirable in industrially-oriented projects, since the aim is the minimization of production costs and the maximization of process productivity. In our case, the *Escherichia coli* K38 pT7-5KE32 was cultured in chemostat reactors and the oxygen supply was controlled as stated (see Materials and Methods section). The L(-)-carnitine productivities obtained are summarized in Table 2. Good L(-)-carnitine productivities were observed under steady state

conditions, though maximal specific productivity was obtained during the earlier stages of the biotransformation, when cells were exponentially growing.

Table 1. L(-)-carnitine yield during biotransformation batch assays. The wild type and transformed with pT7-5KE32 *E. coli* K38 strains are compared. L(-)-carnitine productivities are related to intracellular DNA, RNA and protein concentrations as determined by FCM. Productivities measured after 24 h of culture.

Strain	Y ($g_{car} \cdot g_{biom}^{-1}$)	q_{LCar} ($g_{car} \cdot g_{biom}^{-1} \cdot h^{-1}$)	q_{DNA} ($g_{car} \cdot mg_{DNA}^{-1} \cdot h^{-1}$)	q_{RNA} ($g_{car} \cdot mg_{RNA}^{-1} \cdot h^{-1}$)	$q_{protein}$ ($g_{car} \cdot mg_{prot}^{-1} \cdot h^{-1}$)
E. coli K38	3.502	0.146	1.768	0.616	1.176
E. coli K38 pT7-5KE32	7.870	0.328	3.273	1.296	2.563

Table 2. Specific L(-)-carnitine productivity and macromolecules (DNA, RNA and protein) synthesis rates of *E. coli* K38 pGP1-2 pT7-5KE32 in continuous cultivation systems. Rates were calculated once the steady state had been reached for each dilution rate and before plasmid loss occurred. In the case of free cells, the results are presented for both the transformed strain (GM, *E. coli* K38 pGP1-2 pT7-5KE32) and the control strain (WT, *E. coli* K38)

Cells	Strain D (h^{-1})	q_{LCar} ($g_{car} \cdot g_{biom}^{-1} \cdot h^{-1}$)	q_{DNA} ($fg \cdot cell^{-1} \cdot h^{-1}$)	q_{RNA} ($fg \cdot cell^{-1} \cdot h^{-1}$)	q_{prot} ($fg \cdot cell^{-1} \cdot h^{-1}$)
Freely suspended	GM 0.15 h^{-1}	0.178	8.894	26.508	12.423
	WT 0.15 h^{-1}	0.113	9.085	25.859	10.679
Immobilized	GM 0.20 h^{-1}	0.430	0.404	1.338	0.831
	GM 0.50 h^{-1}	0.801	0.397	1.277	1.014
	GM 1.00 h^{-1}	1.020	0.362	1.224	1.015

The genetic stability of the transformed strain in batch and continuous fermentations was studied, since this is considered a limiting factor in the development of continuous bioprocesses with engineered cells. Samples were withdrawn from the fermentor and plated into selective and non-selective media as stated in Materials and Methods section. In batch cultures, the strains were shown to be genetically stable, and the number of colonies counted in LB and LBKA plates was the same (100%). Further, growth limitation as a result of nutrient shortages or substrate consumption occurred before the plasmids became unstable. However, when the strain was cultured in continuous, a decrease in the number of antibiotic-resistant colonies was observed. After 140 h of operation, a sharp decrease in plasmid bearing cells was observed, after which, only ten per cent of the cells were transformed. By culturing in LBA and LBK plates, it was shown that the first plasmid to be lost, and thus the most unstable, was the high copy number pT7-5KE32, which carries the ampicillin resistance gene. Increasing the antibiotic concentration was not a useful strategy, since plasmid loss was only delayed, but not prevented (results not shown).

Genetic stabilization of the strain upon cell immobilization in carrageenan gels.

Genetic stabilization was attempted by decreasing cell growth rate. An immobilization-based strategy was followed, also aiming at improving the performance of this strain in continuous cultures. Cells were entrapped in κ-carrageenan gel beads using the method of Chibata et al. (1987), as described in Materials and Methods section. Immobilized cells were cultured in the same conditions as in the chemostat, and L(-)-carnitine production and genetic stability were studied. Figure 2 shows *E. coli* K38 pT7-5KE32 cells immobilized in carrageenan beads. High cell densities were reached inside the beads. Plating onto antibiotic-containing media showed that plasmids were not lost during continuous operation for a period longer than 150 h. In order to determine if the strain was stable when operating at higher dilution rates, the medium addition rate was altered. No plasmid loss was assessed even though dilution rates three times higher than the maximum specific growth rate determined for free cells were reached. Thus, the pursued objective of genetic stabilization was considered to be reached.

Regarding L(-)-carnitine production (see Table 1 for batch studies), higher volumetric productivity was attained by increasing the dilution rate (Table 2). Specific productivity was also substantially increased upon immobilization, indicating the high catalytical capacity of the strain.

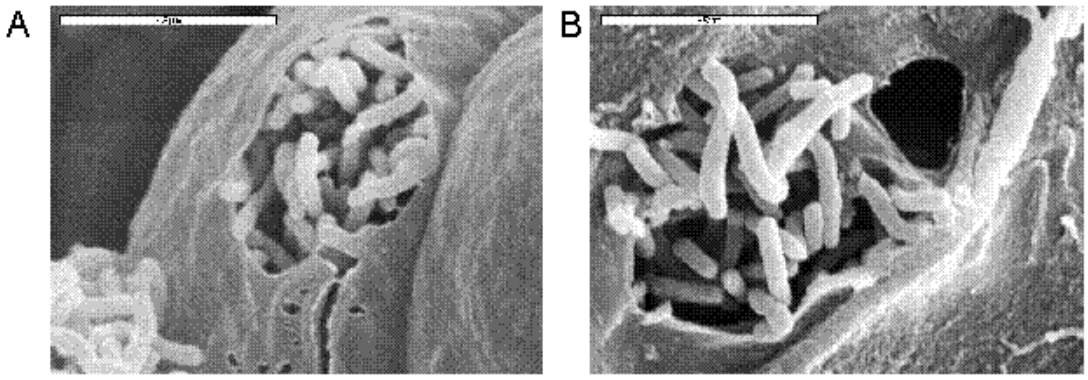

Figure 2. *E. coli* K38 pGP1-2 pT7-5KE32 cells entrapped in carrageenan gel beads. Photographs were taken after 65 (A) and 100 (B) hours of continuous cultivation. High cell densities were reached inside the beads. Size bars are shown in the pictures.

Flow cytometrical study of the impact of immobilization on the presence of plasmids and physiology of *Escherichia coli* cells.

In the rational design of biotechnological processes, the effect of reactor operation on microbial physiology has to be considered, and for this, new methodologies for assessing the cell state would help (Veal et al., 2000). The use of suitable fluorochromes allows us to probe nearly *in vivo* intracellular conditions with flow cytometry. Bearing in mind the objective of analysing the effect of cultivation conditions on the physiology and plasmid maintenance in *E. coli*, this technique was used.

Physiological impact of the presence of plasmids in E. coli cells: comparison of DNA, RNA and protein levels in transformed and non-transformed strains.

Preliminary experiments were performed to determine whether flow cytometry was a suitable technique for detecting differences between transformed and non-transformed *E. coli* strains. For this, wild-type and transformed (pGP1-2, pT7-5KE32) *E. coli* K38 cells were cultured in batch and intracellular levels of DNA, RNA and protein were analyzed following previously optimized protocols (Cánovas et al., 2007). As regards differences between strains, protein and DNA levels were higher for the transformed strain throughout the experimentation time (Fig. 3). The difference in DNA and protein intracellular concentrations between the two strains was around 10 and 26 $fg \cdot cell^{-1}$, respectively, depending on the cultivation time. The presence of the plasmid within the strain was readily detectable from the amount of

cellular DNA. In addition, protein levels were increased as a result of the expression of the cloned genes (around 20-30 fg·cell^{-1} higher in the transformed strain). Nevertheless, RNA levels were very similar for both strains during the exponential growth phase, only being slightly higher (<10 fg·cell^{-1}) for the transformed strain at 24 h, while once the stationary growth phase had begun, the RNA levels decreased more sharply in this strain. RNA levels were similar for the transformed and control strain during the exponential growth phase, when substrate limitation does not occur and cellular energy is still available for RNA and protein synthesis. This suggests strong cellular control over total RNA levels while the decreased level observed in the transformed strain after the medium had been consumed could have been due to higher energy depletion or lower energy storage. It is worthy noting at this point that turnover is much faster for RNA than for other cellular components due to the RNase activity.

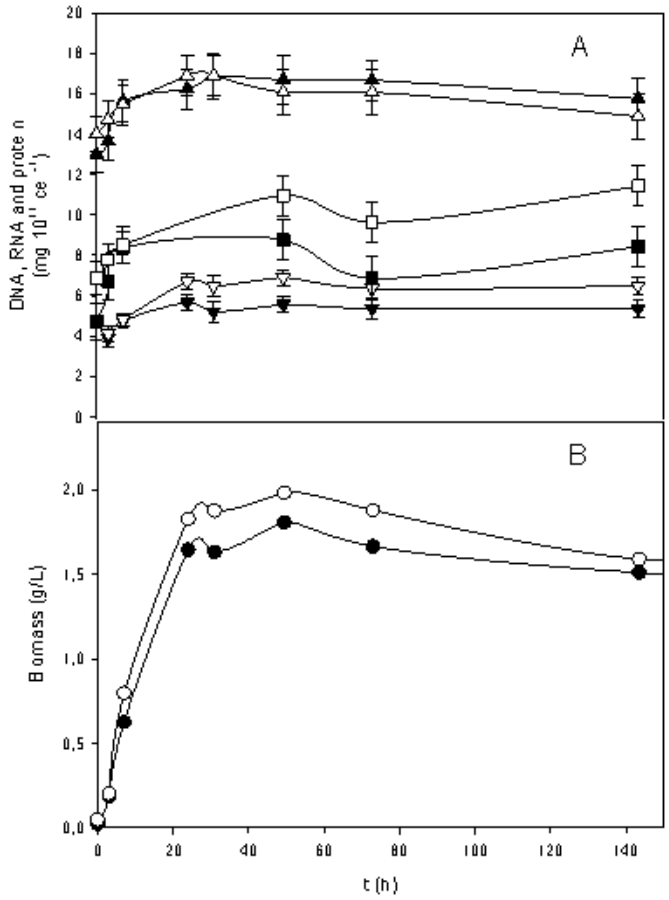

Figure 3. (A) Intracellular DNA (▼), RNA (▲) and protein (■) content and (B) biomass evolution (●) for wild type (solid symbols) and transformed (open symbols) ***E. coli*** K38 cells. Bacteria were grown in batch in MM at 37ºC. Oxygen saturation was kept below 30%.

Table 1 presents productivity values for transformed and non-transformed strains referring to biomass and also to the intracellular macromolecule content, as determined by flow cytometry. These data provide further information about the limiting factors of genetically engineered strains: gene copy number or transcription and translation rates.

DNA, RNA and protein levels during continuous L(-)-carnitine production.

A. Free cells

Transformed *E. coli* cells were cultured in a continuous reactor without cell retention and the intracellular macromolecule content was monitored throughout the experimentation time (Fig. 4). Coinciding with plasmid loss, a maximum of DNA·cell^{-1} was observed after 140 h of operation, meaning an increase in the duplication rate of cell genetic material. From that moment on, cell DNA decreased rapidly, possibly as a result of the increase in the cell division rate. RNA and protein profiles ran almost parallel, with their levels increasing during the pre-steady state and remaining constant after the reactor had reached the steady state. No change in these variables was correlated with plasmid loss. The maximum observed intracellular macromolecule concentration remained almost constant after reaching the steady state. The cellular macromolecule synthesis rates for freely suspended cells were calculated as stated in Stephanopoulos et al. (Stephanopoulos et al., 1998). Steady-state values are shown in Table 2. L(-)-carnitine productivities referring to the intracellular content of DNA, RNA and protein in the steady state are shown in Fig. 6.

B. Immobilization in carrageenan gel beads.

To assess the physiological effects of continuous operation and immobilization on *E. coli* cells, intracellular levels of DNA, RNA and protein were followed during the time course of the experiments (Fig. 5). The DNA and RNA profiles behaved in a similar way to that seen for the continuous reactor with freely suspended cells (Fig. 4). The RNA levels determined were seen to be almost constant independently of the working dilution rate. Surprisingly, the intracellular protein concentration increased steadily during the bioprocess until an apparent maximum at around 140 fg/cell was attained after 180 h of operation. Continuous operation, together with immobilization, seemed to favour protein production, which could be of interest in certain applications. L(-)-carnitine productivities referring to the intracellular content of DNA, RNA and protein for each of the three steady states are shown in Figure 6A. In addition, cellular macromolecule synthesis rates for immobilized cells were

also calculated the steady state values being shown in Table 2 (Stephanopoulos et al., 1998).

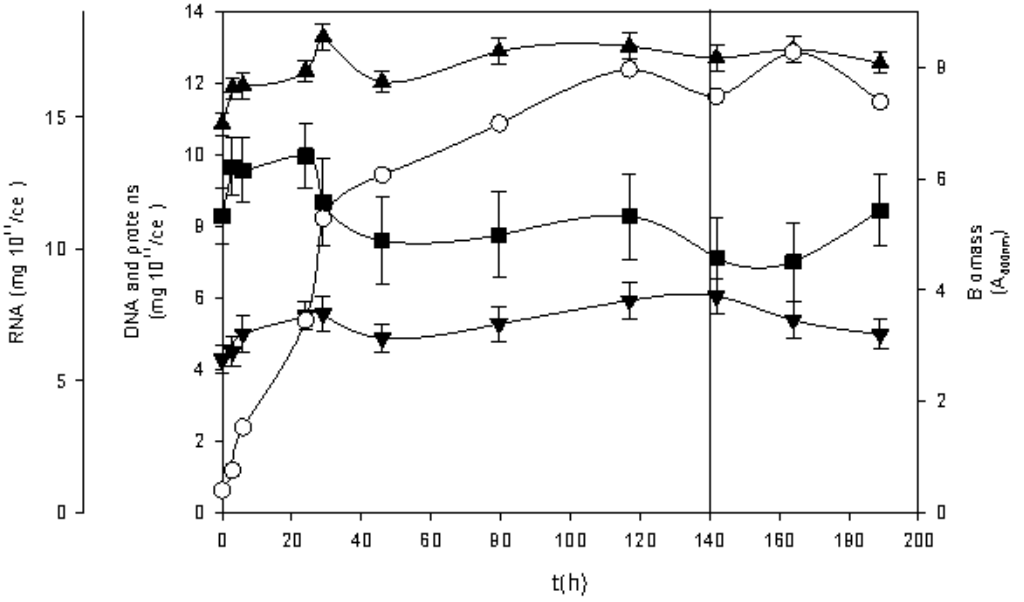

Figure 4. Evolution of biomass (○) and intracellular DNA (▼), RNA (▲) and protein (■) in ***E. coli*** K38 pT7-5KE32 freely suspended cells grown in continuous in MM at 37ºC. Oxygen saturation was kept below 30%. Vertical solid line indicates plasmid loss.

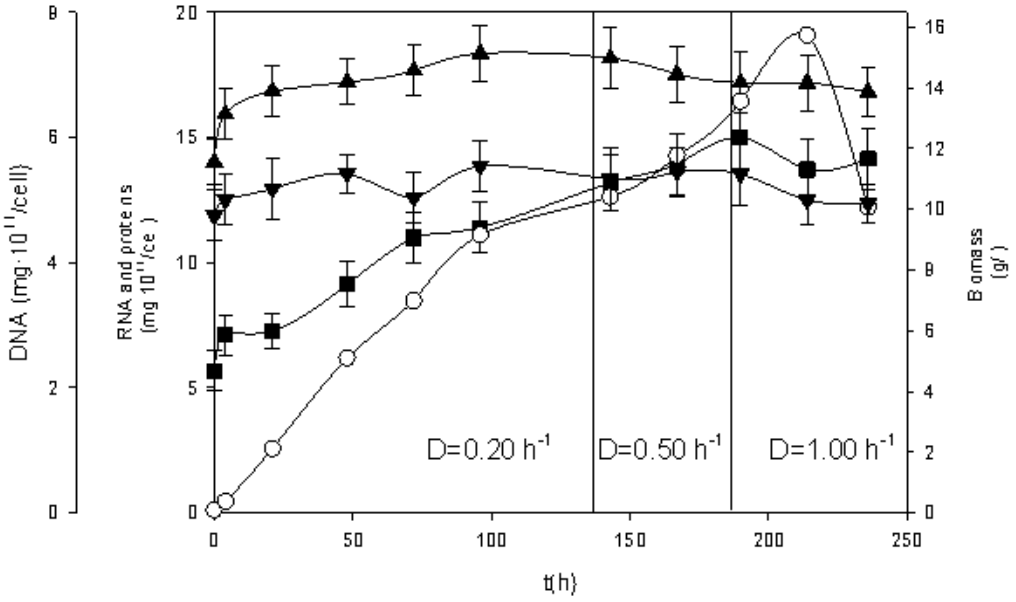

Figure 5. Evolution of biomass (○) and intracellular DNA (▼), RNA (▲) and protein (■) in ***E. coli*** K38 pT7-5KE32 immobilized cells grown in continuous in MM at 37ºC. Oxygen saturation was kept below 30%. The reactor was operated at dilution rates of 0.2, 0.5 and 1.0 h^{-1}. Vertical solid lines indicate switches in dilution rates.

Table 3. Viability of growing *E. coli* K38 pGP1-2 pT7-5KE32 cells immobilized in carrageenan cells. Samples were withdrawn throughout the experiment once that steady states had been reached for each dilution rate. The study was performed using BOX and PI, and cells were de-immobilized as explained in the corresponding Materials and Methods section.

D (h^{-1})	% Dead cells (PI stained)	% Depolarized Cells (BOX stained)	% Living Cells (non stained)
0.20	6.50	85.34	8.05
0.50	0.26	91.07	8.57
1.00	0.94	87.00	11.79

Cell state in carrageenan gel beads.

To further understand how immobilization affects the *E. coli* cell state, double-staining with propidium iodide and bis-oxonol was performed (Table 3). A decrease in the viability of carrageenan gel immobilized *E. coli* cells with experimentation time was observed (from the initial 95% to 8-12% at each of the steady states). Most of the cells (>80%) were BOX-stained and thus, depolarized. Nevertheless, the build up of a large amount of depolarized cells was not followed by a decrease in the biotransformation capacity. From the catalytic point of view, depolarized cells were still active and the biotransformation continued with no sign of decrease. However, the addition of fresh medium led to a recovery in the number of viable cells and, once more, an increase in biomass, meaning that these cells still conserved, at least partly, their reproductive ability (Fig. 5). A decrease in the amount of depolarized and dead cells was assessed upon change in dilution rate. BOX-stained (depolarized) cells decreased from 91% to 62% and PI-BOX stained (dead) cells from 15% to 1%. Nevertheless, the physiological state of the cells was correlated with the biotransforming capacity, and maximum values in L(-)-carnitine productivities were always obtained with exponentially growing cells, coinciding with the "healthier" cell state. This point was observed for both freely suspended and immobilized cells and is easily explainable from the point of view of the energy-dependence of the transport and activation of trimethylammonium compounds in CoA derivatives (CaiC) (Cánovas et al., 2003). This makes the biotransformation more likely to operate in exponentially growing cells.

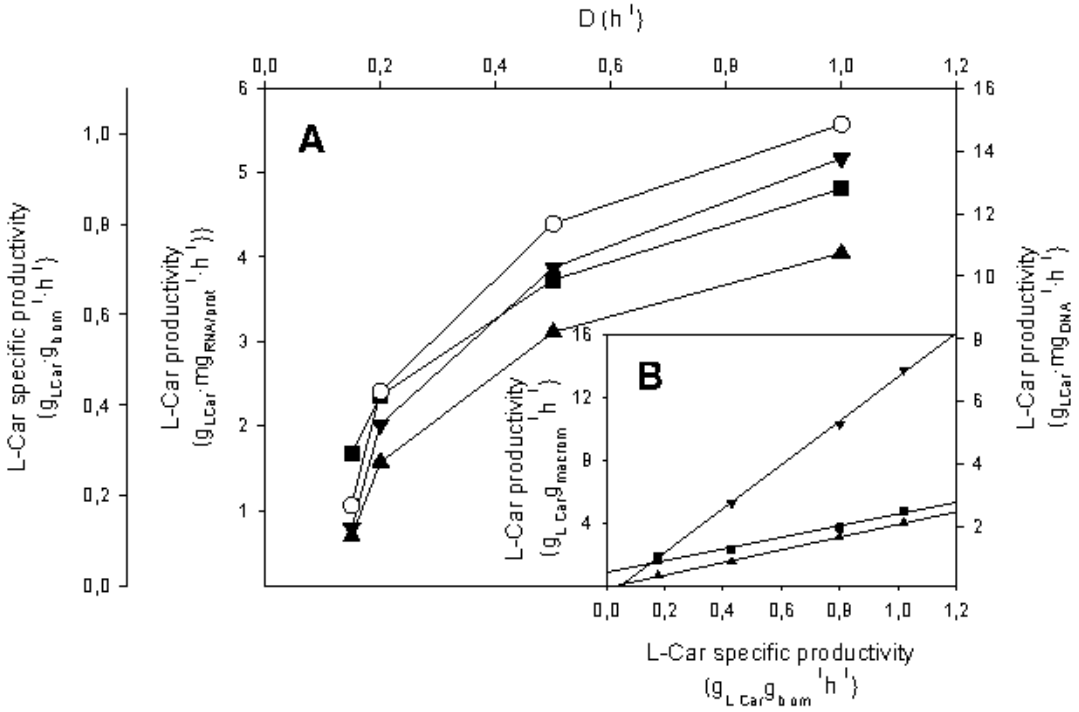

Figure 6. (A) Relationship between L-Car productivities referred to intracellular components DNA (▼), RNA (▲) and protein (■) and biomass (○) with dilution rate (D, h^{-1}). (B) Relationship between L-Car productivities referred to intracellular components DNA (▼), RNA (▲) and protein (■) with specific L-Car productivity. Results refer to freely suspended and immobilized ***E. coli*** K38 pT7-5KE32 cells in continuous cultures in MM at 37ºC.

DISCUSSION

Though in the wild-type *E. coli* O44K74 strain the metabolism of trimethylammonium compounds (Fig. 1) is principally related to anaerobic conditions (Eichler et al., 1994; Elssner et al., 2001), L(-)-carnitine can also be produced in aerobiosis (Cánovas et al., 2003). High productivities of L(-)-carnitine have been observed when using *E. coli* K38 pT7-5KE32 strain (Castellar et al., 2001).

When comparing the transformed and non-transformed (control) strains in terms of L(-)-carnitine production in aerobic conditions, a nearly three-fold increase in L(-)-carnitine production was ascertained (Table 1). The expression of carnitine metabolism enzymes, coded by the *cai* operon, is differently regulated by oxygen in Enterobacteriaceae (Cánovas et al., 2003). Further, leaky expression has been described for this expression system, even at 37ºC (Castellar et al., 2001) and the expression level achieved was sufficient to allow the overexpression of carnitine metabolism. In addition, the expression of the plasmid-coded *caiF* arose from its own promoter region (Eichler et al., 1996). As a result of the joint effect of oxygen and fumarate as electron acceptors (Cánovas et al., 2005), high productivities were attained, since the formation of γ-butyrobetaine was prevented (see Fig. 1).

High productivities at industrial scale can be obtained in *E. coli* cultures by minimizing the accumulation of metabolites and achieving high cell densities, however, genetic stability is a major drawback when employing recombinant microorganisms. It is important that the vector can be kept stable by the transformed cell, even in the absence of selective pressure. In our work, batch cultured cells did not lose plasmids since cell growth stopped as substrate depletion occurred. In the case of continuous cultures, cells grew at a constant rate once the steady state had been reached. In steadily-growing freely suspended cells, plasmid loss occurred; nevertheless, though immobilized cells were also growing steadily the plasmids were not lost. Some authors have stated that phosphate and/or carbon limitation provoke an increase in plasmid instability (Caulcott et al., 1987). To analyze operational effects on cell stability, dilution rates even three times higher than the maximum specific growth rate of free cells were checked and no plasmid loss was observed (Fig. 5). Thus, the pursued objective of genetic stabilization was considered to be reached by cell immobilization. In addition, not only volumetric but also specific productivity of the strain was increased (Table 2). Thus, the transformed strain showed itself to be suitable for continuous process operation and even capable of being subjected to dynamic stress, which may be useful for control and optimization strategies.

Little is known about the physiological changes effected by cell immobilization during bioprocesses (Junter and Jouenne, 2004). The lack of information is a major inconvenience for the correct development of bioprocesses. Application of flow cytometry in microbiology has shown itself to be capable of providing highly valuable information (Davey and Kell, 1996; Nebe von Caron et al., 2000) since nearly *in vivo* intracellular conditions can be studied, independently of cell culturability (Hewitt et al., 1999). Flow cytometry allowed the comparative analysis of macromolecule levels in the transformed and non-transformed strains. It must be noted that the technique gives a quantitative picture of the intracellular DNA, which allows the effect of experimental constraints on plasmid content to be investigated. Application of FCM greatly reduces analysis time compared with classical plating-based methods to assess plasmid loss. Preliminary experiments showed that the presence of the plasmid was readily detectable by flow cytometry in batch growing cells (Fig. 3). In addition, increased protein levels resulted from the expression of the cloned genes. Interestingly, RNA levels were similar for both strains during exponential growth, reflecting the tight control on cellular RNA levels (Fig 3). The slightly decreased RNA levels after consumption of the media in the case of the transformed strain could be explained by higher energy depletion or lower cell energy storage. Differences observed in the levels of DNA and proteins between the two strains were significant, while, RNA levels were almost the same for both strains and the slight differences assessed remained within the error interval of the technique (Fig. 3).

With the aim of analyzing cell physiology in a production-driven environment, the levels of macromolecules were also followed in continuous cultures. Continuous reactors with freely suspended and immobilized cells were studied and plasmid maintenance was followed in parallel. The genetically modified *E. coli* strain was far more stable when immobilized in κ-carrageenan gels than when used as freely suspended cells. Moreover, physiological responses to continuous operation and immobilization could be assessed. Thus, high RNA and protein levels were observed, pointing to the high activity of the transcriptional and translational machinery (Figs. 4 and 5). For immobilized cells particularly, protein levels showed a constant increase until an apparent maximum value was achieved (Fig. 5). In addition, RNA levels were slightly higher than those found in exponentially growing batch cultured cells. Further, steady state concentrations were mostly constant, independently of the medium addition rate. This, together with the observation that RNA levels were almost the same in batch cultures for the transformed and non-

transformed strains, lends weight to the idea that the RNA pool is subjected to tight control in the cellular metabolic bulk. However, it has been long known that RNA turnover is much higher than the turnover of proteins. Since most cellular RNA comprises transference and ribosomal RNA, the increased levels point to an increased translational activity during continuous cultivation, reflecting a more active cell metabolic state.

An increase in DNA levels has previously been assessed for batch stationary phase cells (Akerlund et al., 1995; Akermann et al., 1995; Hewitt et al., 2000). However, medium addition during continuous cultivation keeps cells in exponential growth and the steady-state DNA levels of freely suspended and, particularly, immobilized cells (Fig. 4 and Fig. 5) were much lower than those observed for batch-grown stationary phase *E. coli* cells (Fig. 3). This would indicate that batch stationary phase cells were not actively dividing and that multiple copies of the cellular genome were present, while continuously-cultured cells were dividing and the DNA content per unit of cell was lower. Moreover, no correlation of DNA levels with growth rate was observed, since the specific growth rate for freely suspended cells was 20 to 25-fold higher than that of immobilized cells (0.150 vs. 0.007 h^{-1}). Thus, not only a decrease in the genome copy-number but also in plasmid copy-number seems a feasible fact, especially considering the assessed instability of the strain.

Metabolic burden imposed by plasmid maintenance and the expression of plasmid-coded proteins has been considered as an important factor favouring plasmid segregational loss (Jones and Keasling, 1998; Flores et al., 2004). In fact, flux limitation through the oxidative branch of the pentose phosphate pathway (PPP) is related to the observed changes in the growth rate of cells harbouring plasmids, because of the limiting availability of building blocks for the biosynthesis of nucleotides, recombinant proteins or aromatic metabolites. When flux through this pathway was increased, at least partial recovery of the cell growth rate occurred (Flores et al., 2004). This indicates that the availability of building blocks synthesized through the PPP has a positive effect on the biosynthesis of multicopy plasmid DNA, mRNA and encoded proteins and helps in cell stability. Moreover, metabolic burden is greatly increased in induction conditions, especially when cells are steadily growing. Further, when the overexpressed proteins are enzymes or regulatory proteins, the cell physiology may also have to cope with a higher metabolic demand due to the alteration of metabolic fluxes. Though this is not usually considered when defining metabolic burden, it should be taken into account, especially when the final

aim is the production of metabolites in bioreactors. In this work, the quantification of the metabolic burden during a bioprocess is attempted for the first time. Flow cytometry allowed us to quantify the alteration in the synthesis rate of macromolecules. An estimation of cellular DNA, RNA and protein synthesis rates can be worked out, since, in continuous cultures, the macromolecule synthesis rate is directly proportional to the specific growth rate (Stephanopoulos et al., 1998). Consequently, low specific growth rates in immobilized cells correlated with low specific DNA, RNA and protein synthesis rates (Table 2), which throw further light on the stabilization of the strain. As a consequence of continuous operation, the DNA cellular content decreased in both freely-suspended and immobilized cells. This fact, together with the higher growth rate of the freely suspended cells, increased the segregational instability of the strain, while the lowered macromolecules synthesis rate of the immobilized cells meant a much lower metabolic burden.

Modifications to the overall DNA and RNA contents in immobilized cells have previously been reported (Doran and Bailey, 1986; Kiy and Tiedtke, 1993; Lyngberg et al., 1999), though almost no data exist for *E. coli*. In the case of *T. thermophila*, the macronucleus doubled its size and DNA content upon immobilization in Ca-alginate, an effect probably related with cell immobilization (Kiy and Tiedtke, 1993). The stabilization of RNA levels in latex-immobilized *E. coli* cells has also been reported, even when kept in phosphate buffered media (Lyngberg et al., 1999). Thus immobilization may stabilize the protein synthesis capacity of bacteria, as reflected by the total RNA content, but also by the increased protein content. The high intracellular protein concentration might also be a result of the low cell division rate (Fig. 5).

Although the L(-)-carnitine yield during the biotransformation was higher in the case of batch operation, higher volumetric productivities were obtained with continuous systems (Table 2). Furthermore, both volumetric and specific productivities were increased with the feed rate for immobilized cells (Fig. 6A). Data obtained through FCM could also provide deeper knowledge of the link between cell physiology and process performance. L(-)-carnitine productivity was calculated by reference to the intracellular macromolecules content (DNA, RNA and protein) for each of the reactor configurations studied (Fig. 6A). The productivity values referred to the macromolecule content did not allow us to further clarify the limitations involved, although RNA and protein levels are the most feasible limiting factors. In addition, although specific productivity increased with the dilution rate, no linear

relationship could be established (Fig 6A). This meant that with increasing dilution rates, the production machinery kinetics became saturated. In addition, when these productivities were plotted against specific productivity referring to biomass, a linear relationship was shown. This demonstrated two things: first, the goodness of the data obtained and, second, that an equivalent cellular physiological state was attained at the different dilution rates.

Double staining studies with PI and BOX pointed to a decrease in cell viability in immobilized cells (Table 3). Though heterogeneity in the growth and evolution of bacterial populations is the norm (Hewitt et al., 2000; Nebe von Caron et al., 2000; Looser et al., 2005), the performance of bioprocesses is largely determined by the cell state. The amount of damaged or dead cells within carrageenan gel beads was high, despite the fact that biotransformation levels remained high (Table 2). As cells become stressed as a consequence of energy depletion the cytoplasmic membrane loses its transmembrane potential, finally resulting in cell permeabilisation and death. From the biotransformation point of view, the sequential steps from polarised to depolarised membrane and then to permeabilised cell have a detrimental effect on reactor performance. In addition, as has previously been suggested, a cell with no membrane potential is difficult to understand (Hewitt et al., 1999), and it is thought that this intermediate state is quite ephemeral with cells progressing towards membrane permeabilisation and death or even re-polarization in the presence of a fresh energy source. In our case, as a result of substrate limitations, cells lost their membrane potential. Membrane depolarization was a reversible phenomenon, since the numbers of non-stained cells recovered upon the addition of fresh medium. The results here presented further support the idea that cell depolarisation indicates a decline in cell functionality, because of energy depletion, but does not imply cell death. Using multicolour FCM and cell sorting, Nebe von Caron et al. (2000) also demonstrated the ability of BOX-stained sorted cells to grow when transferred to fresh media. The potential of *E. coli* cells to recover their biotransformation capacity upon re-energization has been demonstrated (Cánovas et al., 2007).

Summarizing, many factors affect plasmid maintenance and the stability of genetically engineered immobilized cells. The importance of lower growth rate, the limitation in maximum retained biomass, and lower metabolic burden in immobilized cells have been underlined by this work. When the continuous production of metabolites with growing cells is the goal, a compromise solution between adequate gene expression, induction and cell physiology must be achieved. This means that

the choice of an adequate expression system is of crucial importance for the design and application of living, genetically engineered cells as catalysts in bioprocesses. Thus, stable expression systems, together with the optimization of media for the growth and induction, allowing lower metabolic burden and a suitable environment for cells, would allow an improvement and optimization of bioprocesses employing recombinant microorganisms.

CONCLUSIONS

Flow cytometry was used to study a bioprocess using genetically-engineered cells, extending the technique to the analysis of immobilized cells for the first time. This work provides a quantitative picture of the metabolic burden caused by plasmid maintenance and protein overexpression in *Escherichia coli*, which should be considered for the optimization of bioprocesses. Further work should be carried out to obtain a comprehensive understanding of the adaptations undertaken by the immobilized cell populations. State-of-the-art techniques for "omics" analysis, in relationship with the information provided by FCM analysis, should provide highly valuable information as regards the physiological adaptations suffered by microbial cells. Thus, a multidisciplinary approach is required to further our knowledge of the suitability of cell biocatalysts for any given bioprocess.

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Capítulo 4

Link between primary and secondary metabolism in the biotransformation of trimethylammonium compounds by *Escherichia coli*.

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ABSTRACT

The aim of this work was to understand the steps controlling the process of the biotransformation of trimethylammonium compounds into L(-)-carnitine by *Escherichia coli* and the link between the central carbon or primary and the secondary metabolism expressed. Thus, the enzyme activities involved in the biotransformation process of crotonobetaine into L(-)-carnitine (crotonobetaine hydration reaction and crotonobetaine reduction reaction), in the synthesis of acetyl-CoA (pyruvate dehydrogenase, acetyl-CoA synthetase and ATP:acetate phosphotransferase) and in the distribution of metabolites for the tricarboxylic acid (isocitrate dehydrogenase) and glyoxylate (isocitrate lyase) cycles were followed in batch with both growing and resting cells and during continuous cell growth in stirred tank and high-cell density membrane reactors. Further, the levels of carnitine, crotonobetaine, γ-butyrobetaine, ATP, NADH/NAD^+ and acetyl-CoA/CoA ratios were measured in order to know how metabolic fluxes were distributed in the catabolic system. The results provided the first experimental evidence to demonstrate the important role of the glyoxylate shunt during resting cells biotransformation and the need for high levels of ATP to maintain metabolite transport and biotransformation (2.1-16.0 $mmol \cdot L_{cell}/mmol_{ATP} \cdot L_{reactor} \cdot h$). Moreover, the results obtained for the pool of acetyl-CoA/CoA indicated that it is correlated with the biotransformation process too. The main metabolic pathway operating during cell growth in the high-cell density membrane reactor was that related to isocitrate dehydrogenase (during start up) and isocitrate lyase (during steady operation), together with phosphotransacetylase and acetyl-CoA synthetase. More important, the link between the central carbon and L(-)-carnitine metabolism at the level of the ATP pool was also confirmed.

INTRODUCTION

In order to optimize the bioprocesses performed by whole cells, it is necessary to decipher and quantify the kinetic and regulatory structure of the metabolic pathways involved. In the past, this type of experimental data was seldom available and stoichiometric analysis of the bioprocess was considered a valuable substitute (Varma et al., 1993). Attempts have been made to optimize metabolic dynamics but progress appears to have been hampered by the lack of kinetic and regulatory information regarding the functioning of all the enzymes in a particular cell. However, it is now possible to identify the optimal redirection of metabolic fluxes by means of mathematical tools (See et al., 1996; Torres et al., 1997; Stephanopoulos 1998; Stephanopoulos et al., 1998), while analytical tools have allowed the development of methodologies to reveal active metabolic pathways (Sauer et al., 1999; Chassagnole et al., 2002; Yoon et al., 2003). Nowadays, modern experimental methods of genomics, proteomics and metabolic profiling are rapidly changing the "playing field" (Voit 2002), and comprehensive quality data are being made available for integrative analysis and optimization. Although genes and proteins in simple organisms such as *Escherichia coli* have been identified, it is still impossible to predict how this microorganism will respond in untested environments and how its metabolism related to energy production, growth and maintenance is linked to its secondary metabolism in certain biotechnological applications. Therefore, if whole cell bioprocess optimization is one of the aims, biocatalyst metabolism in different scenarios (reactor configurations, media and physical conditions) must be understood before undertaking optimization strategies. Moreover, if increasing productivity in continuous processes by retaining cell catalysts is another aim, a reactor outlet membrane barrier or any other cell immobilization method should be used (Drioli and Giorno, 2000; Cánovas et al., 2002). However, this latter method will strongly affect cell biochemistry and physiology not only in the steady-state but also under perturbations (Cánovas et al., 2003a), which complicates the matter.

L(-)-carnitine (R(-)-3-hydroxy-4-trimethylaminobutyrate) transports long-chain fatty acids through the inner mitochondrial membrane, which is why several clinical applications for L(-)-carnitine have been identified. Consequently, the demand for L(-)-carnitine has increased worldwide (Seim et al., 2001) and chemical and biological processes have been developed for its production (Cavazza, 1981; Kulla, 1991; Hoeks et al., 1996; Kleber, 1997). Strains belonging to the genera *Escherichia*, *Proteus* and *Salmonella* racemice D(+)-carnitine, a waste product and

an environmental problem resulting from the L(-)-carnitine chemical synthesis, and/or biotransform crotonobetaine (dehydrated D(+)-carnitine) to produce L(-)-carnitine (Kleber, 1997; Castellar et al., 1998; Obón et al., 1999; Cánovas et al., 2002). However, there is a need for the bioprocesses involved to be further studied and optimised. In *E. coli,* the protective carnitine or trimethylammonium compounds metabolism has been studied, since this allows cells to withstand osmotic up-shocks, although it is still not totally understood (Eichler et al., 1994a, 1994b; Kleber, 1997; Elssner et al., 2001). This secondary metabolism has been used for L(-)-carnitine production with *E. coli* cells in growing and resting state (Cánovas et al., 2002). In brief, crotonobetaine is transformed into L(-)-carnitine by the involvement of CoA esters and two new enzymes, an enoyl-CoA hydratase and a CoA-transferase, while it is reduced to γ-butyrobetaine by a crotonobetaine reductase and a CoA-transferase (Preusser et al., 1999; Elssner et al., 2001) which are induced anaerobically in the presence of D,L-carnitine mixture and/or crotonobetaine (see Central and Carnitine Metabolisms in *E. coli* section). Genetic studies have been conducted to elucidate carnitine metabolism in *E. coli* O44K74 (Kleber, 1997). Two divergent structural operons, ***caiTABCDE*** and ***fix***ABCX, are coexpressed, the former being responsible for the enzymes of carnitine metabolism and the latter being required to provide electrons for carnitine reduction (Eichler et al., 1996; Walt and Kahn, 2002). Although the pathway/genome data base of *E. coli* is available (Karp et al., 2002), if the cell biochemistry and physiology to optimize this bioprocess is to be understood, it will be necessary to decipher the links, quantify the kinetics and determine the regulatory structure of the metabolic pathways involved in the different scenarios, such as reactor type used and the cell either in growing or resting state. Resting cell processes are also important since biotransformation occurs in a simple medium containing only the substrate dissolved in buffer, thus lowering production costs and simplifying product purification compared with growing cell processes.

The present work represents a first approach to understanding the link between the central carbon or primary metabolism and the metabolism of the secondary trimethylammonium compounds involved in the production of L(-)-carnitine by *E. coli* in growing and resting states. The choice of appropriate biocatalyst state and reactor configuration is of prime importance for L(-)-carnitine formation and process optimization. Therefore, the bioprocess was carried out in batch and continuous stirred tank and high-cell density membrane reactors. To gain insight into the kinetics of the whole bioprocess and to ascertain the connection

between both metabolisms, the activity of certain enzymes involved in the central metabolism, such as isocitrate dehydrogenase (Krebs cycle), isocitrate lyase (glyoxylate shunt) and pyruvate dehydrogenase, acetyl-CoA synthetase, ATP: acetate phosphotransferase and the levels of acetyl-CoA and CoA (acetyl-CoA metabolism), were followed and the cellular energy was determined as cell ATP and reducing power level ($NADH/NAD^+$ ratio), throughout the bioreactor runs. In addition, since the L(-)-carnitine metabolism activity to biotransform the exogenously added substrate crotonobetaine is mainly induced under anaerobic conditions, the metabolism of *E. coli* was followed by determining the levels of acetate, formate, lactate, pyruvate and fumarate. Furthermore, we also characterize the metabolism of the trimethylammonium compounds in *E. coli* by following the enzyme activities involved in the production of L(-)-carnitine in different growing and resting conditions.

MATERIALS AND METHODS

Bacterial strain and culture media

The bacterial strain used, *E. coli* O44K74 (DSM 8828), contained the complete *cai* and *fix* operons and was stored in a minimal medium containing glycerol (20%) at –20ºC. The minimal medium (MM) was that described by Obón et al. (1999). Complex medium (CM) contained (g/L): bacteriological peptone, 20; NaCl, 5; glycerol (carbon source), 12.6; and crotonobetaine, 4. The pH of both media was adjusted to 7.5 with 1 *M* KOH prior to autoclaving.

Reactor operation

Growth of the bacteria

Batch and continuous experiments were performed in reactors equipped with temperature, pH, oxygen and pump controllers (Biostat B, Braun Biotech International GMBH, Melsungen, Germany). A 1 L culture vessel with 0.5-0.8 L working volume was used. *Escherichia coli* O44K74 was grown under different conditions, in order to optimise the induction of the carnitine metabolism enzymes. The culture was inoculated with a 3% (v/v) of the liquid culture stored at -20ºC in 20% (v/v) glycerol, while the medium employed was the CM mentioned above. The cells were grown in batch or continuous feeding under both aerobic and anaerobic conditions at 37ºC. Anaerobic conditions were maintained to induce the enzymes involved in the carnitine metabolism, while D,L-carnitine mixture, D(+)-carnitine or crotonobetaine were supplied as inducers. Air and nitrogen was used to maintain a non-limiting oxygen concentration or to ensure anaerobiosis during the experiments.

Resting cells experiments

For the resting cell experiments, aerobic or anaerobic cultures were harvested at the end of the exponential growth phase, centrifuged at 16,000 x g for 10 min and washed twice with 75 m*M* phosphate buffer, pH 7.5. The final pellet was re-suspended in the same initial volume but containing 50-500 m*M* crotonobetaine in 50 m*M* phosphate buffer, pH 7.5 at 37ºC and left in the reactor system for 15-24 hours. All experiments were performed at least in triplicate and under sterile conditions. The values reported are the means of the assays performed.

Membrane reactor operation

The reactor vessel was also coupled to a cross-flow filtration module (Minitan, Millipore, USA) equipped with four 0.1 μm hydrophilic polyvinylidene difluoride Durapore plates of 60 cm^2 area (Millipore, USA) (Cánovas et al., 2002). The cell

broth was recycled into the reactor with a peristaltic pump adjusted to a high flow rate (70 mL/min) to minimise membrane fouling. *E. coli* cells for the inoculum were grown as explained previously and transferred to the fermenter. Continuous operation was set at 37 ºC and started by feeding with the CM medium (anaerobically by bubbling nitrogen or aerobically by feeding air previously passed through a water trap).

Enzyme assays

The crotonobetaine hydration reaction (CHR) assay was carried out according to Jung et al., (1989), and the crotonobetaine reduction reaction (CRR) assay according to Preusser et al., (1999), both started by using crotonobetaine as substrate. Enzyme activity was defined either as the total μmols of substrate consumed per minute (U) or specific activity, μmol of substrate consumed per minute and mg of protein (U/mg). In certain experiments fumarate was used to inhibit the CRR activity and then increase the L(-)-carnitine production.

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

Isocitrate dehydrogenase (ICDH)

The method was that of Bennet and Holms (1975). The extraction buffer was 64.5 m*M* potassium phosphate, pH 7.5, and 6.45 m*M* Mg_2Cl. The increase in NADPH absorbance at 340 nm (ε_{NADPH}=6.220 $M^{-1}cm^{-1}$) was followed in a GBC 918 U/V (Australia) spectrophotometer at 37 ºC. One unit of enzyme activity was that required for the generation of 1μmol of NADPH per min.

Isocitrate lyase (ICL)

The assay was that of Dixon and Kornberg (1959) using the same extraction buffer as above. The increase in absorbance due to the complex formed from the glyoxylate produced and the fenylhydracine added at 324 nm ($\varepsilon_{complex}$=17,000 $M^{-1}cm^{-1}$), was followed in a spectrophotometer GBC 918 U/V (Australia) at 37ºC. One unit of enzyme activity was that required to generate 1μmol of complex per min.

Acetyl-CoA synthetase (ACS)

The method used was that established by Brown et al. (1977). The extraction buffer was 64.5 m*M* potassium phosphate, pH 7.5 with 1 m*M* β-mercaptoethanol. The Acetyl-CoA synthetase activity was followed as the increment in the NADH absorbance at 340 nm (ε_{NADPH}=6,220 $M^{-1}cm^{-1}$) and 45 ºC. Enzyme activity unit was the enzyme required for the generation of 1μmol of NADH per min.

Phosphotransacetylase (PTA)

The assay was carried out as in Lundie and Ferry (1989). The extraction buffer was 50 m*M* HEPES, pH 7.5 with 1 m*M* β-mercaptoethanol. The enzyme activity was followed as the increment in NADH absorbance at 340 nm (ε_{NADPH}=6,220 $M^{-1}cm^{-1}$) and 37 ºC, one unit being taken as the enzyme required for the generation of 1μmol of NADH per min.

Pyruvate dehydrogenase complex (PDH)

The method was that of De Graef et al., (1999). The extraction buffer was 50 m*M* potassium phosphate, pH 7.5, EDTA 0.1 m*M*, β-mercaptoethanol 5 mM and $MgCl_2$ 3 m*M*. The enzyme activity was followed as the decrease in potassium ferricyanide absorbance at 430 nm ($\varepsilon_{ferricande}$=1,030 $M^{-1}cm^{-1}$) and 37 ºC. Enzyme activity unit was the enzyme required for the generation of 2 μmol of ferricyanide per min.

Substrate consumption for growth and biotransformation processes

L(-)-carnitine concentration was determined enzymatically with the carnitine acetyl transferase method (Jung et al., 1989). Glycerol, crotonobetaine and γ-butyrobetaine were analysed by HPLC with a Tracer Spherisorb-NH_2 column, 3μm, 25 x 0.46 cm, supplied by Teknokroma (Barcelona, Spain) as reported (Obón et al., 1999). The isocratic mobile phase was 0.05 mol/L acetonitrile/H_3PO_4 (65/35) pH 5.5 at a flow rate of 1 mL/min. Bacterial growth was followed spectrophotometrically at 600 nm, using a Novaspec II from Pharmacia-LKB, (Uppsala, Sweden), and converted to dry weight accordingly.

Determination of central metabolite concentration

ATP content and NADH/NAD⁺ ratio. The energy content per unit of cell was determined as the ATP level and NADH/NAD^+ ratio throughout the experiments. For ATP measurement, the HS II bioluminescence assay kit from Boehringer (Mannhein, Germany), based on the luciferase enzyme using a FluoStar fluorimeter (BGP, Germany) in the conditions stated and after cell DMSO lysis, was used.

Reducing power as $NADH/NAD^+$ ratio was calculated as in Snoep et al., (1990). The measurements were made using an enzymatic method based on the alcohol dehydrogenase enzyme. The extraction of NADH and NAD^+ was carried out by two different methods, involving alkali or acid extraction depending on whether the reduced or the oxidized form was obtained. The cell content was determined after biomass optical density transformation as dry weight and assuming either an intra-cellular volume of 1.63 µL/mg (Emmerling et al., 2000) or 1.72 mLx 10^{-13}/cell (worked out by flow cytometry in this work.).

Acetyl-CoA and CoA cell content. The cell level of both metabolites was determined by HPLC using the method described by De Buysere and Olson (1983). The system was that used in Section 4, equipped with a µ-Bondapak™ C18 (Millipore), 4.5 mm x 25 cm column. The mobile phase was as follows: phase A (85%), 120 m*M* phosphate buffer, pH 4 and 0.05% β-mercaptoethanol and phase B (15%), 98% methanol with 2%clorophorm eluted at 0.8 mL/min. Acetyl-CoA and CoA were detected at 254 nm.

E. coli anaerobic metabolite production

The acetate, fumarate, lactate, formate and pyruvate contents of the bulk liquid reactor were determined by HPLC using the same system as in Section 4. A Tracer Spherisorb-NH_2 (3µm, 25 x 0.46 cm) column supplied by Teknokroma (Barcelona, Spain) was used. The isocratic mobile phase was 0.05 mol/L acetonitrile/H_3PO_4 (65/35) pH 4.7 at a flow rate of 0.5 mL/min. The effluent was monitored at 207 nm. Samples were withdrawn from the reactor and centrifuged at 12,000 x g for 10 min at 4 ^{0}C. The supernatant was used for analyses.

Transport assays

The uptake of carnitine in *E. coli* O44K74 was measured using L[*N-methyl* ^{14}C]carnitine (56 mCi/mmol) as substrate. Cell samples were re-suspended in the L(-)-carnitine uptake assay at an A^{600} of approximately 0.5, containing 10-20 µ*M* L[*N-methyl* ^{14}C]carnitine (5.6 mCi/mmol) in a total reaction volume of 3 mL. Samples (0.3 mL) were taken at various times and filtered through 0.45 µm-pore-size filters of 25 mm diameter. The filters were then washed with 20 mL of isotonic minimal salts and the radioactivity retained was determined with 2.5 mL of scintillation liquid in a Liquid Scintillation Counter (Wallak 1409, USA). All the experiments and assays were performed in triplicate at least.

CENTRAL AND CARNITINE METABOLISMS IN *E. coli*

Since carnitine metabolism in *E. coli* is repressed by glucose, we will consider as the central carbon or primary metabolism that arising from glycerol as the carbon source (Materials and Methods). Furthermore, although this secondary metabolism in *E. coli* is repressed in the presence of high levels of oxygen (Kleber 1997; Cánovas et al., 2002), aerobic conditions will also be considered to gain deeper insight into its link with the central metabolism.

Central carbon metabolism of *E. coli*.

Glycerol, an intermediary product resulting from lipid metabolism, enters in the glycolytic pathway, where it is transformed into glyceraldehyde-3-phosphate. Pyruvate is converted into acetyl-CoA, which enters the Krebs cycle (TCA), producing reducing power which will be transformed into ATP within the electron transport chain during aerobiosis. The glyoxylate shunt is associated to the TCA, short-cutting the metabolic flow and forming oxaloacetate from acetyl-CoA (Fig. 1, EcoCyc-MetaCyc, Karp et al., 2002). However, when *E. coli* grows under anaerobiosis or under a limiting oxygen supply, the reducing power comes from the mixed-acid fermentation for ATP formation, reducing pyruvate to acid metabolites such as lactate, succinate and formate, which will render CO_2 and H_2, while acetyl-CoA will be reduced to acetate and ethanol (neutral) (Varma et al., 1993). These reducing paths also allow the production of ATP, although at lower levels than during aerobiosis. From the central (primary) metabolism, cells obtain energy and intermediates for cell growth and turn-over. Moreover, when carnitine metabolism is expressed, certain intermediates would also be addressed to this (generally termed) secondary metabolism. In fact *E. coli* cells do not need this metabolism for their life cycle unless they are under osmotic up-shocks and/or in anaerobiosis, in which case crotonobetaine acts as an electron acceptor.

E. coli carnitine metabolism

Different enterobacteriaceae, such as *Escherichia coli*, *Proteus vulgaris* and *Proteus mirabilis*, are able to convert L(-)-carnitine via crotonobetaine into γ-butyrobetaine in the presence of carbon and nitrogen sources under anaerobic and/or aerobic conditions (Kleber, 1997).

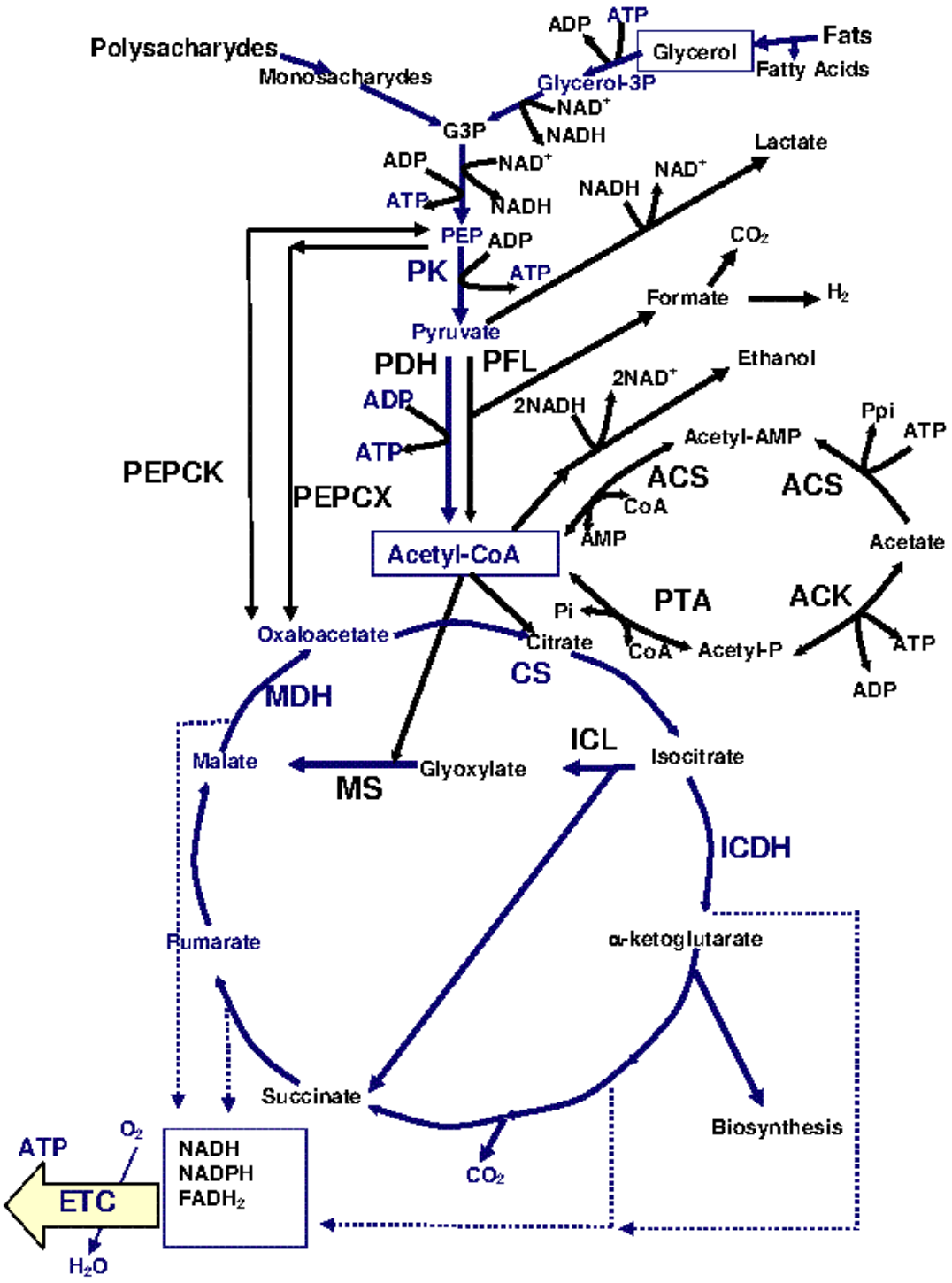

Figure 1. Central metabolism of *E. coli*.

ACK: Acetate kinase, ACS: Acetyl-CoA synthetase, CS: Citrate synthetase; ETC: Electron transport chain, ICDH: Isocitrate dehydrogenase, ICL: Isocitrate lyase, LDH: Lactate dehydrogenase, PEP: Phosphoenolpyruvate, PEPCK: PEP carboxykinase, PEPCX: PEP carboxylase, PFL: Pyruvate formate lyase: PK: Pyruvate kinase and PTA: Phosphotransacetylase. (EcoCyc-MetaCyc-2002, USA, Karp et al. 2002)

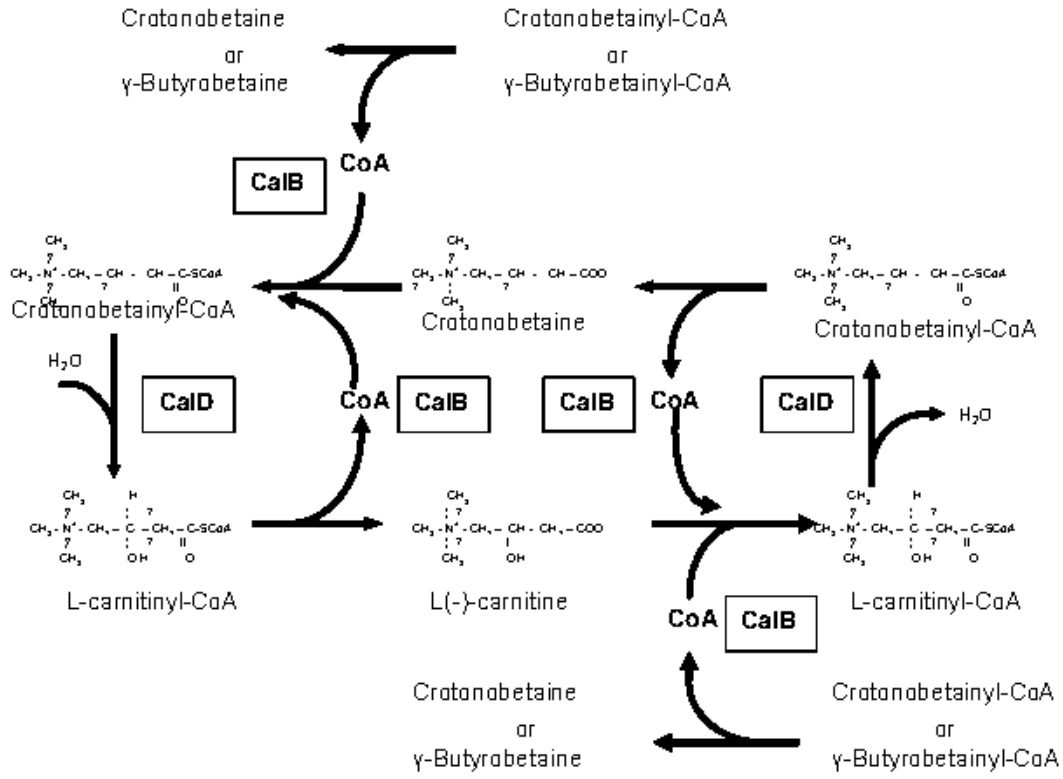

Figure 2. Anaerobic metabolism of L(-)-carnitine in *E. coli*. CaiB: acetyl-CoA/CoA transferase, CaiD: enoyl-CoA hydratase (previously known as L(-)-carnitine dehydratase). After Elssner et al., (2001).

In *E. coli,* operons ***caiTABCDE*** and *fix*ABCX are modulated positively by general regulators, such as the AMPc receptor protein (CRP) or the transcriptional regulator responsible for anaerobic induction (FNR), and negatively by the DNA-binding protein H-NS, glucose or nitrate (Unden and Trageser 1991; Eichler et al., 1994a). In addition, it has been proposed that a positively controlled ***caiF*** gene, 3' adjacent region to the ***cai*** operon, acts as a specific transcriptional regulator for carnitine metabolism (Eichler et al., 1996). This pathway is detectable not only in cells previously grown anaerobically but also in some species, such as *E. coli* ATCC 25922 and DSM 8828, *P. vulgaris* and *P. mirabilis*, grown under aerobiosis in the presence of inducers such as D,L-carnitine mixture or crotonobetaine (Kleber, 1997; Obón et al., 1999; Elssner et al., 2000; Cánovas et al., 2002). It was first postulated that L(-)-carnitine dehydratase reversibly catalyzed L(-)-carnitine into crotonobetaine

and that crotonobetaine reductase non-reversibly transformed crotonobetaine into γ-butyrobetaine as an electron sink (Jung et al., 1989; Roth et al., 1994; Kleber, 1997), even though this latter in *E. coli* can be inhibited by fumarate addition as another electron sink (Obón et al, 1999). Now that functions have been assigned to each putative protein of the *cai* operon, it is known that CaiT is an exchanger (antiporter) for L(-)-carnitine and γ-butyrobetaine in *E. coli* (Jung et al. 2002), which also transport crotonobetaine, requiring ATP (Cánovas et al., 2002; Cánovas et al., 2003b). The enoyl-CoA hydratase (CaiD) is composed of two identical subunits, requiring a CoA-transferase activity (CaiB) (Fig.2), while the crotonobetaine reductase activity requires two proteins: CaiB (one dimer) and CaiA (one tetramer). It has been verified that the hydration reaction of crotonobetaine to L(-)-carnitine (CHR) proceeds at the CoA-level in two steps: the protein CaiD-catalyzed hydration of crotonobetainyl-CoA to L-carnitinyl-CoA, followed by CoA-transfer from L-carnitinyl-CoA to crotonobetaine, catalyzed by CaiB (Elssner et al., 2001) (Fig. 2). Thus, CaiD and CaiB from *E. coli* have been found to catalyze the reversible biotransformation of crotonobetaine to L(-)-carnitine in the presence of a co-substrate, either γ-butyrobetainyl-CoA or crotonobetainyl-CoA (Elssner et al., 2001). CaiD was also postulated to be involved in racemisation of D(+)-carnitine (Eichler et al., 1996). Further, CaiC has been suggested as a CoA-trimethylammonium ligase (Eichler et al., 1996), activating crotonobetaine/γ-butyrobetaine/L(-)-carnitine when they reach the cell. The function of protein CaiE is not totally understood and further studies must be undertaken. With all this information, we have proposed a model to describe the whole activity of *E. coli* able to produce L(-)-carnitine from crotonobetaine under both anaerobic and aerobic conditions in bioreactors (Figure 3).

Biotransformation with resting cells in batch reactors.

In resting cell processes no carbon-growing substrate is fed to the reactor, and therefore the cell-stored material is the only carbon source for the synthesis of the enzymes involved in cell turn-over/maintenance and the biotransformation of crotonobetaine (Castellar et al., 1998; Obón et al, 1999). Therefore, the model presented above (Fig. 3) also depicts the biotransformation of the externally added crotonobetaine by resting cells, using their energy-storage material.

Biotransformation in continuous stirred tank and high-cell density membrane reactors.

The *E. coli* central carbon and carnitine metabolism can be affected by cell growth in continuous high-cell density membrane reactors, as a result of cell metabolism stress due to high bulk liquid viscosity and nutrient shortages (Lee, 1996; Canovas et al., 2002). Therefore, for comparison, biotransformation studies also were carried out with both continuous stirred tank and high-cell density membrane reactors.

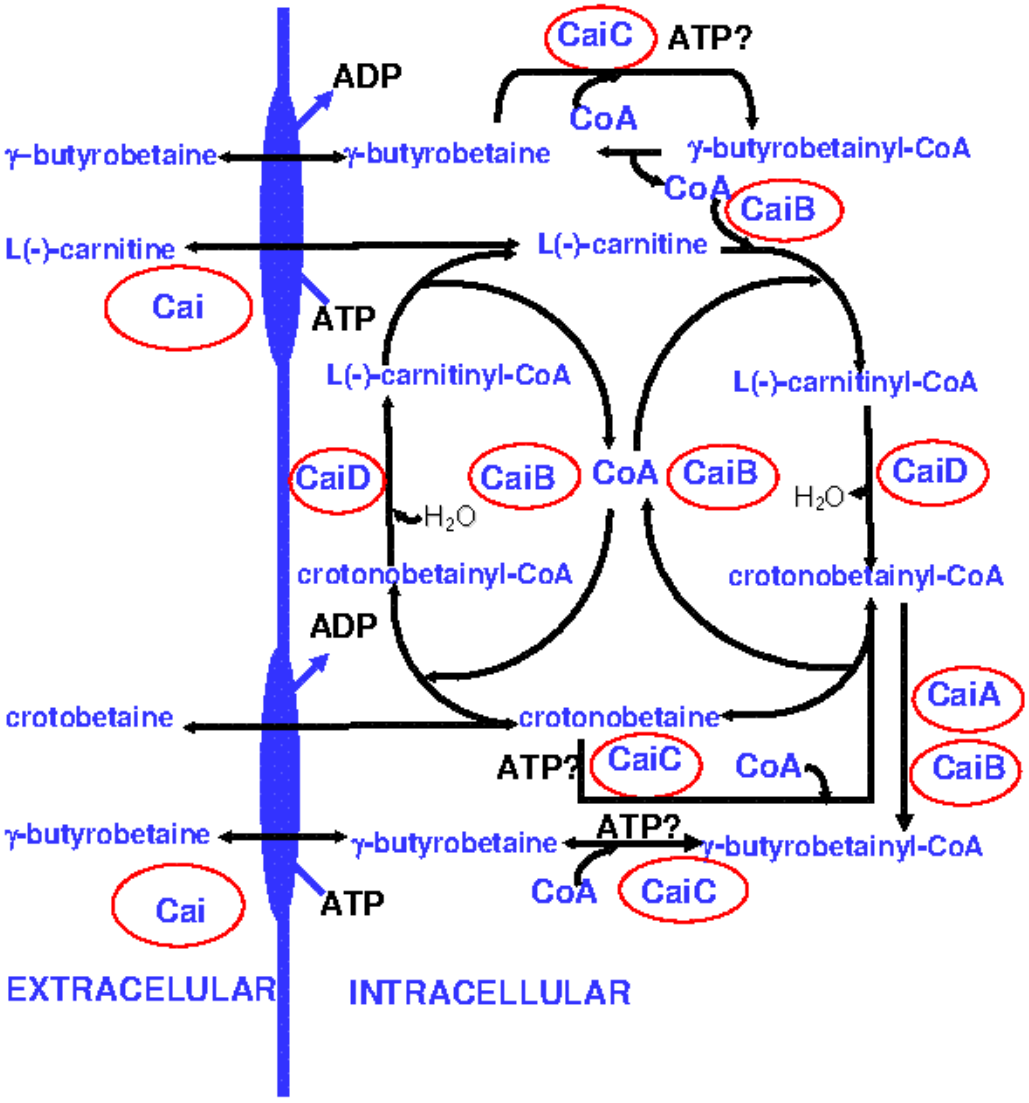

Figure 3. Anaerobic metabolism of L(-)-carnitine in *E. coli*. CaiT, L(-)-carnitine/γ-butyrobetaine/crotonobetaine protein transporter; CaiA, CaiB, crotonobetaine reduction reaction; CaiB, acetyl-CoA/CoA transferase; CaiC, L(-)-carnitine/γ-butyrobetaine/crotonobetaine CoA ligase; CaiD, enoyl-CoA hydratase or D(+)-carnitine racemase activity.

Link between the central metabolism and the L(-)-carnitine metabolism in E. coli.

It is of prime importance to find the link between the central carbon (primary) and the carnitine (secondary) metabolisms of *E. coli*, if the biotransformation process is to be optimized. Bearing in mind the described metabolisms in *E. coli* (Figs. 1 and 3), the link between both is postulated at the level of the pool of ATP and the pool of Acetyl-CoA/CoA.

RESULTS

Biotransformation with growing cells of *E. coli* in batch reactors

Central metabolism during cell growth

Cells were fed on the CM, containing crotonobetaine as biotransformation substrate. When the ICL enzyme activity was studied, the importance of the glyoxylate shunt was clear (Fig. 1) from its high activity, which doubled from 15.8 to 34.9 mU/mg protein in 15 h before to decreasing to 9.0 mU/mg protein after 72 h (Fig. 4C). It was also shown that the enzyme ICDH, producing α-ketoglutarate and NADPH, did not significantly vary during the experiments, with levels of 43.9-53.2 mU/mg protein (Fig. 4C).

The acetyl-CoA metabolism showed that the pyruvate dehydrogenase complex (PDH) did not present any activity (Fig. 4B and Table 1). In fact the acetate would have been produced from the activity of the enzyme pyruvate-formate lyase (PFL), which in anaerobiosis gives acetyl-CoA and formate (Knappe and Sawers, 1990). A high concentration of formate was observed (15 g/L) five hours into the experiments decreasing to levels of 1.2 g/L after 72 h (Fig. 5A). Table 1 shows the level of the PDH complex for aerobic and anaerobic conditions. The decrease in formate must have been the result of formate metabolization to CO_2 and H_2 by the inducible enzyme formate-hydrogen lyase, expressed in anaerobiosis and regulated by the system Ph1A (Unden and Trageser, 1991). The results also showed that under anaerobiosis the PTA enzyme presented a higher level during the first 24 h (a specific activity of 41.7 mU/mg protein) and then decreased to levels of 5.3 mU/mg protein during the last 48 h (Fig. 4B), while was not expressed in aerobiosis (Table 1). This pathway renders ATP, which reached the highest level (0.87 mM) within the first 15 h, decreasing to half this level after 72 h (Fig. 5C). The activity of the ACS enzyme catalysing the production of acetyl-CoA form acetate fell four-fold between 15 and 24 h, before increasing to 60.10 mU/mg protein during the last 48 h (Fig. 4B). This was accompanied by an increase in acetate levels at the beginning of the biotransformation, decreasing after 15 h to a level that was maintained until the end (72 h). The level of acetyl-CoA, related to the ACS, PTA and the PFL enzyme activities, showed an increase from 40 to 100 mM between 5 and 15 h to decrease to 40 mM after 48 h (Fig. 5B). The increase in acetyl-CoA also coincided with a fall in ACS activity, which is subject to a negative regulation by feedback of acetyl-CoA as enzyme product (Kumari et al., 2000).

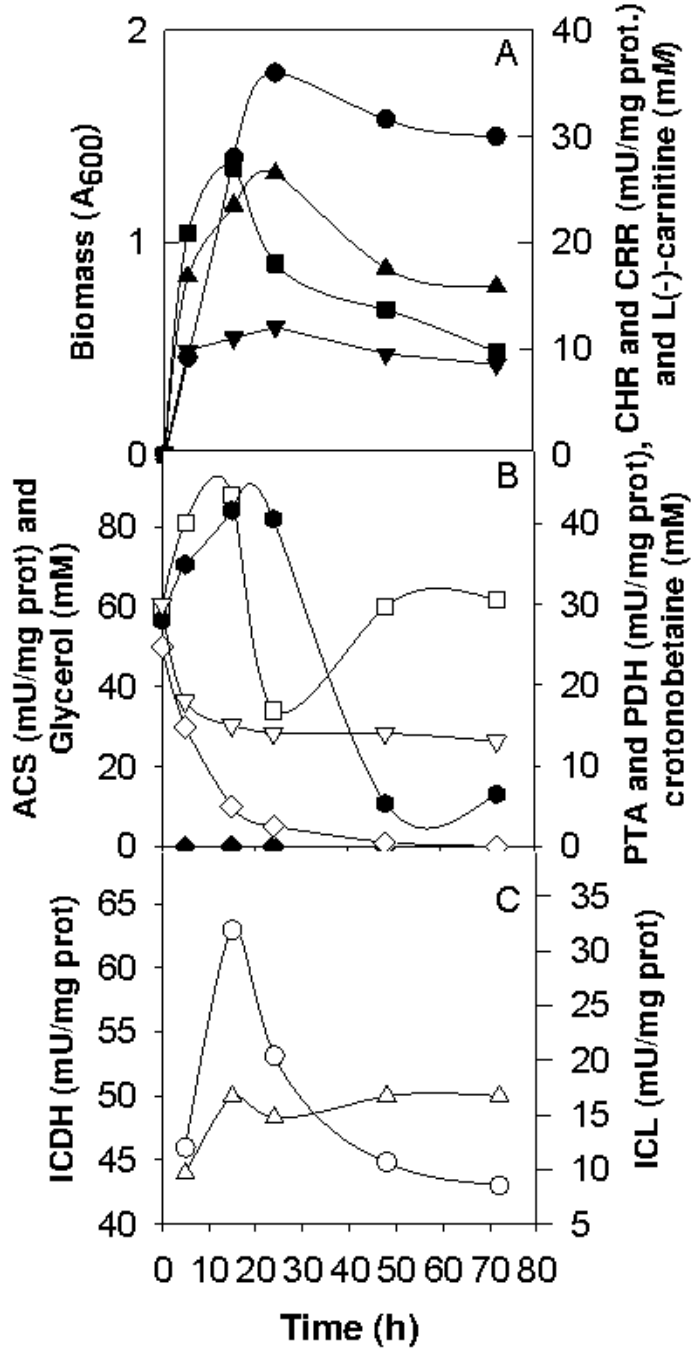

Figure 4. Evolution of *E. coli* O44K74 metabolism on a complex medium in anaerobic conditions and batch systems. A) (●) Biomass (A^{600}), (■) CRR (crotonobetaine reduction reaction), (▲) CHR (crotonobetaine hydration reaction) in mU/mg prot and (▼) L(-)-carnitine in m*M*, B) (◇) Glycerol, (▽) crotonobetaine, (□) ACS (acetyl-CoA synthase), (●) PTA (phosphotransacetylase) and (◆) PDH (pyruvate dehydrogenase) in mU/mg prot and C) (△) ICDH (isocitrate dehydrogenase) and (○) ICL (isocitrate lyase) in mU/mg prot.

However, the CoA levels remained nearly constant (30 mM) throughout the experiment, its production being in equilibrium with consumption. One important fact is that the ACS activity was very low under aerobiosis (Table 1), indicating the preferred Krebs cycle carbon flow. Besides, results showed that during the first 15 h, lactate concentration was 1.2 g/L, decreasing to 0.3 g/L until the end of the culture (Fig. 5A). Finally it can be seen that the cell reducing power, as reflected by the NADH/NAD^+ ratio, was lower during the first 15 h (0.48) and progressively increased during the experiment to 0.80, while cellular ATP level decreased (Fig. 5C).

Secondary metabolism during the biotransformation of crotonobetaine into L(-)-carnitine

The results obtained for the secondary metabolism showed that L(-)-carnitine was produced during the first 15 h. Moreover, it was during this period that the enzymes involved, enoyl-CoA hydratase:CoA transferase (CaiD:CaiB, crotonobetaine hydration reaction, CHR) and crotonobetaine reductase:CoA transferase (CaiA:CaiB, crotonobetaine reduction reaction, CRR) reached their maximum values of 26.5 and 27.0 mU/mg protein, respectively, to gradually decrease during the last 48 h (Figs. 3, 4A). Table 1 shows the differences observed between anaerobic and aerobic conditions for the biotransformation.

Table. 1. Specific activities for the enzymes under aerobiosis and anaerobiosis after 15 h of culture in batch reactors. Activity is expressed as in mU/mg protein. Values are the mean of three measurements.

	Batch reactor	
Enzyme	**Anaerobiosis**	**Aerobiosis**
CHR	22.4±1.5	8.4±1.9
CRR	20.3±2.1	ND
ICL	28.9±3.1	20.8±2.5
ICDH	49.9±4.1	59.7±3.9
PDH	ND	183.8±5.1
PTA	41.6±3.2	ND
ACS	88.0±4.1	13.7±3.1

ND. Not detected. ICDH: Isocitrate dehydrogenase, ICL: Isocitrate lyase, PTA: Phosphotransacetylase, CHR (crotonobetaine hydration reaction, CaiD:CaiB), CRR (crotonobetaine reduction reaction, CaiA:CaiB), ACS (acetyl-CoA synthase) and PDH (pyruvate dehydrogenase) in mU/mg prot.

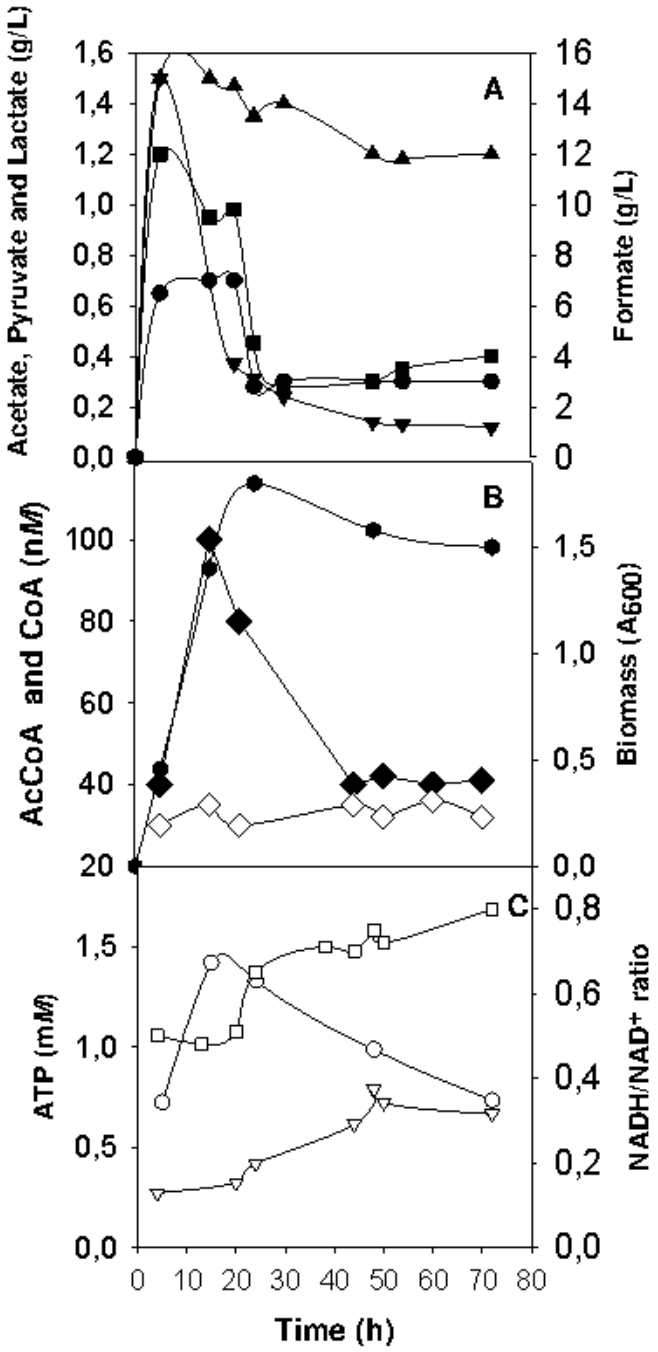

Figure 5. *E. coli* O44K74 metabolism evolution on a complex medium in anaerobic conditions in batch systems. **A)** (▲) pyruvate, (■) lactate, (●) acetate and (▼) formate, **B)** (◆) acetyl-CoA, (◇) CoA and (●) biomass, and **C)** (○) ATP concentration and NADH/ NAD^+ ratio, (□) anaerobiosis and (▽) aerobiosis.

Biotransformation with resting cells in batch reactors

Central metabolism during resting cells

E. coli O44K74 cells were grown as described previously in anaerobic conditions to allow maximum carnitine metabolism induction, then centrifuged and fed in the reactor on a biotransformation medium, composed of crotonobetaine in phosphate buffer (Fig. 6A). In this way, cell metabolism was only devoted to cell maintenance, performing the parallel biotransformation. From 5 to 24 h, the ICL activity doubled reaching levels as high as 12.9 mU/mg protein (Fig. 7B). With respect to the Krebs cycle, after a slight increase of the ICDH to 90.3 mU/mg protein during the first 5 h, decreased to 19.8 mU/mg protein at 35-48 h (Fig. 7B). Further, the energetic status of the cells showed that the ATP concentration decreased from 0.7 to 0.31 m*M* after 24 h, decreasing slowly afterwards (Fig. 8B). Moreover, the activities PTA and ACS decreased throughout the biotransformation, PTA not being detected after 5 h, while the ACS was five-fold lower after 24 h and not detected at 72 h (Fig. 7A). With respect to the PDH complex, it doubled its levels during the first 24 h to reach 19.60 mU/mg protein, and then fell to 15.6 mU/mg protein during the last 48 h (Fig. 7A). This was accompanied by a two fold increase in acetyl-CoA at 24 h, decreasing to the initial levels towards the end of the process. Furthermore, CoA paralleled the acetyl-CoA behaviour, the acetyl-CoA/CoA ratio increasing from 1.1 to 1.7 to decrease to the previous level at the end of the biotransformation (Fig. 8A).

Secondary metabolism during the biotransformation with resting cells

The biotransformation studies in these conditions showed that the CRR activity (CaiA:CaiB) decreased from 18.0 to 3.0 mU/mg protein, while the CHR activity (CaiD:CaiB) decreased sharply during the first 5-10 h from 48.4 to 9.0 mU/mg protein, remaining close to 10.0 mU/mg protein during the rest of the process (Fig. 6B). As regards L(-)-carnitine production (Fig. 6A), the yield reached high levels after the first 24 h, paralleling the consumption of crotonobetaine. The yield (50 %), was higher than that obtained in growing conditions (40%) (Fig.4A). The reducing power slightly increased, though to a lesser extent than in growing cells (Fig. 8A). One important fact seen from studies performed on the transporter CaiT during biotransformation, using N(*methyl-*^{14}*C*)-carnitine to determine the L(-)-carnitine transport in cells sampled from the biotransformation reactor bulk, is that throughout the biotransformation time the rate of transport decreased parallel to the ATP cell level (Figs. 8B and 8C), which would affect the production of L(-)-carnitine (Fig 6A) from crotonobetaine.

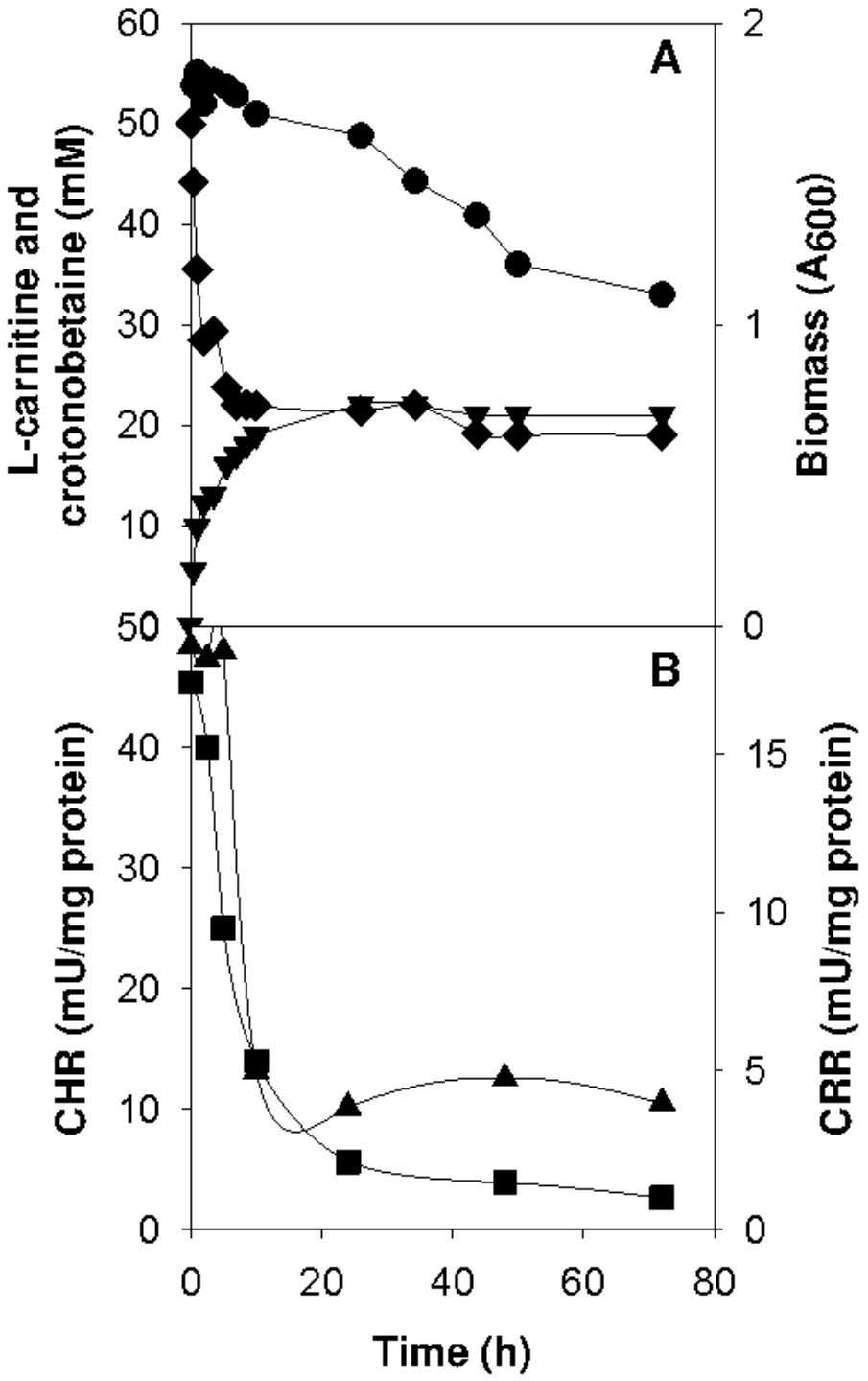

Figure 6. A) Biotransformation of crotonobetaine (◆) into L(-)-carnitine (▼) and biomass (●) in batch reactors with *E. coli* O44K74 resting cells. The specific activities of the following enzymes, **B)** (■) CRR (crotonobetaine reduction reaction) and (▲) CHR (crotonobetaine hydration reaction) in mU/mg prot, respectively, in the biotransformation process are shown.

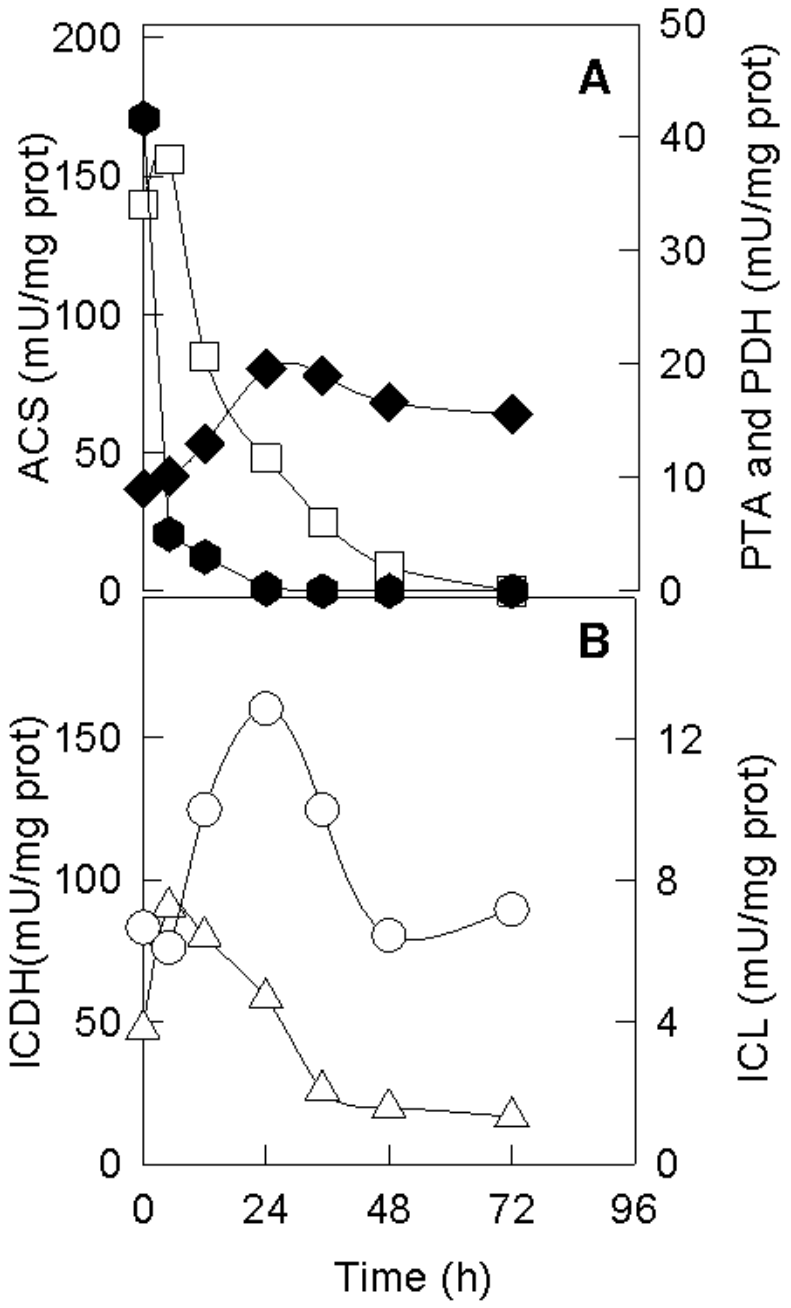

Figure 7. Biotransformation of crotonobetaine into L(-)-carnitine in batch reactors with *E. coli* O44K74 resting cell systems. The specific activities of the following enzymes are shown: **A)**: (□) ACS (acetyl-CoA synthase), (●) PTA (phosphotransacetylase) and (◆) PDH (pyruvate dehydrogenase) and **B)**: (△) ICDH (isocitrate dehydrogenase) and (○) ICL (isocitrate lyase) in mU/mg prot.

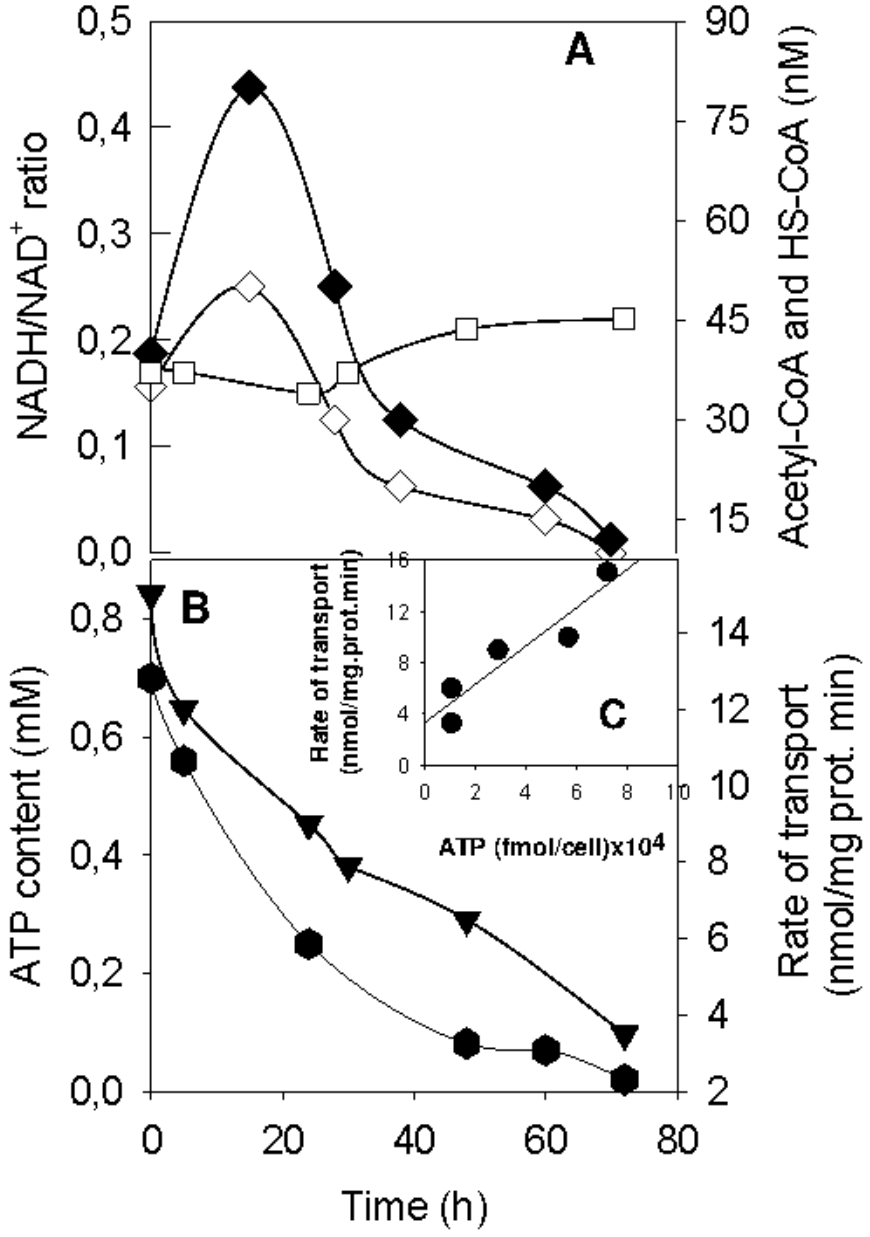

Figure 8. Biotransformation of crotonobetaine into L(-)-carnitine in batch reactors with resting *E. coli* O44K74 cell systems. **A)** (□) NADH/ NAD^+ ratio, (◆) acetyl-CoA and (◇) CoA, **B)** (●) ATP concentration and (▼) uptake rate of L[*N-methyl* ^{14}C]carnitine by *E. coli* O44K74 cells performing the biotransformation of crotonobetaine in resting and **C)** (●) relationship between uptake rate of L[*N-methyl* ^{14}C]carnitine and the ATP/cell. The L[*N-methyl* ^{14}C]carnitine concentration for uptake assays was 20 μM (5.6 $mCi.mmol^{-1}$). Assays were carried out at 37°C.

Biotransformation in continuous reactors

Central metabolism during growth and biotransformation in continuous systems

In order to characterize the metabolism of the cell population in continuous processes, experiments were carried out in stirred tank and membrane high-cell density reactors at different dilution rates under anaerobiosis using CM and fumarate to inhibit the CRR activity (Kleber 1997). Figure 9 shows the activity levels for ICDH, ICL, ACS, PTA, CHR and PDH in a continuous reactor with and without cell retention, and in batch systems using either growing or resting cells. The results were obtained during the period that the L(-)-carnitine concentration did not change in batch (growing and resting conditions) and during steady operation for the continuous systems. ICDH was slightly higher in the continuous systems, while ICL activity was slightly higher in both the batch (growing and resting cells) and the high-cell density membrane systems. With respect to enzymes PTA and ACS, which are connected with acetate metabolism, the continuous processes resulted in higher activity than the batch systems, indicating high production of acetate and as a result acetyl-CoA.

In Figure 10B, the ICDH/ICL ratio is depicted throughout the experimental period. The ratio increased until the steady state was reached, then the glyoxylate shunt ICL increased with respect to the Krebs ICDH decreasing the ratio. More importantly, the ATP per unit of cell increased during the reactor start up and decreased as the steady state was reached (Fig. 10B). Table 2 shows that the activity levels of the enzymes related with acetate and acetyl-CoA synthesis increased substantially with respect to the corresponding activities in batch reactors. This indicates that the bulk accumulated acetate is being transformed into acetyl-CoA, to be used for cell metabolism. However, during the steady state, ICL expression increased in cells in the high-density cell recycle reactor.

Secondary metabolism during biotransformation in continuous systems

The level of γ-butyrobetaine was close to zero when the CRR activity was inhibited by fumarate addition to the CM, while L(-)-carnitine production reached values ranging from 18-22 m*M* within the outstream of the reactor due to the CHR activity (Fig. 10A).

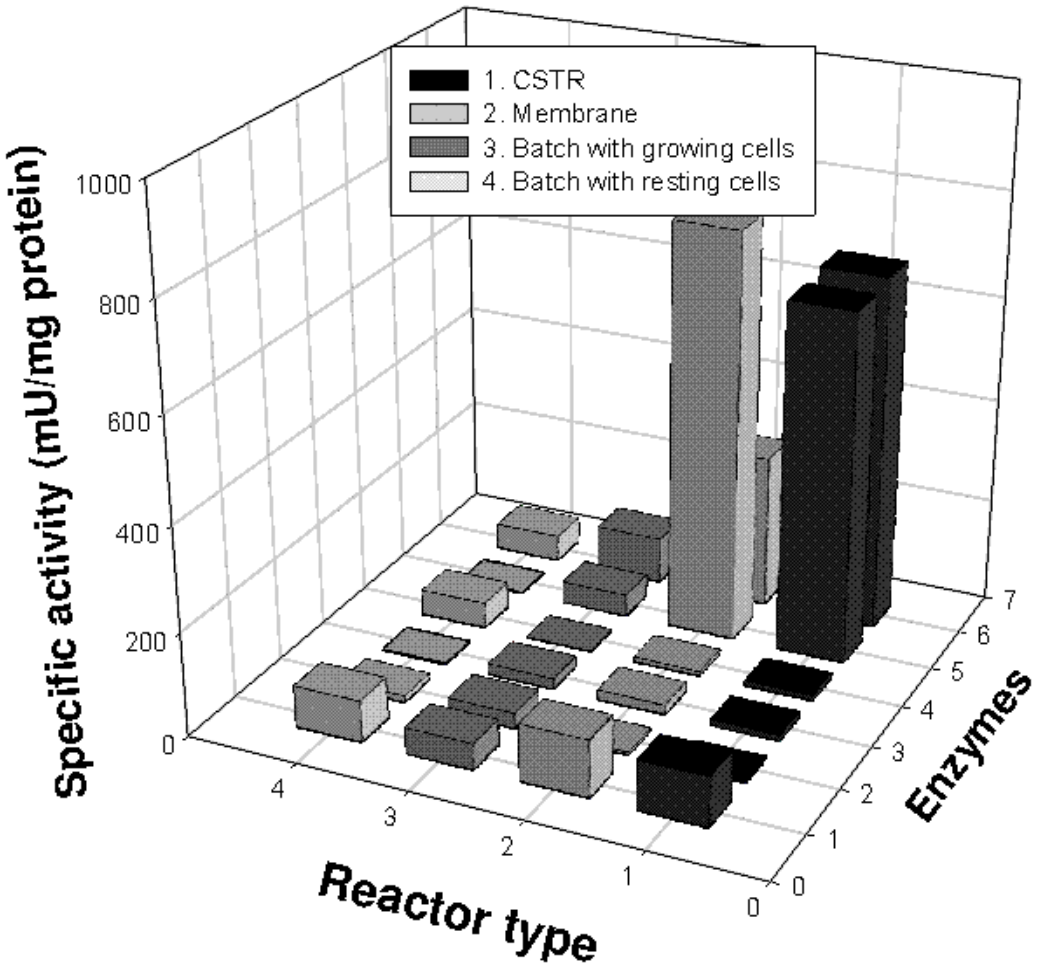

Figure 9. Specific activities of the following enzymes in batch, continuous stirred tank and membrane reactors are shown: 1) ICDH (isocitrate dehydrogenase) and 2) ICL (isocitrate lyase), 3) CHR (crotonobetaine hydration reaction), 4) PDH (pyruvate dehydrogenase), 5) PTA (phosphotransacetylase) and 6) ACS (acetyl-CoA synthase). Experiments were performed with 75 m*M* glycerol, 50 m*M* crotonobetaine, 25 m*M* fumarate, an initial biomass 0.02 A^{600}, at 37 °C and a dilution rate of 0.4 h^{-1} for the continuous reactors while 50 mM glycerol, 50 mM crotonobetaine, 25 mM fumarate and the same initial biomass and temperature for batch growing and only 75 mM crotonobetaine in 50 mM phosphate buffer pH 7.5 at 37 °C and 2.0 A^{600} initial biomass for resting experiments. Results were obtained at steady state for continuous systems and at L(-)-carnitine steady value for batch systems.

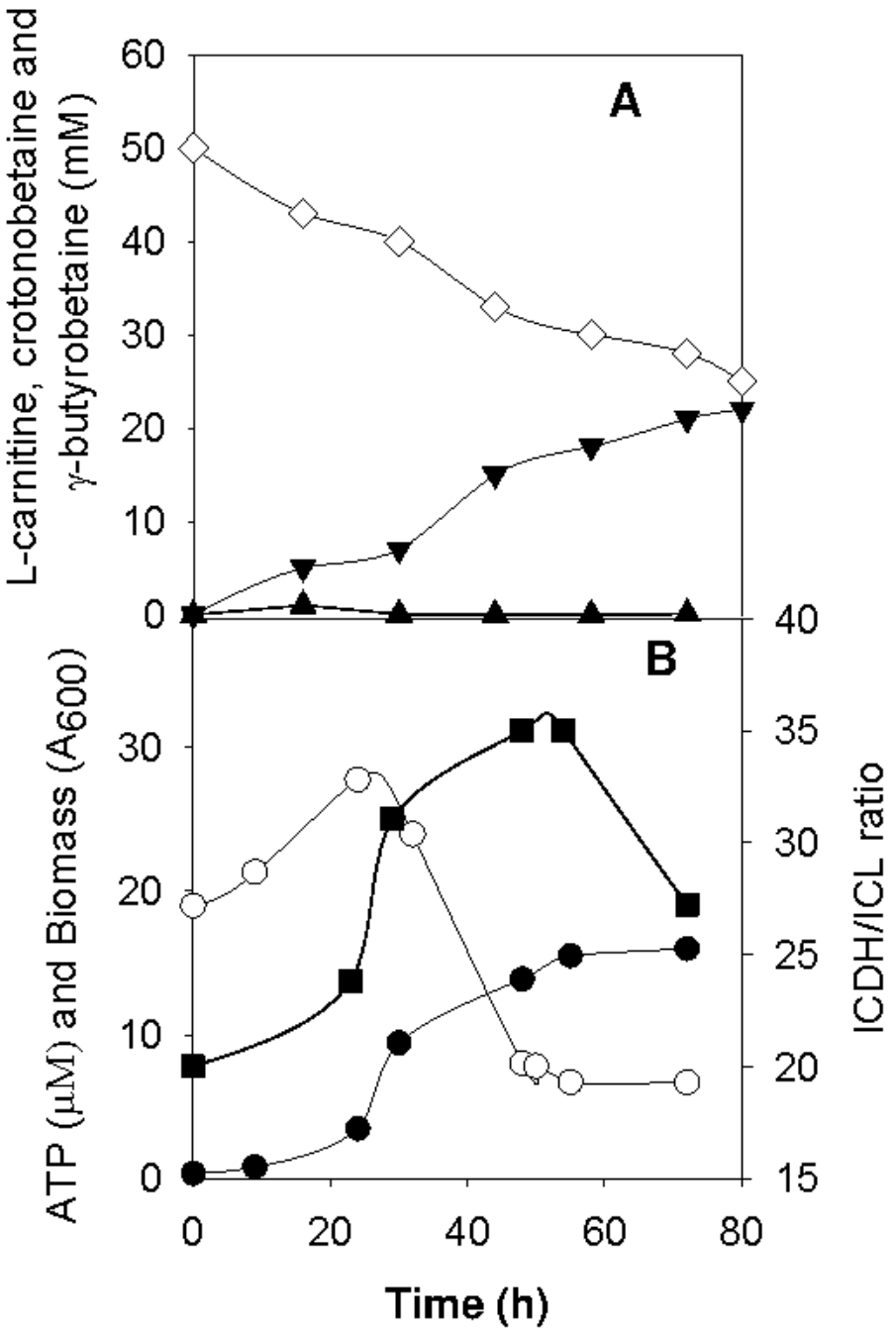

Figure 10. *E. coli* O44K74 metabolism evolution on a complex medium in anaerobic conditions in a high-density cell recycle membrane reactor. A) (◇) crotonobetaine into (▼) L(-)-carnitine and (▲) γ–butyrobetaine in m*M* and **B)** (○) ATP concentration, (■) ICDH/ICL ratio and (●) biomass *E. coli* O44K74 growing on a complex medium containing 75 m*M* of glycerol under anaerobic conditions.

Table. 2. Specific activities for the enzymes under anaerobic conditions and steady L(-)-carnitine level in the batch with growing and resting cells (without keeping strict anaerobic conditions) and membrane reactor. Activity is expressed as in mU/mg protein. Values are the mean of three measurements.

	Batch system		Continuous high-cell density
Enzyme	**Growing**	**Resting**	
PTA	42.1±3.2	2.2±1.2	785.2±10.3
ACS	88.0±2.3	50.3±5.0	394.1±9.2
PDH	ND	20.8±2.7	10.0±2.1
ICDH	50.5±5.3	80.4±5.1	110.2±8.9
ICL	29.2±2.3	12.3±1.1	5.2±2.3
CHR	22.1±5.1	15.1±2.1	18.0±4.3

ND: not detected. ICDH: Isocitrate dehydrogenase, ICL: Isocitrate lyase, PTA: Phosphotransacetylase, CHR (crotonobetaine hydration reaction, CaiD:CaiB), ACS (acetyl-CoA synthase) and PDH (pyruvate dehydrogenase) in mU/mg prot.

DISCUSSION

The ICL and ICDH levels observed during the bioprocess in anaerobic batch growing cells emphasised the importance of the glyoxylate shunt compared with the Krebs cycle (Figs. 1 and 4C), since both compete for the same substrate. Competition was also mentioned by Cronan and La Porte (1996), who described the existence of an aceBAK operon, codifying for the glyoxylate enzymes (ICL: aceA and MS: aceB) and a third enzyme (ICDH-kynase/phosphatase: aceK), which performs a transcriptional modification of ICDH, inactivating it and improving opportunities for ICL.

With respect to PTA, acetyl-CoA was transformed into CoA and acetyl-phosphate, rendering acetate and ATP (Figs. 4B and 5) by the acetate kynase enzyme (ACK) (Kleman and Strohl, 1994). Acetate can be used by ACS to produce acetyl-CoA and this pathway is considered to be repressed by catabolites, which are induced by acetate and a high affinity system acting at low concentrations of acetate (Brown et al., 1977). In fact, the changes in acetate level suggest it is produced through the PTA-ACK pathway during *E. coli* growth and consumed at the beginning of the transition towards the stationary phase, being activated to acetyl-CoA by the ACS enzyme (Kumari et al., 2000), rendering energy and biosynthetic compounds. Therefore, after 20 h the metabolism adjusts to the nutrient shortages and acetate starts to be consumed (Fig. 5A). Further, the evolution of acetyl-CoA and CoA provided information on the expression of the PTA-ACK and ACS metabolisms in anaerobiosis (Fig. 1). The level of acetyl-CoA was related to ACS, PTA (Figs. 4B and 5B) and possibly PFL enzyme activities, the two former showing an increase during the first period of growth. The acetyl-CoA increase also coincided with a lowering of the ACS activity, which is subjected to a negative regulation by feed-back of the acetyl-CoA as the enzyme product (Kumari et al., 2000). However, the CoA levels remained almost constant (30 m*M*), with a slight increase and decrease, since production was nearly in equilibrium with consumption. The rest of the metabolites studied provides information on reducing power, since lactate, which is produced from pyruvate by the lactate dehydrogenase (LDH) enzyme, consumes NADH to produce NAD^+, while the glyoxylate shunt generates reducing power (Figs. 1 and 5C).

Escherichia coli is unable to assimilate the carbon or the nitrogen from L(-)-carnitine (Kleber, 1997). Results concerning the production of L(-)-carnitine and the levels of CHR (CaiD:CaiB) and CRR (CaiA:CaiB) activities and those concerning the central metabolism (ATP and acetyl-CoA/CoA pools) strongly suggest the points of

both metabolisms connection (Figs. 1 and 3). Thus, the results show that the best biotransformation conditions were associated with the growth phase, where higher production of energy from the reducing power and higher levels of acetyl-CoA were evident (Fig.5). During this time, the acetyl-CoA/CoA ratio increased from 1.1 to 3.3, declining at the end of the exponential growth (Fig. 5B). Furthermore, ATP is necessary for the transport protein CaiT (Cánovas et al., 2003b) and since energy is required for the action of other ligases (Vessey and Kelley, 2001), energy would be also necessary for the action of the enzyme crotonobetaine/butyrobetaine-CoA ligase (CaiC) that synthesizes the crotonobetainyl-CoA or γ-butyrobetainyl-CoA required for the activation of the trimethylammonium compounds (Elssner et al., 2000) (see Figs. 2 and 3).

Besides, the CHR activity was always high because the ***cai*** operon (responsible for the expression of carnitine metabolism) was controlled by proteins regulators such as FNR (transcriptional regulator under anaerobic conditions), catabolic repression via protein receptor (CRP) of AMPc, histones (H-NS) and the transcriptional factor σ^2 (RpoS), which is expressed when the cell reaches the stationary phase of growth and inhibits carnitine metabolism (Eichler et al., 1996). All these factors would have a negative effect under aerobiosis (Table 1). Further, the enzyme ACS is positively regulated by CRP (generating acetyl-CoA), high levels of AMPc and FNR (at low oxygen partial pressure), while it is negatively by the σ^2 factor (Kumari et al., 2000). Also, the glyoxylate shunt has been related with acetate metabolism, a protein, IclR, repressing the expression of the ICL and the ACS (Shin et al., 1997). However, the presence of PEP from glycerol metabolisation and the neoglucogenic pathway (Fig. 1) would prevent the binding of the IclR protein to the promoter region of the glyoxylate shunt ***ace***BA***K*** operon (Cortay et al., 1991). Other factors too, such as ArcAB (regulator of the anaerobic and aerobic metabolism, inhibiting the Krebs cycle, the electron transport chain and the PDH activity in anaerobic conditions) activate the PFL enzyme (De Graef et al., 1999), which is also regulated by FNR. Furthermore, the NADH/NAD^+ ratio (Fig 5C) regulates the PDH (Fig. 4B) and PFL enzymes. A low ratio means low levels of reducing power and higher PDH enzyme activity, while high ratios inactivate the PDH and activate PFL (De Graef et al., 1999). Our study confirms that the NADH/NAD^+ ratio was higher in anaerobiosis than in aerobiosis, and that the PDH activity was inhibited (Table 1). Therefore, results demonstrate the relationship between the central carbon and the carnitine metabolism both under anaerobiosis and aerobiosis, also showing the

importance of the glyoxylate and acetate metabolism during the biotransformation in anaerobiosis.

The biotransformation in batch with resting cells (crotonobetaine only in phosphate buffer medium) also showed the importance of the glyoxylate shunt, since the ICL activity doubled (Fig. 7B), using the cell stored material. Further, the cell energetic status (ATP level) decreased since it is used in cell maintenance and in the biotransformation (Cánovas et al., 2003b) (Fig. 8B). Besides, the activities PTA and ACS decreased throughout the biotransformation process, partly due to the lack of carbon source and partly because these enzymes belong to the *E. coli* anaerobic metabolism; the biotransformation with resting cells was performed in the absence of aeration, since the process does not require it, although strict anaerobiosis was not maintained. Furthermore, notice the importance of the pools of ATP, since the decrease of this variable coincided with the cessation of L(-)-carnitine production and a sharp decrease in CHR activity (CaiD:CaiB, Figs. 2, 3 and 6B) at 15-18 h. Another fact is the correlation between the acetyl-CoA/CoA ratio and carnitine metabolism, which might indicate its importance in carnitine metabolism since enzyme trimethylammonium:CoA transferase requires CoA from the pool.

Moreover, a very important observation when using N(*methyl-*^{14}*C*)-carnitine to study the CaiT transporter during biotransformation was the decrease in the transport rate throughout the biotransformation process, paralleling the ATP cell content (Fig. 8C, r^2: 0.989). This confirms that, as a result of ATP cell content shortages, the transport of both the substrate (crotonobetaine) and the product (L(-)-carnitine) decreased. In fact we calculated a production rate of 2.1 mmol L(-)-carnitine·L cellular volume/L reactor·mmol ATP·h, with a higher rate during the first 10 h., 16 mmol L(-)-carnitine·L cellular volume/L reactor·mmol ATP·h. Assuming that at the beginning ATP is only used for transport, a rate of 0.04 fmol L(-)-carnitine/fmol ATP·min can be stated. Previous results (Jung et al., 1990; Cánovas et al., 2003b) indicated that trimethylammonium compounds transporter, CaiT, requires ATP. Furthermore, during resting processes the importance of the cell stored material was also obvious, since the level of acetyl-CoA was maintained, with even high activities of the enzymes ICL and PDH (Fig. 7 and 8) which might point to the synthesis of cell-maintenance compounds and energy. In fact, an acetyl-CoA/CoA ratio ranging from 1.0 to 1.7 was observed throughout the biotransformation process in resting cells, the maximum ratio coinciding with the maximum biotransformation rate (Figs. 3, 6A and 8A). Besides this, cell metabolism was not devoted to the production of

new cells, since the A_{600} decreased slowly (1.8 to 1.2) during the 72 h of biotransformation, and the cell ATP levels went down quickly from the start. These facts give a possible explanation for the high yield of L(-)-carnitine, since during the first 22 h the energy and material stored within the cell was devoted to the biotransformation in resting state. The ATP level decrease may be the possible limiting step of the biotransformation. Moreover, it is also possible that the enzyme crotonobetainyl/γ-butyrobetainyl-CoA ligase (CaiC) required ATP to activate crotonobetaine or γ-butyrobetaine (Fig. 2 and 3), which are necessary for the enzymes involved in L(-)-carnitine synthesis (Elssner et al., 2001). This suggestion arises from the similarities with other CoA-ligases (Vessey and Kelley, 2001), although confirmation is required.

When comparing the *E. coli* metabolism in the different systems used (Fig. 9), the ICL was expressed at higher levels in the high-cell density reactor and even higher in batch systems with growing and resting cells. This indicates that the glyoxylate shunt is important in systems where high-cell density stress and/or possible stress due to nutrient shortages and cell metabolite accumulation is observed. However, in the steady state the acetate metabolism enzymes, PTA and ACS, presented higher activities in the continuous systems. This would imply high steady levels of reactor bulk acetate (Kleman and Strohl, 1994; Lee, 1996) produced from acetyl-CoA (PTA-ACK pathway, producing ATP) and the parallel production of acetyl-CoA from acetate (ACS pathway, consuming ATP and producing Ppi, which is hydrolysed by a pyrophosphatase, generating energy) for the glyoxylate shunt and/or other pathways.

Moreover, in order to compare the relative importance of the Krebs cycle with respect to the glyoxylate shunt during the high-cell density membrane reactor operation, the ICDH/ICL ratio was determined. The results showed that the glyoxylate shunt was less important at the beginning of cell growth than when biomass achieved its maximum level in the steady state stressing conditions due to high-density cell population. Furthermore, the ATP per unit of cell increased during the start up of the reactor and decreased as cell population reached a steady state, in which less energy is required to keep metabolism at a lower rate. These results demonstrate that the glyoxylate shunt was as important as in batch systems when the steady state was being approached. Therefore, when the membrane reactor enzyme specific activities of the central metabolism are compared with the corresponding activities in batch, the glyoxylate shunt activity was lower; secondly, the activity through the TCA cycle increased and, thirdly, there was an increase in

acetate metabolism enzyme activities such as the PTA and ACS, probably induced by the anaerobic conditions and/or the acetate level. Finally, as shown in Fig. 10A, when fumarate is used to supplement the CM no γ-butyrobetaine is produced since the CRR activity (CaiA:CaiB) is inhibited (Kleber, 1997; Obón et al., 1999). Therefore, under these conditions the model presented in Figure 3 would work by a L(-)-carnitine/crotonobetaine antiport with CaiB and CaiC being the biotransformation motion force to keep CaiD producing L(-)-carnitine from crotonobetaine

CONCLUSIONS

The model sketched herein shows for the first time in *E. coli* the link between cell metabolic production of a secondary metabolite, such as L(-)-carnitine, and the primary or central carbon metabolism, providing the basis for further work to optimise its production. This study also substantiates, for the first time, that the ATP level is a critical variable for the biotransformation, not only due to the transporter, CaiT, but for the postulated trimethylammonium-CoA ligase activity (CaiC, Eichler et al., 1996). Moreover, in resting studies, it seems that the gluconeogenesis/consumption of stored material plays an important role, since the activity of the glyoxylate shunt was seen to increase and there was a correlation between the intra-cellular acetyl-CoA/CoA ratio and the biotransformation of crotonobetaine. Further, we also hypothesize that another limiting step for the biotransformation might be the acetyl-CoA/CoA ratio at the level of the transferase enzyme, CaiB, and the enzyme crotonobetaine/butyrobetaine-CoA ligase (CaiC), which would also use ATP as substrate. The connection of both metabolisms sketched in Figure 1 and 3 suggests the existence of control points where it would be possible to act to redirect the metabolic fluxes. However, analysis of the different fermentations flux analysis with respect to the primary carbon metabolism and the carnitine metabolism, as well as a study of the still kinetically unknown enzymes (CaiC and CaiB), is essential to draw precise conclusions. This work is being undertaken by our group to fully understand the biotransformation within the *E. coli* metabolism. Therefore, future studies to optimize the biotransformation should be also addressed at redirecting the metabolic fluxes towards an increase in energy levels and the levels of metabolites required for the biotransformation, and then over-express activities associated to the biotransformation (CHR, ICL, PDH, CoA-ligase:CaiC and transport protein CaiT), with the help of strain selection and the use of the recombinant technologies.

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NOMENCLATURE

aceBAk: Operon acetate formation,

ACK: acetate kinase,

ACS: acetyl-CoA synthetase,

AMPc: Cyclic AMP,

ArcAB: Regulation of the anaerobic/aerobic metabolism that in anaerobic inhibits Krebs, electron transport chain and PDH and activate PFL,

CHR: Crotonobetaine hydration reaction (CaiD:CaiB),

CRR: Crotonobetaine reduction reaction (CaiA:CaiB),

CRP: Catabolic repression via protein receptor AMPc,

CS: Citrate synthetase,

ETC: Electron transport chain,

FNR: Transcriptional regulator of the ***cai*** operon under anaerobiosis,

H-NS: Histone protein,

ICDH: Isocitrate dehydrogenase,

ICL: Isocitrate lyase,

IclR: Represor of ICL and activator of ACS,

LDH: Lactate dehydrogenase,

MS : Malate synthetase,

PEP: Phosphoenolpyruvate,

PEPCK: PEP carboxykinase,

PEPCX: PEP carboxylase,

PFL: Pyruvate formate lyase,

PK: Pyruvate kinase,

PTA: Phosphotransacetylase,

σ^2 RpoS: Transcriptional regulator expressed when cells get into stationary phase, inhibiting carnitine metabolism,

caiTABCDE **: Carnitine operon,**

CaiT: L(-)-carnitine protein transporter,

CaiA: Crotonobetaine reductase,

CaiB: Acetyl-CoA/HS-CoA transferase,

CaiC: Crotonobetaine, L(-)-carnitine or γ-butyrobetaine: acetyl-CoA/HS-CoA ligase,

CaiD: Enoyl-CoA hydratase activity,

CaiE: Protein related to cofactors: crotonobetainyl-CoA/L-carnitinyl-CoA/γ-butyrobetainyl-CoA,

CaiF: ***caiTABCDE*** activator in presence of L(-)-carnitine/D-carnitine mixture or crotonobetaine.

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Capítulo 5

Salt stress effects on the central and carnitine metabolisms of *Escherichia coli*.

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ABSTRACT

The aim was to understand how interaction of the central carbon and the secondary carnitine metabolisms is affected under salt stress and its effect on the production of L(-)-carnitine by *Escherichia coli*. The biotransformation of crotonobetaine into L(-)-carnitine by resting cells of *E. coli* O44K74 was improved by salt stress, a yield of nearly two-fold that for the control being obtained with 0.5 M NaCl. Crotonobetaine and the L(-)-carnitine formed acted as an osmoprotectant during cell growth and biotransformation in the presence of NaCl. The enzyme activities involved in the biotransformation process (crotonobetaine hydration reaction and crotonobetaine reduction reaction), in the synthesis of acetyl-CoA/acetate (pyruvate dehydrogenase, acetyl-CoA synthetase and ATP:acetate phosphotransferase) and in the distribution of metabolites for the tricarboxylic acid cycle (isocitrate dehydrogenase) and glyoxylate shunt (isocitrate lyase) were followed in batch with resting cells both in the presence and absence of NaCl and in perturbation experiments performed on growing cells in a high density cell recycle membrane reactor. Further, the levels of carnitine, crotonobetaine, γ-butyrobetaine and ATP and the NADH/NAD^+ ratio were measured in order to know how the metabolic state was modified and coenzyme pools redistributed as a result of NaCl's effect on the energy content of the cell. The results provided the first experimental evidence of the important role played by salt stress during resting and growing cell biotransformation (0.5 M NaCl increased the L(-)-carnitine production in nearly 85%), and the need for high levels of ATP to maintain metabolite transport and biotransformation. Moreover, the main metabolic pathways and carbon flow operating during cell biotransformation was that controlled by the isocitrate dehydrogenase/isocitrate lyase ratio, which decreased from 8.0 to 2.5, and the phosphotransferase/acetyl-CoA synthetase ratio, which increased from 2.1 to 5.2, after a NaCl pulse five-fold the steady state level. Resting *E. coli* cells were seen to be made up of heterogeneous populations consisting of several types of subpopulation (intact, depolarized and permeabilized cells) differing in viability and metabolic activity as biotransformation run-time and the NaCl concentration increased. The results are discussed in relation with the general stress response of *E. coli,* which alters the NADH/NAD^+ ratio, ATP content and central carbon enzyme activities.

INTRODUCTION

The occurrence of stress conditions during fermentation processes is an important issue for the design and optimization of bioprocesses. As fermentations proceed, one of the problems that microorganisms undergo is the progressive modification of the environment. The accumulation of low and high molecular weight molecules as a result of cell metabolic activity not only affects cells due to the build up of inhibitory levels but also due to osmotic changes. Stress responses are of fundamental interest as they often have a determining effect on process performance (Lindley, 2003) since the triggering of molecular response mechanisms (Hengge-Aronis, 1999) will affect the cell physiology and metabolism.

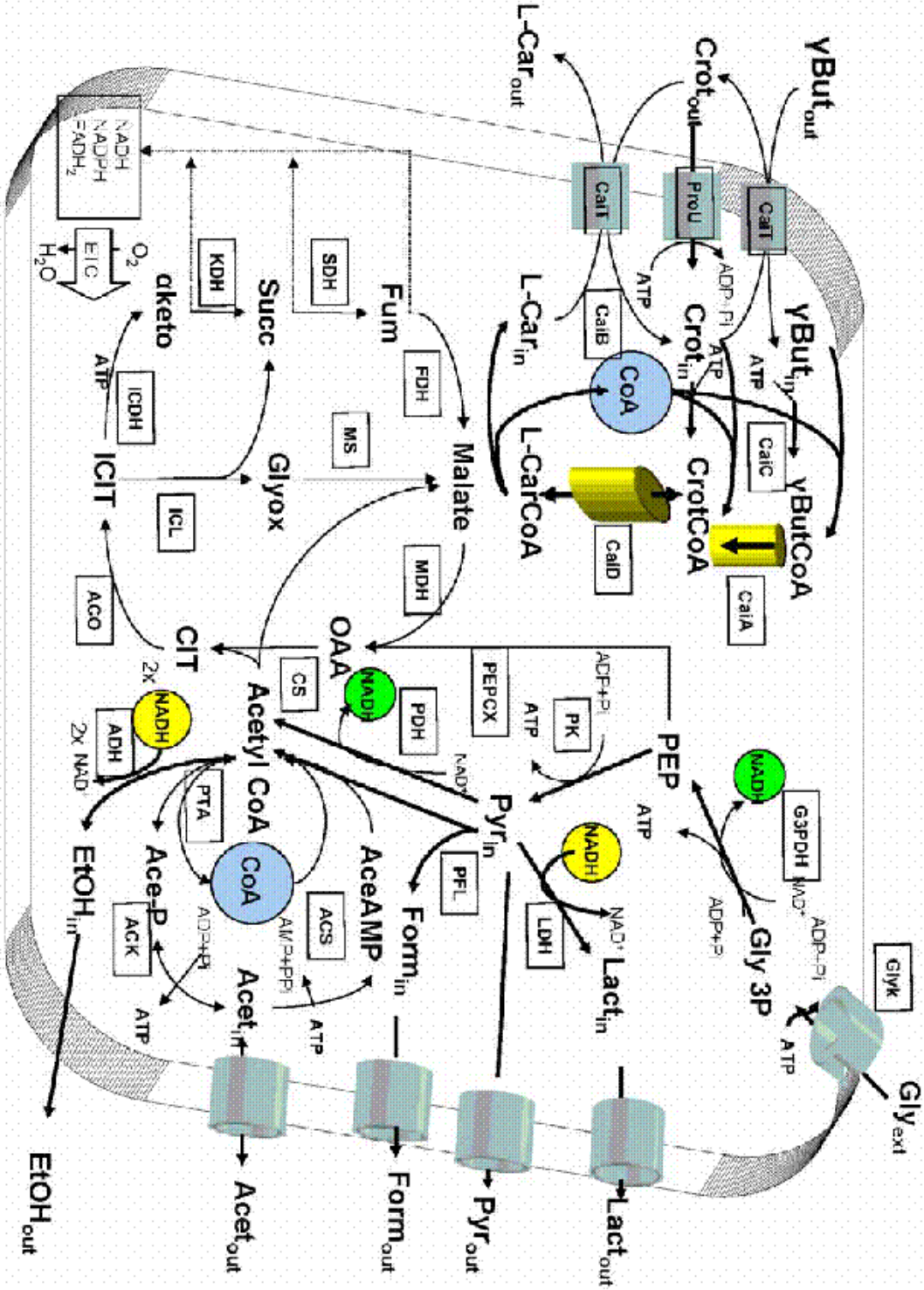
General stress response is triggered when *Escherichia coli* is exposed to a variety of stress conditions affecting the environment, being mainly determined by the upregulation of RpoS, a sigma RNApolymerase subunit which is associated with entry into stationary phase, starvation and stress conditions (Loewen and Hengge-Aronis, 1994; Hengge-Aronis, 2002) all of which strongly modify cell physiology. In *E. coli*, when the osmolarity of the growth medium is increased, osmoadaptation occurs, the phases of which have been summarized previously (Wood, 1999). To avoid dehydration, the osmotic pressure inside the cells is increased (Le Rudulier et al., 1984), certain solutes being accumulated at high cytosolic levels, either by uptake from the medium or through *de novo* synthesis (Csonka 1991, Verheul et al., 1998). 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) (Kappes and Bremer, 1998). Two transporters, ProP and ProU, have been mentioned as being primarily responsible for the uptake of osmoprotectant betaines in enteric bacteria under conditions of hyperosmolarity (Verheul et al., 1998). ProP is a H^+-antiporter, and its activity is K^+ dependent (Macmillan et al., 1999), while ProU belongs to the ATP-binding cassette (ABC) family of transport systems, and the mediated betaine transport is highly osmotically stimulated at the level of gene expression (Csonka 1991). Additionally, CaiT is an exchanger (antiporter) for L(-)-carnitine and γ-butyrobetaine in *E. coli* (Jung et al., 2002), although it does not play a significant role in osmoregulation (Verheul et al., 1998). Despite the research effort devoted to unravelling the responses displayed by *Escherichia coli* when exposed to osmotic stress conditions (Hengge-Aronis, 1996 and 2002), the mechanisms through which the central and energetic metabolism of the bacteria is adapted to such conditions remain unstudied. In *Corynebacterium glutamicum,* a

rearrangement of energetic metabolism has been shown to take place in response to increasing osmolality (Varela et al., 2003, 2004), reflecting the increase in cell energy needs. In *E. coli,* the protective role of carnitine (trimethylammonium compound) metabolism has been studied, since this allows cells to withstand osmotic up-shocks, although it is still not totally understood (Eichler et al., 1994a, 1994b; Kleber, 1997; Elssner et al., 2000, 2001). This secondary metabolism has been used for L(-)-carnitine production from crotonobetaine or D(+)-carnitine with *E. coli* cells in growing and resting state (Castellar et al., 1998; Obón et al., 1999; Cánovas et al., 2002). In brief, the carnitine pathway enzymes are induced in anaerobiosis in the presence of D,L-carnitine and/or crotonobetaine (see the "Central and carnitine metabolism in *E. coli*" section, Materials and Methods and Fig. 1). Cánovas et al. (2003c) observed that salt stress improved the production of L(-)-carnitine from crotonobetaine (dehydrated D(+)-carnitine) by resting cells and characterized the transport of L(-)-carnitine in *E. coli* O44K74. Resting cell processes are also important since biotransformation occurs in a simple medium containing only the substrate dissolved in buffer, thus lowering production costs and simplifying product purification compared with growing cell processes. However, to optimize this bioprocess is necessary to determine the regulatory structure of the metabolic pathways involved in the biotransformation carried out by cells in growing or resting state and in the presence or absence of NaCl. Moreover, little information is available concerning the effect of salt stress on the central and carnitine metabolism of resting and growing *E. coli* cells performing biotransformations. Further, since both substrates (crotonobetaine and D(+)-carnitine) and products (L(-)-carnitine and γ-butyrobetaine) of the biotransformation (Fig. 1) are osmoprotectants, a deeper insight into metabolic adaptations and/or modifications under osmotic stress conditions becomes more necessary for improving strains to be used in biotechnological processes before undertaking optimization strategies.

The aim of this work is to report a first approach to understanding the metabolism of the secondary carnitine and the primary or central carbon metabolism involved in the biotransformation of crotonobetaine into L(-)-carnitine under salt stress conditions, since no information is available concerning the effect of salt stress on resting and growing cells metabolism when substrates and products are osmoprotectants. To gain insight into the kinetics of the whole bioprocess in the presence of NaCl, the activity of certain enzymes involved in the central metabolism, such as isocitrate dehydrogenase (Krebs cycle), isocitrate lyase (glyoxylate shunt) and pyruvate dehydrogenase, acetyl-CoA synthetase, ATP: acetate

phosphotransferase and the levels of acetyl-CoA and CoA (acetyl-CoA metabolism), were followed and the cellular energy was determined as cell ATP and reducing power levels ($NADH/NAD^+$ ratio), throughout the bioreactor runs in batch and a high density cell recycle membrane reactor. The latter was also studied after the perturbation of the NaCl bioreactor level as well as the perturbation of the crotonobetaine bioreactor level in the presence of a steady state NaCl concentration during the continuous biotransformation process. Furthermore, we also characterized the metabolism of the trimethylammonium compounds in *E. coli* by following the enzyme activities involved in the production of L(-)-carnitine and the cell population heterogeneity in different resting conditions, such as the presence or absence of NaCl within the reactor.

Figure 1. Central metabolism of *E. coli*. ACK: Acetate kinase, ACS: Acetyl-CoA synthetase, CS: Citrate synthetase; ETC: Electron transport chain, ICDH: Isocitrate dehydrogenase, ICL: Isocitrate lyase, LDH: Lactate dehydrogenase, PEP: Phosphoenolpyruvate, PEPCK: PEP carboxykinase, PEPCX: PEP carboxylase, PFL: Pyruvate formate lyase: PK: Pyruvate kinase and PTA: Phosphotransacetylase. (EcoCyc-MetaCyc-2002, USA, Keseler et al., 2004). Anaerobic metabolism of L(-)-carnitine in *E. coli*. CaiT: L(-)-carnitine/γ-butyrobetaine/crotonobetaine protein transporter, CaiA: CaiB crotonobetaine reduction reaction, CaiB: acetyl-CoA/CoA transferase, CaiC: crotonobetaine, L(-)-carnitine or γ-butyrobetaine CoA ligase, CaiD: enoyl-CoA hydratase or D(+)-carnitine racemase activity.


γBut_out
Crot_out
L-Car_out
CaiT
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CaiB
CoA
L-Car_in
L-CarCoA
CaiD
ATP
ADP+Pi
NADH
G3PDH
NAD+
Gly 3P
Glyk
Gly_ext
PEP
PK
PEPCX
Pyr_in
PFL
LDH
Lact_in
Lact_out
Pyr_out
Form_in
Form_out
AceAMP
ACS
AMP+PPi
Acet_in
Acet_out
Acetyl CoA
PTA
Ace-P
ACK
EtOH_in
EtOH_out
ADH
2x NADH
2x NAD+
CIT
ACO
ICIT
ICL
Glyox
MS
MDH
Malate
OAA
CS
PDH
FDH
Fum
SDH
Succ
KDH
aketo
ICDH
NADH
NADPH
FADH2
ETC
O2
H2O

MATERIALS AND METHODS

Bacterial strain and culture media

The strain *E. coli* O74K74 (DSM 8828) was used. This strain contains the complete *cai* and *fix* operons. The strain was stored in a minimal medium containing crotonobetaine 50 mM and 20 % glycerol at –20ºC. The minimal medium (MM) was that described by Obón et al. (1999), while the standard complex medium (CM) used contained (g/L): bacteriological peptone, 20; NaCl, 5; glycerol (carbon source), 12.6; crotonobetaine, 4; and fumarate, 2 (to inhibit crotonobetaine reductase, Kleber (1997)). The NaCl concentration was varied as stated in the text. The pH of media was adjusted to 7.5 with 1 M KOH prior to autoclaving.

Batch and continuous reactor operation

Growth of the bacteria.

Batch experiments were performed in reactors equipped with temperature, pH and oxygen probes. For continuous operation, pump controls of Biostat B (Braun Biotech International GMBH, Melsungen, Germany) were employed. A 1 L culture vessel with 0.5-0.8 L working volume was used. *Escherichia coli* O44K74 was grown under the different conditions stated in the text. The culture was inoculated with a 3% (v/v) of the liquid culture stored at -20ºC in 20% (v/v) glycerol, while the medium employed was the CM mentioned above unless stated otherwise. The cells were grown in batch at 37ºC, under anaerobic conditions, while D,L-carnitine mixture, D(+)-carnitine or crotonobetaine were supplied as inducers of the enzymes involved in the carnitine metabolism. Nitrogen was used to ensure anaerobiosis during the experiments.

Resting cells experiments.

The strain was grown either in a minimal (MM) or complex medium (CM) in anaerobiosis, as in Castellar et al. (1998). L(-)-carnitine metabolism (*caiTABCDE* operon) was always induced in anaerobiosis in the presence of crotonobetaine. For the resting cell biotransformations, cultures were harvested at the end of the exponential growth phase, centrifuged at 16,000 x g for 10 min and washed twice with 75 mM phosphate buffer, pH 7.5. The cell pellet was resuspended in 50 mM phosphate buffer, containing 50-500 mM crotonobetaine (depending on experiment) at pH 7.5 at 37ºC and left in the reactor system for 24-72 h without aeration. Therefore, microaerophilic conditions were maintained since non strict anaerobiosis

was kept. All experiments were performed at least in triplicate and under sterile conditions. The values reported are the means of the assays performed.

Continuous membrane reactor operation.

For continuous operation, the reactor vessel was coupled to a cross-flow filtration module (Minitan, Millipore, Billerica, MA, USA) equipped with four 0.1 μm hydrophilic polyvinylidene difluoride Durapore plates of 60 cm^2 area (Millipore, USA) (Cánovas et al., 2003a, 2003b). We used this type of bioreactor, since it is the one that is currently used for L(-)-carnitine production in the bioprocess we are optimizing. We believe that results present both academic and industrial interest. The cell broth was recycled into the reactor with a peristaltic pump adjusted to a high flow rate (70 mL/min) to minimise membrane fouling. *E. coli* cells for the inoculum were grown as explained previously and transferred to the fermenter. Continuous operation was set at 37 ºC and started by feeding with the CM medium. Anaerobiosis was maintained through the experimentation by bubbling nitrogen previously passed through a water trap. The reactor was run at a dilution rate of 0.3 h^{-1} until steady state was reached at a biomass dry weight of 6 g/L. For the pulse experiments, an additional injection pump was employed to supply the concentrated component to be perturbed. The perturbation was performed when the culture was around the steady state and the pulse supplied a small volume (20-25 mL) in less than 3 s (7 to 9 $ml.s^{-1}$). Samples of 2 mL for metabolites and 5 mL for enzyme activities were withdrawn from the high cell density reactor at different times after the pulse. In order to ensure that the reactor runs were strictly under anaerobic conditions, especially during the pulse additions, we decided to maintain strict anaerobiosis not only with respect to the carnitine metabolism (Kleber, 1997) which is expressed under anaerobiosis but also to avoid misleading results. Thus, the reactor was continuously purged with nitrogen, while the pulsed metabolite was similarly purged before the perturbation. A sampling valve with a minimal dead volume was adjusted to the reactor vessel. The sampling time was less than 5 s, and the complete procedure was computer controlled. The valve was flushed with water to clean the tubing. The reactor was left to recover and, after 20 to 30 reactor residence times (so as to ensure that a new steady state was reached), a new perturbation experiment was carried out. Samples were collected in test tubes kept at -20 ºC and immediately centrifuged at 16,000 x g at 4 ºC. The rotor was precooled at -20 ºC. Supernatant was used for external metabolites, whereas pellets were used for enzyme activity and ATP cell content and $NADH/NAD^+$ ratio measurements.

Enzyme assays

The enzyme activity assays were optimized for the conditions and media. Enzyme activity was defined as μmol of substrate consumed per minute and mg of protein (U/mg). In each case, reactor bulk liquid samples were withdrawn and centrifuged at 16,000 x g at 4 °C. The supernatant was removed and cells were resuspended with the corresponding extraction buffer. Cells were sonicated on 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 16,000 x 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., (1951).

Isocitrate dehydrogenase (ICDH)

The method was that of Bennet and Holms (1975). The extraction buffer was 64.5 mM potassium phosphate, pH 7.5, and 6.45 mM $MgCl_2$. The increase in NADPH absorbance at 340 nm (ε_{NADPH}=6,220 $M^{-1}cm^{-1}$) was followed in a GBC 918 U/V (Melbourne, Australia) spectrophotometer at 37 °C. One unit of enzyme activity was that required for the generation of 1μmol of NADPH per min.

Isocitrate lyase (ICL)

The assay was that described by Dixon and Konberg (1959), using the same extraction buffer as above. The increase in absorbance at 324 nm due to the reaction of the glyoxylate produced with phenylhydracine ($\varepsilon_{comp\ ex}$=17,000 $M^{-1}cm^{-1}$), was followed in a spectrophotometer GBC 918 U/V (Melbourne, Australia) at 37 °C. One unit of enzyme activity was taken as that needed to generate 1μmol of adduct per min.

Acetyl-CoA synthetase (ACS)

The method used was that established by Brown et al. (1977). The extraction buffer was 64.5 mM potassium phosphate, pH 7.5 with 1 mM β-mercaptoethanol. The acetyl-CoA synthetase activity was followed as the increment in NADH absorbance at 340 nm (ε_{NADPH}=6,220 $M^{-1}cm^{-1}$) and 45 °C. Enzyme activity unit was defined as the enzyme required for the generation of 1μmol of NADH per min.

Phosphotransacetylase (PTA)

The assay was carried out as in Lundie and Ferry (1989). The extraction buffer was 50 mM HEPES, pH 7.5 with 1 mM β-mercaptoethanol. The enzyme activity was followed as the increment in NADH absorbance at 340 nm

(ε_{NADPH}=6,220 $M^{-1}cm^{-1}$) and 37 °C, one unit being taken as the enzyme required for the generation of 1μmol of NADH per min.

Pyruvate dehydrogenase complex (PDH)

The method was that of De Graef et al., (1999). The extraction buffer was 50 mM potassium phosphate, pH 7.5, EDTA 0.1 mM, β-mercaptoethanol 5 mM and $MgCl_2$ 3 mM. The enzyme activity was followed as the decrease in potassium ferricyanide absorbance at 430 nm ($\varepsilon_{ferricyanide}$=1,030 $M^{-1}cm^{-1}$) and 37 °C. One enzyme activity unit was taken to be the enzyme required to generate 2 μmol of ferricyanide per min.

Enzymes of L(-)-carnitine metabolism (CHR and CRR)

The crotonobetaine hydration reaction (CHR) assay was carried out according to Jung et al., (1989), and the crotonobetaine reduction reaction (CRR) assay according to Preusser et al., (1999). Crotonobetaine was employed as substrate in both cases.

Substrate consumption for growth and biotransformation processes

L(-)-carnitine concentration was determined enzymatically with the carnitine acetyl transferase method (Jung et al., 1989). Glycerol, crotonobetaine and γ-butyrobetaine were analysed by HPLC with a Tracer Spherisorb-NH_2 column, 3μm, 25 x 0.46 cm, supplied by Teknokroma (Barcelona, Spain) as previously reported (Obón et al., 1999). The isocratic mobile phase was acetonitrile/50 mM phosphate buffer pH 5.5 (65/35) at a flow rate of 1 mL/min. Bacterial growth was followed spectrophotometrically at 600 nm, using a Novaspec II from Pharmacia-LKB, (Uppsala, Sweden), and converted to dry weight accordingly.

***E. coli* anaerobic metabolite production.**

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

Determination of ATP content and NADH/NAD^+ ratio

The energy content per unit of cell was determined as the ATP level and NADH/NAD^+ ratio throughout the experiments. For ATP measurement, the HS II bioluminescence assay kit from Boëhringer (Mannhein, Germany), based on the

luciferase enzyme using a FluoStar fluorimeter from BMG LABTECH (Offenburg, Germany) in the conditions stated and after cell DMSO lysis, was used.

Reducing power, considered as the NADH/NAD^+ ratio, was calculated as in Snoep et al., (1990). The measurements were made using an enzymatic method based on the alcohol dehydrogenase enzyme. The extraction of the reduced or the oxidized forms was carried out by two different methods, involving alkali or acid extraction. The cell content was determined after biomass optical density transformation as dry weight and assuming either an intra-cellular volume of 1.63 µL/mg (Emmerling et al., 2000) or 1.72 $mL \cdot 10^{-13}$/cell (worked out by flow cytometry in this work).

Flow-cytometric determination of cell viability

To determine cell viability by FCM, double staining was performed, using 5 $\mu g \cdot ml^{-1}$ propidium iodide (PI) (from a 1 mg·ml-1 stock solution) and 10 $\mu g \cdot ml^{-1}$ bis-oxonol (BOX) (from a 2 $mg \cdot ml^{-1}$ stock solution in dimethylsulfoxide). Staining was performed in the dark at room temperature for 10 min. Samples were run in an Analyzer cytometer mod. Epics XL from Beckman Coulter (Fullerton, CA, USA), equipped with an argon laser of 15 mW for the excitation of the fluorophores at a wavelengh of 488 nm. Fluorescence detection was carried out at 525 nm for BOX and 675 nm for IP, and spectral overlap was corrected by using a numerical compensation. Positive (heat-treated *E. coli* cells) and negative (either non-stained or stained exponentially growing *E. coli* cells) controls were undertaken in order to avoid false positives. The BOX and PI fluorescence was compensated according to Hewitt et al., (1999, 2000), using heat stressed cells treated at 60 ^{0}C for 5 min as positive stained control. The green fluorescence channel (FL1) for BOX stained cells was plotted versus the red fluorescence channel (FL3) for PI/BOX stained cells. Results are presented as the percentage of stained population.

Transport assays

The uptake of carnitine in *E. coli* O44K74 was measured using L[*N-methyl* ^{14}C]carnitine (56 mCi/mmol) as substrate. Cell samples were re-suspended in the L(-)-carnitine uptake assay at an A^{600} of approximately 0.5, containing 10-20 mM L[*N-methyl* ^{14}C]carnitine (5.6 mCi/mmol) in a total reaction volume of 3 mL. Samples (0.3 mL) were taken at various times and filtered through 0.45 µm pore size filters of 25 mM diameter. The filters were then washed with 20 mL of isotonic minimal salts and the radioactivity retained was determined with 2.5 mL of scintillation liquid in a

Liquid Scintillation Counter mod. Wallac 1409 (Perkin Elmer, Wellesley, MA, USA). All the experiments and assays were performed in triplicate at least.

Central and carnitine metabolisms in *E. coli*

Since carnitine secondary metabolism in *E. coli* is repressed by glucose, and in the presence of high levels of oxygen (Kleber 1997; Cánovas et al., 2002; Cánovas et al., 2003a), we chose glycerol as the carbon source and maintained anaerobic or microaerophilic conditions for this study. Glycerol enters in the glycolysis as glyceraldehyde-3-phosphate, before conversion to pyruvate. This is converted into acetyl-CoA, which enters the Krebs cycle (TCA), producing reducing power, which will be transformed into ATP within the electron transport chain during aerobiosis, and metabolic intermediates through anaplerotic reactions. The glyoxylate shunt is associated to the TCA, short-cutting the metabolic flow and forming oxaloacetate from acetyl-CoA (Fig. 1, EcoCyc-MetaCyc, Keseler et al., 2004). However, when *E. coli* grows under anaerobiosis or under a limiting oxygen supply, the reducing power comes from the mixed-acid fermentation for ATP formation, reducing pyruvate to acid metabolites such as lactate, succinate and formate and acetyl-CoA, which will be further reduced to acetate and ethanol (Varma et al., 1993). These reducing paths also allow the production of ATP, although at lower levels than during aerobiosis. In the presence of alternative electron acceptors, such as fumarate, anaerobic respiration allows more effective cell redox cofactor regeneration and provides a higher energetic yield, which is finally manifested as higher biomass levels. Moreover, when carnitine metabolism is expressed, certain intermediates, such as ATP and the pool of acetyl-CoA/CoA are addressed to this (generally termed) secondary metabolism (Cánovas et al., 2003a), linking it with the central metabolism. The physiological role of L(-)-carnitine and related trimethylammonium compounds in *E. coli* is involved in the protection against various stress conditions, since protectant behaviour has been observed upon uptake and cytosolic accumulation (Kleber, 1997). Further, trimethylammonium compounds can be metabolized, in which case crotonobetaine acts as an alternative electron acceptor (Fig.1). The carnitine metabolization pathway depends on the expression of the *caiTABCDE* operon which is induced when cells are grown in the presence of some compounds, such as D,L-carnitine mixtures or crotonobetaine, not only in anaerobiosis but, in some species, such as *E. coli* ATCC 25922 and DSM 8828, *P. vulgaris* and *P. mirabilis*, also when grown under aerobiosis (Kleber, 1997; Obón et al., 1999; Elssner et al., 2000; Cánovas et

al., 2002). L(-)-carnitine metabolism is repressed in growing cells subjected to osmotic stress (Jung et al., 1990), since it does not provide any advantage for the cell and its uptake depends on the activation of genes other than the ***cai*** operon. Nowadays, it is known that CaiT is an exchanger (antiporter) for L(-)-carnitine and γ-butyrobetaine in ***E. coli*** (Jung et al., 2002), which also transports crotonobetaine (Cánovas et al., 2002, 2003a). The enoyl-CoA hydratase (CaiD) requires a CoA-transferase activity (CaiB), CaiB and CaiD both being dimers (Fig.1), while the crotonobetaine reductase activity requires two proteins: CaiB (one dimer) and CaiA (one tetramer). It has been verified that the hydration reaction of crotonobetaine into L(-)-carnitine (CHR) proceeds at the CoA-level in two steps: the protein CaiD-catalyzed hydration of crotonobetainyl-CoA (Crot-CoA) to L-carnitinyl-CoA (L-Car-CoA), followed by CoA-transfer from L-carnitinyl-CoA to crotonobetaine, catalyzed by CaiB (Elssner et al., 2001; Cánovas et al., 2003a). Thus, CaiD and CaiB from ***E. coli*** have been found to jointly catalyze the reversible biotransformation of crotonobetaine to L(-)-carnitine. Further, it has been suggested that caiC codifies a CoA:trimethylammonium ligase (Eichler et al., 1994a), which activates crotonobetaine/γ-butyrobetaine/L(-)-carnitine when they reach the cell. The function of protein CaiE is not totally understood and further studies must be undertaken. Cánovas et al., (2003a) have proposed a model to describe the whole activity of ***E. coli*** able in producing L(-)-carnitine from crotonobetaine under both anaerobic and aerobic conditions in bioreactors (Fig. 1). In resting cell processes, no carbon source is fed to the reactor and so the cell-stored material is the only carbon source for the synthesis of the enzymes involved in cell turnover/maintenance and the biotransformation of crotonobetaine (Castellar et al., 1998; Obón et al, 1999).

RESULTS

Growth of *E. coli* in the presence and absence of NaCl and using crotonobetaine as osmoprotectant

Cells were grown on the MM and CM media in anaerobiosis either in the presence or absence of 0.5 M NaCl and in the presence or the absence of crotonobetaine as an osmoprotectant. As can be observed for both media, the final biomass concentration was lower in the presence of salt (Fig. 2), an effect that was overcome by the addition of crotonobetaine which acted as an osmoprotectant. The best conditions for *E. coli* growth were observed when only crotonobetaine was added to the system.

Biotransformation with resting cells

After growth in anaerobic conditions and to allow maximum induction of the carnitine metabolism in the presence and absence (control) of NaCl, cells were resuspended in the biotransformation medium, composed of crotonobetaine in phosphate buffer. Cell incubation was performed in microaerophilic conditions, since the reactor was kept as an open system with agitation, while neither air nor nitrogen was supplied to the reactor. Biotransformation assays were performed in the presence and absence (control) of NaCl (see Materials and Methods). As shown in Figure 3A, the addition of NaCl to the culture medium used for cell growth prior to the collection of cells for the biotransformation process, reduced the biotransformation yield with respect to those grown in its absence. In fact, salt adaptation studies showed that the growth of cells under salt stress provoked a decrease in the biotransformation capacity of resting cell processes carried out both with and without added NaCl. Nevertheless, cells subjected only to salt shock during the biotransformation process increased L(-)-carnitine production with respect to unshocked cells (Fig. 3A). Control resting cells achieved maximum L(-)-carnitine yields in the range of 20-40%, depending on the substrate employed and its concentration (Castellar et al., 1998). In this work, when NaCl was increased to 0.5 M, L(-)-carnitine production from crotonobetaine with resting cells reached a yield of 65-70%, while high salt concentrations had a deleterious effect. In Table 1, biotransformation variables and specific growth rate at different concentrations of NaCl are depicted.

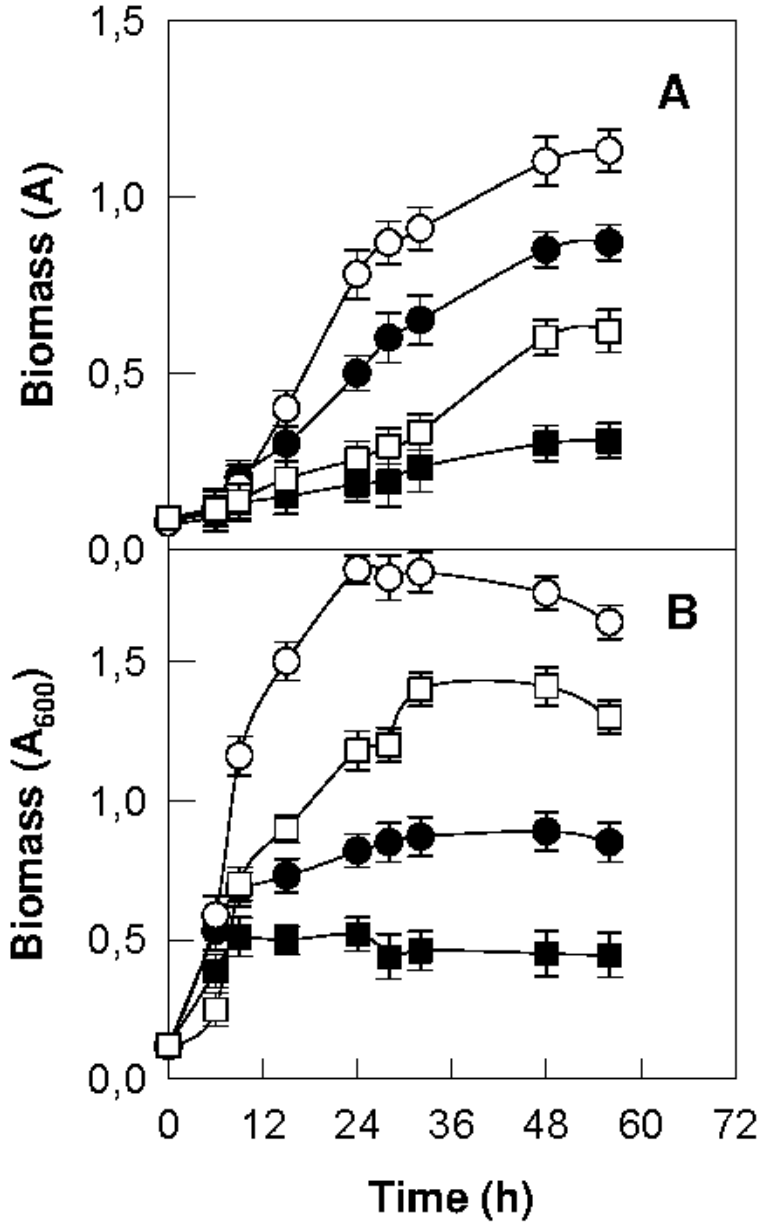


Figure 2. Effect of salt stress on the *E. coli* growth with (■,□) and without (●,○) NaCl (0.5 M), and in the presence (○,□) and absence (●,■) of crotonobetaine (50 mM), respectively in **A**) MM and **B**) CM media.

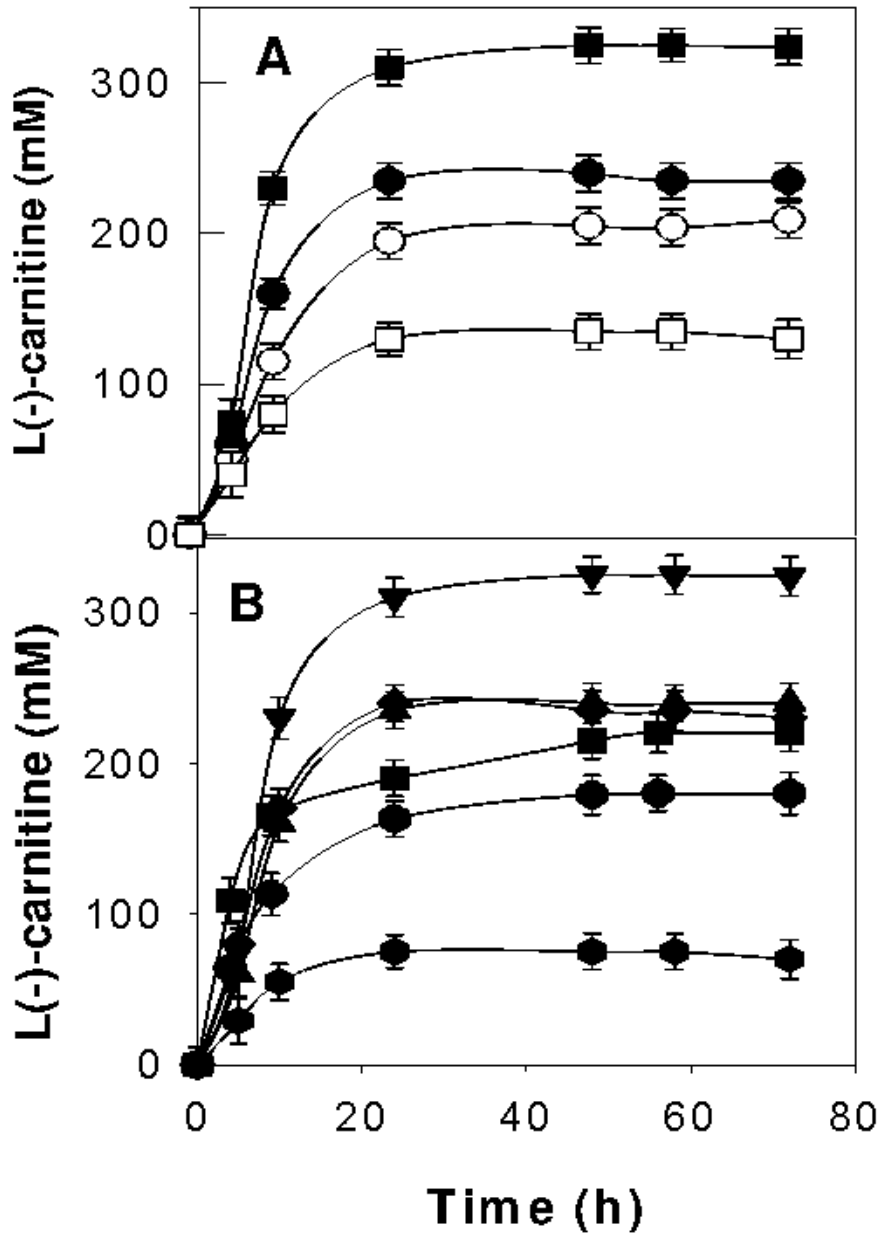


Figure 3. A) Effect of salt stress on the biotransformation of crotonobetaine (500 mM) into L(-)-carnitine with cells grown with (○,□) and without (●,■) NaCl (0.5 M), and during the resting process in the presence (■,□) and absence (●,○) of NaCl (0.5 M), respectively. **B)** *E. coli* L(-)-carnitine production under different NaCl concentrations, (●) control, ■) 0.25, ▲) 0.35, ▼) 0.5, ◆) 0.85 and ●) 1.85 M. Assays were carried out with 4.3 $g \cdot l^{-1}$ biomass at pH 7.5 and 37ºC.

Table. 1. Biotransformation variables and specific growth rates obtained at different concentrations of NaCl. Values are the mean of three measurements.

	NaCl (M)					
	Control	0.25	0.35	0.5	0.85	1.5
Conversion (%)	40±1.2	45±1.5	55±2.1	68.5±1.3	52.4±1.5	22.4±1.9
Productivity (mM/h)	8.4±0.5	10±1.2	12±0.8	14±1.1	12.3±2.1	5±0.7
Specific growth rate (h^{-1})x10	6.2±0.3	4.2±0.2	2.8±0.2	2.3±0.3	1.5±0.1	0.8±0.5

Central metabolism during resting cell processes

To unravel the metabolic consequences of the presence of NaCl in resting biotransformation media, the profile of metabolites and enzymes of *E. coli* O44K74 were recorded both in the presence and in the absence of 0.5 M NaCl. Metabolism in resting cells is only devoted to cell maintenance and the parallel biotransformation (Fig. 6), since no carbon source is fed to the culture. From 5 to 24 h, the control ICL activity doubled, reaching 12.9 mU/mg protein (Fig. 4A). Considering the Krebs cycle, after a slight increase of the control ICDH to 90.3 mU/mg protein during the first 5 h, decreased to 19.8 mU/mg protein at 35-48 h (Fig. 4C). The central metabolism responded to the presence of salt in a different manner to that observed before. The ICL activity increased at higher levels with respect to the control (25.6 mU. mg $prot^{-1}$), and kept increasing gradually up to the end of the biotransformation, contrary to the control (Fig. 4A). Furthermore, the energetic status of the control cells showed that the ATP concentration decreased from 0.7 to 0.31 mM at 24 h, decreasing slowly thereafter (Fig. 4B). In the presence of NaCl the concentration of ATP also decreased from the levels shown by the control, though the fall was steeper. The NADH/NAD^+ ratio of NaCl-treated cells was three-fold that of the control at the end of the experiment (Fig. 4B). Moreover, the PTA and ACS control activities decreased throughout the biotransformation, PTA not being detected after 5 h, while ACS was five-fold lower after 24 h and not detected at 72 h (Fig. 5A). In the presence of NaCl, the ACS and PTA activities decreased during the first hours of the biotransformation (Fig. 5A and 5B), although the latter (ACS) with slightly lower values than in the control experiment. These results indicated that, in resting cells, carbon flux through the Krebs cycle was more relevant and that no acetate

was produced. With respect to the PDH complex, it doubled its levels in the control during the first 24 h, reaching 19.60 mU/mg protein, and then fell to 15.6 mU/mg protein during the last 48 h (Fig. 4A), while in the presence of NaCl the increase in activity was slightly lower.

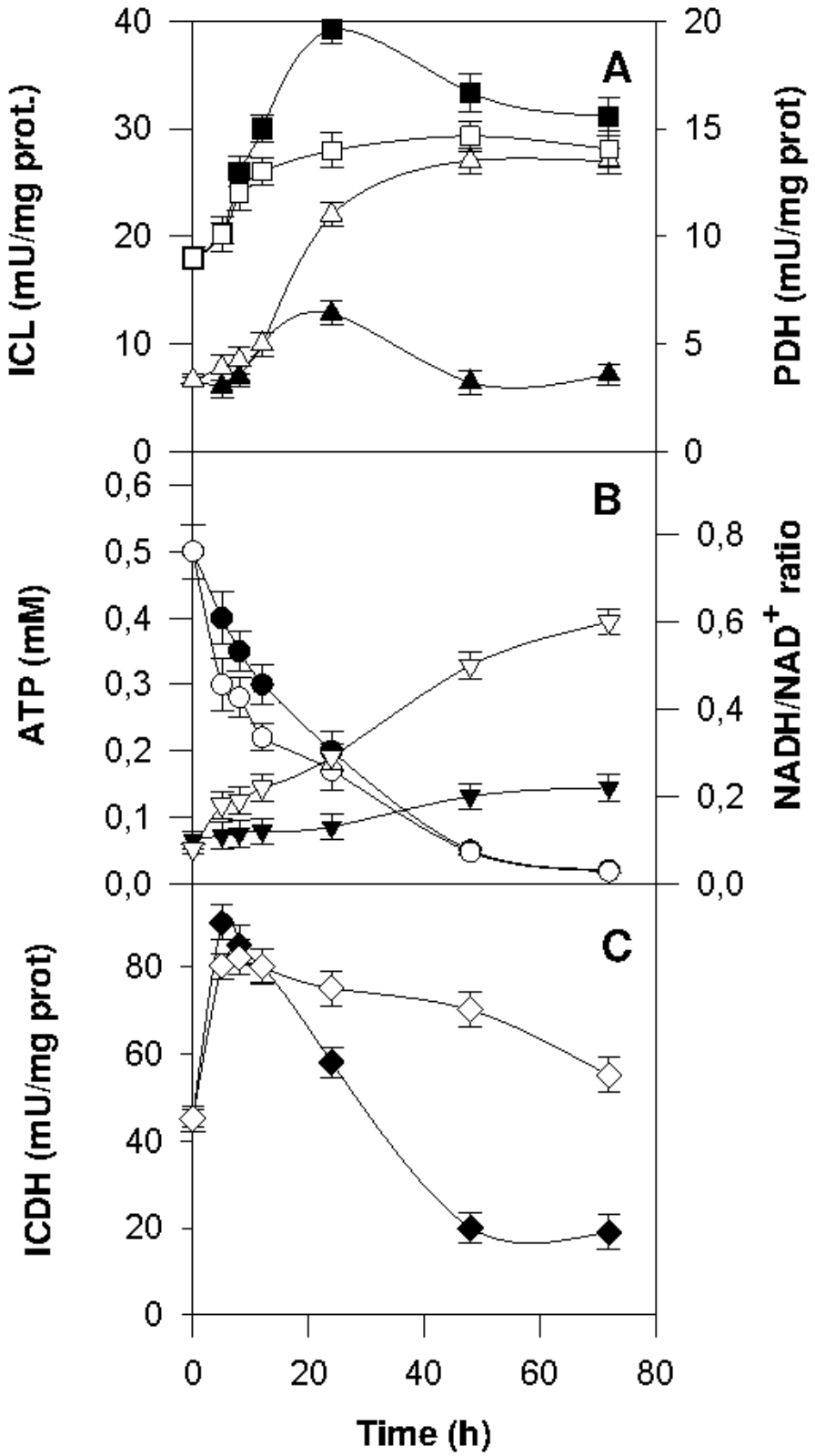
Secondary metabolism during the biotransformation with resting cells

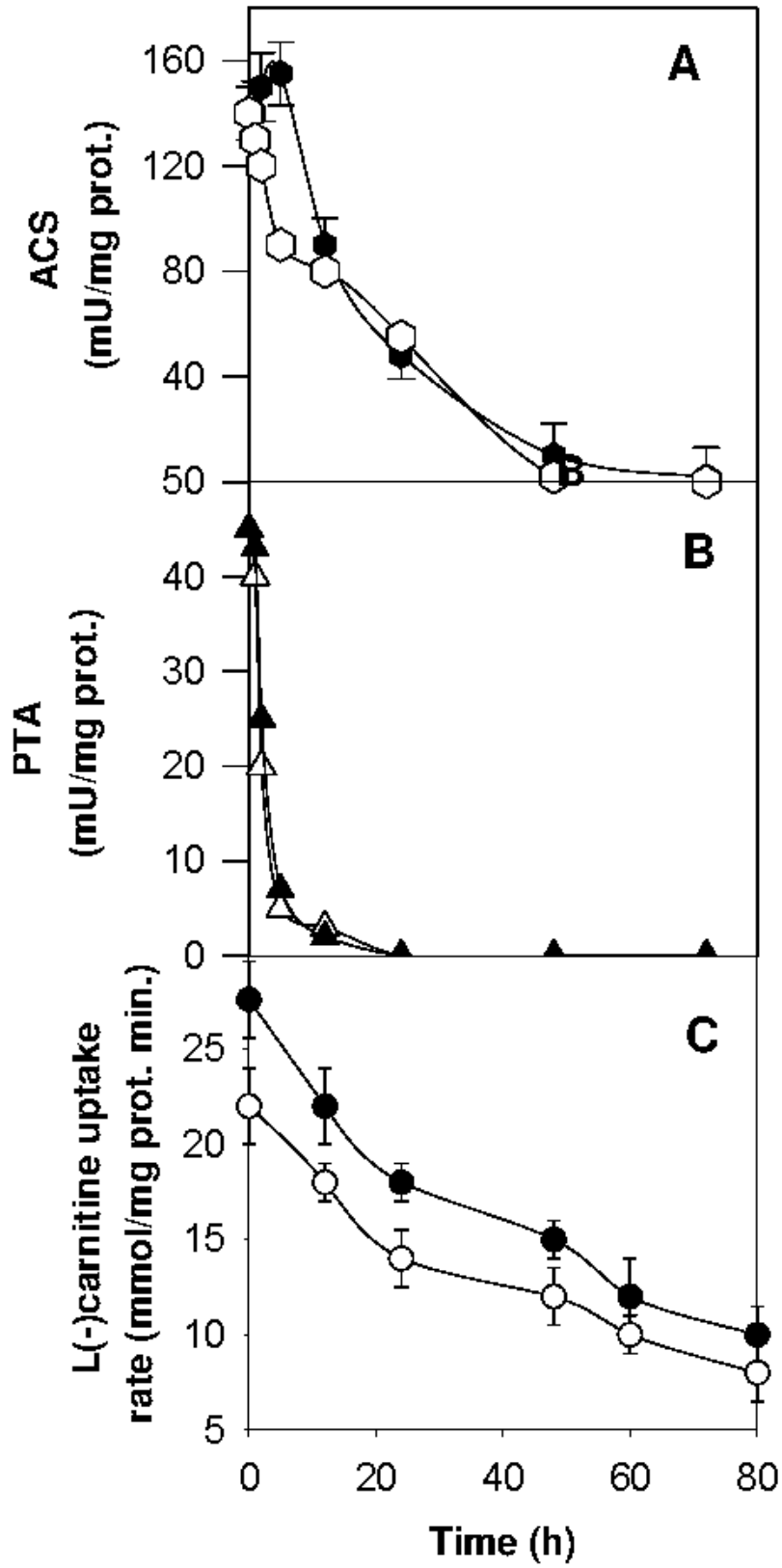
The presence of 0.5 M NaCl increased the L(-)-carnitine yield from crotonobetaine biotransformation (Fig. 6). For transport assays, the decrease in N(*methyl-* ^{14}C)-carnitine uptake is shown in Figure 5C for resting cells both in the presence and absence of NaCl at different points of the process. The presence of NaCl slightly decreased the rate of transport. Moreover, the uptake decreased as the biotransformation evolved. Most importantly, the transport rate decreased with time, paralleling the previously described decrease of ATP for both control and NaCl-treated cells (Fig. 4B).

The biotransformation studies in these conditions showed that the CRR activity (CaiA:CaiB) decreased from 18.0 to 3.0 mU/mg protein, while the CHR activity (CaiD:CaiB) decreased sharply during the first 5-10 h from 48.4 to 9.0 mU/mg protein, remaining close to 10.0 mU/mg protein during the rest of the process (Fig. 6A), for both control and in the presence of NaCl. As regards L(-)-carnitine production (Fig. 6A), the yield reached high levels after the first 24 h, paralleling the consumption of crotonobetaine (Fig. 6B). The yield (65-70 %), was higher than that obtained in the absence of NaCl (40%) (Fig. 6A).

Figure 4. (*Pag. 187*) *E. coli* O44K74 metabolism evolution on a resting biotransformation medium in batch systems. **A)** ICL (▲) and PDH (■) activities in mU/mg prot, **B)** ATP concentration (●) and NADH/ NAD^+ ratio (▼), and **C)** ICDH activity (◆), control (black) and NaCl (open) treated cells, in mU/mg prot.

Figure 5. (*Pag. 188*) Evolution of *E. coli* O44K74 metabolism on a resting biotransformation medium in aerobic conditions and batch systems. **A)** ACS activity (●), **B)** PTA activity (▲), both in mU/mg prot., and **C)** *N(methyl-*^{14}C*)*-carnitine specific uptake (●) during the biotransformation of crotonobetaine into L(-)-carnitine, at different experimental times. Control (black) and NaCl (open) treated cells.


A
B
C
ICL (mU/mg prot.)
PDH (mU/mg prot)
ATP (mM)
NADH/NAD+ ratio
ICDH (mU/mg prot)
Time (h)
0
10
20
30
40
5
15
0,1
0,2
0,3
0,4
0,5
0,6
0,8
60
80


A
B
C
ACS
(mU/mg prot.)
PTA
(mU/mg prot.)
L(-)carnitine uptake
rate (mmol/mg prot. min.)
Time (h)
160
120
80
40
50
40
30
20
10
0
25
20
15
10
5
0
20
40
60
80

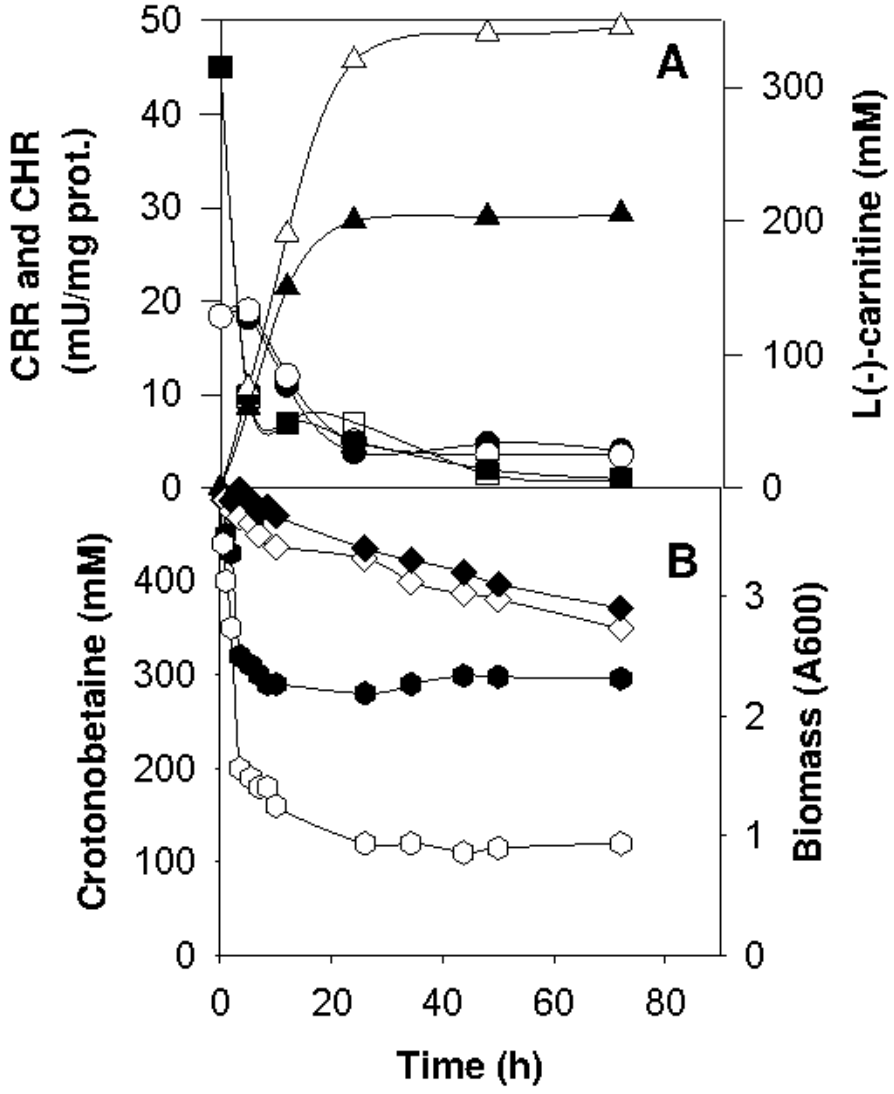


Figure 6. Specific activities of the following enzymes, **A)** (■) CRR (crotonobetaine reduction reaction) and (●)CHR (crotonobetaine hydration reaction) in mU/mg prot and (▲) L(-)-carnitine production, and **B)** (●) crotonobetaine and (◆) biomass concentrations in batch reactors with *E. coli* O44K74 resting cells in the biotransformation process, control (black) and NaCl (open) treated cells.

Resting cells evolution during the biotransformation in the presence of NaCl

Cell heterogeneity during the biotransformation with the resting control and NaCl treated cells was recorded using a flow cytometric method (Hewitt et al., 1999). At zero time, 100% cell viability and only one cell population were assessed by simultaneous staining (Table 2), while three sub-populations appeared after 24 h.

In the control, 20% of the cell population were depolarised (BOX-stained cells), while the percentage of permeabilised and dead cells (simultaneously PI/BOX-stained) was 5.9%. Moreover, after 48 h, 40.8% of the population was depolarised, while dead cells represented less than 1%. This low percentage was probably due to cell disappearance (cell lysis) and to the occurrence of phantom cells. After 72 h, depolarised cells numbered almost 70%, while dead cells remained in the range of 1-2%. However, in the presence of NaCl (0.5 M), 26.6% of the cells had lost their membrane potential and were BOX-stained after 24 h, while 32.0% were dead cells. After 48 h, 50.1% of the cell population was stained with BOX and the dead cells represented 2.3% of the cell population. Thus, most of the population would be composed of phantom cells only stained with BOX (Nebe-Von Caron et al, 1998). At the end of the process 80.4% of the population was BOX-stained and 1.5% of the population were dead cells. The percentage of BOX-stained cells during biotransformation was always higher for NaCl-treated cells than for the control, despite the higher L(-)-carnitine yield obtained (Fig. 3B).

Table 2: Cell viability evolution in presence of NaCl during the biotransformation of crotonobetaine into L(-)-carnitine using resting cells of ***E. coli*** O44K74 harvested from an anaerobic CM. The green fluorescence channel (FL1, BOX stained cells) was plotted versus the red fluorescence channel (FL3, PI/BOX stained cells). Results are presented as percentage of stained population. Samples were taken from the reactor at 0, 24, 48, and 72 h. The study was performed using BOX and PI, as explained in Materials and Methods.

	Percentage of stained cells (%)			
	0 h	**24 h**	**48 h**	**72 h**
Control				
Intact cells	100	74.1	58.3	30.7
BOX		20.0	40.8	68.0
PI/BOX		5.8	0.88	1.3
NaCl treated				
Intact cells	100	42.0	47.7	18.0
BOX		26.6	50.0	80.4
PI/BOX		32.0	2.3	1.5

Metabolic state of growing cells in a high cell density membrane reactor after NaCl and crotonobetaine pulses

Since the response of *E. coli* cells to salt stress was fundamentally distinct in the growing and resting production systems, further experiments were designed to gain further insight into the salt stress response. To discriminate the resting and growing cell responses, pulses were performed around the steady state within a high cell density membrane continuous reactor.

Osmotic upshift pulse

The osmotic upshift pulse consisted of a sudden rise (or pulse) of 0.3 M NaCl (below 0.5 M, to avoid deleterious effects of cell lysis in the biotransformation, Fig. 3B). The cell metabolic state was followed after the pulse, recording the L(-)-carnitine production pathways and the central and energetic routes. To allow the higher expression of the latter metabolism the process was carried out under strict anaerobiosis (Cánovas et al., 2003a).

Fast responses were recorded in the intracellular cofactor pools within the first few minutes following the osmotic upshock. Figure 7A depicts the decreasing trend of the NADH/NAD^+ ratio, while a steep increase in the ATP levels was evident (Fig. 7C). The final products of the central fermentative pathways also modified their levels, with a decrease of the lactate and ethanol levels (from 60 mM to 37 mM, 20 min after the pulse) as the cell addressed its metabolism towards the acetate synthesis (Fig. 7B). This would respond to the occasional increase in the cell needs to obtain ATP and to regenerate NAD^+ (Fig 1). ATP levels started to decline, and initial levels were recovered.

Figure 7A also shows that at the beginning of the osmotic shock there was a decrease in the enzymatic activity of ICDH and ICL, both enzymes probably being inhibited. Beside this, the NADH/NAD^+ ratio decreased since the cell addressed the metabolic fluxes through fermentative pathways (e.g. the acetate producing pathway) rather than through anaerobic-respiratory and anaplerotic routes (Fig.1 and 7A). Coinciding with the trends observed for the fermentation products, the enzymes of the acetate anaerobic metabolism were also regulated, since the level of PTA activity increased while that of ACS showed the opposite trend (Fig. 7B).

Figure 7C shows that the CHR activity increased until approximately 100 minutes after the NaCl pulse, reaching four-fold the original level, and then, fell to recover its steady state level. The L(-)-carnitine level in the bulk reactor also

increased, by almost 40% of the steady state level. This increase paralleled the decrease in ATP levels (Fig. 7C).

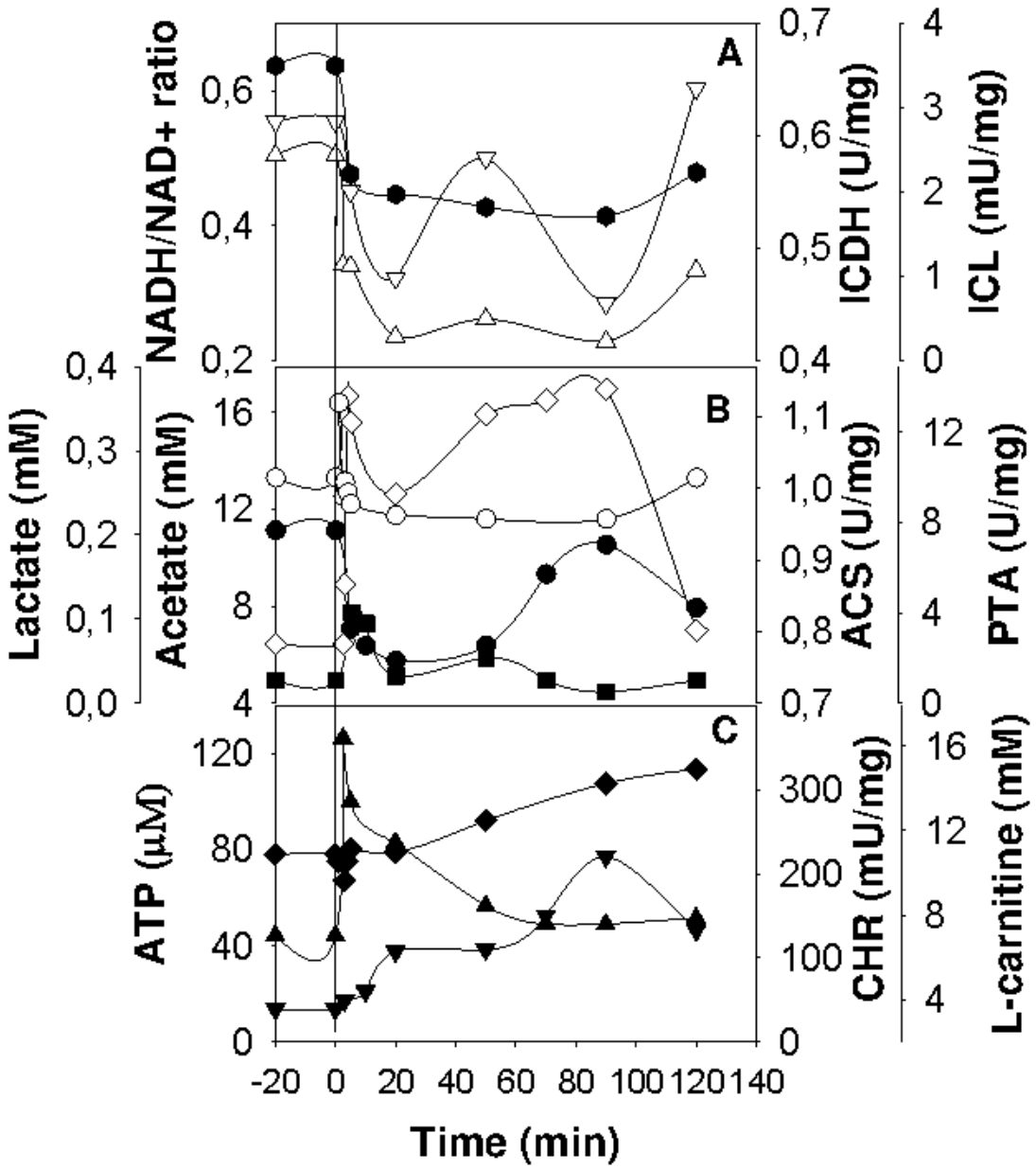


Figure 7. Evolution of *E. coli* O44K74 metabolism growing on a complex medium containing 75 mM of glycerol in a high density cell recycle membrane reactor system under anaerobic conditions and after a pulse of NaCl five-fold that of the basal steady state at t=0. **A)** (△) NADH/NAD$^+$ ratio, (▽) ICDH activity, (●) ICL (isocitrate lyase), **B)** (◇) acetate, (○) lactate, (■) PTA activity, (●) ACS activity and **C)** (▲) ATP, (▼) CHR activity and (◆) L(-)-carnitine.

Substrate pulse in steady-state salt stressed cells

Since the presence of NaCl within the reactor increased the L(-)-carnitine production by approximately 75% when using resting cells (Fig. 3B), it was also decided to determine the effect of a biotransformation substrate (crotonobetaine) (Fig. 1) pulse in the presence of a steady state osmotic stress. To avoid deleterious effects on L(-)-carnitine metabolism expression, 0.3 M NaCl was chosen (five-fold higher than that currently used). The pulse consisted of a sudden five-fold rise in the crotonobetaine concentration (0.3 M) in a high cell density membrane continuous reactor around the steady state.

Again, the faster responses were observed in the coenzyme pools. During the first minutes following the crotonobetaine pulse, the cell was devoted to lactate and ethanol synthesis (around 0.1 and 90 mM, respectively), thus being able to re-generate NAD^+. However, the NADH/NAD^+ ratio increased since a higher carbon flux was devoted to acetate/ATP synthesis, the steady state basal level being reached after the perturbation (Fig. 8A and B). Since the pulse consisted of a sudden rise in the crotonobetaine (Crot) concentration, cellular ATP diminished, having been consumed during transport and even during activation into crotonobetainyl-CoA (Crot-CoA) by CaiC activity (Fig. 1 and 8C) as well as in other metabolic processes. With respect to the Krebs cycle and the glyoxylate shunt, Figure 8A depicts an increase in ICDH enzyme activity, while the ICL activity fell sharply, even though NADH levels rose during the first minutes. Actually, if the initial activities (Fig. 8A) for both enzymes are compared with those in the previous pulse not involving a steady state osmotic stress (Fig. 7A), it is observed that the levels in the previous pulse were lower, especially in the case of ICL. Further, Figure 8B shows that there was a decrease in acetate levels during the first few minutes following the pulse, although they increased afterwards, with a parallel slight increase of the PTA activity, generating also ATP and a decrease in ACS activity. Therefore, the ICDH/ICL and PTA/ACS ratios recorded must be a consequence of the osmotic stress.

After the crotonobetaine pulse, L(-)-carnitine production increased and energy consumption was directed to the transport and activation of crotonobetaine (Fig.8C) as well as for other processes. The altered ATP levels recovered after approximately the first 20 min. (Fig.8C). At the same time, as shown in Figure 8C and coinciding with the higher availability of substrate, the CHR activity increased, either as a consequence of higher induction or as a consequence of the higher

availability of the cofactors necessary for its activity (Crot-CoA, L-Car-CoA). The consequence of this increase in activity was the higher L(-)-carnitine production.

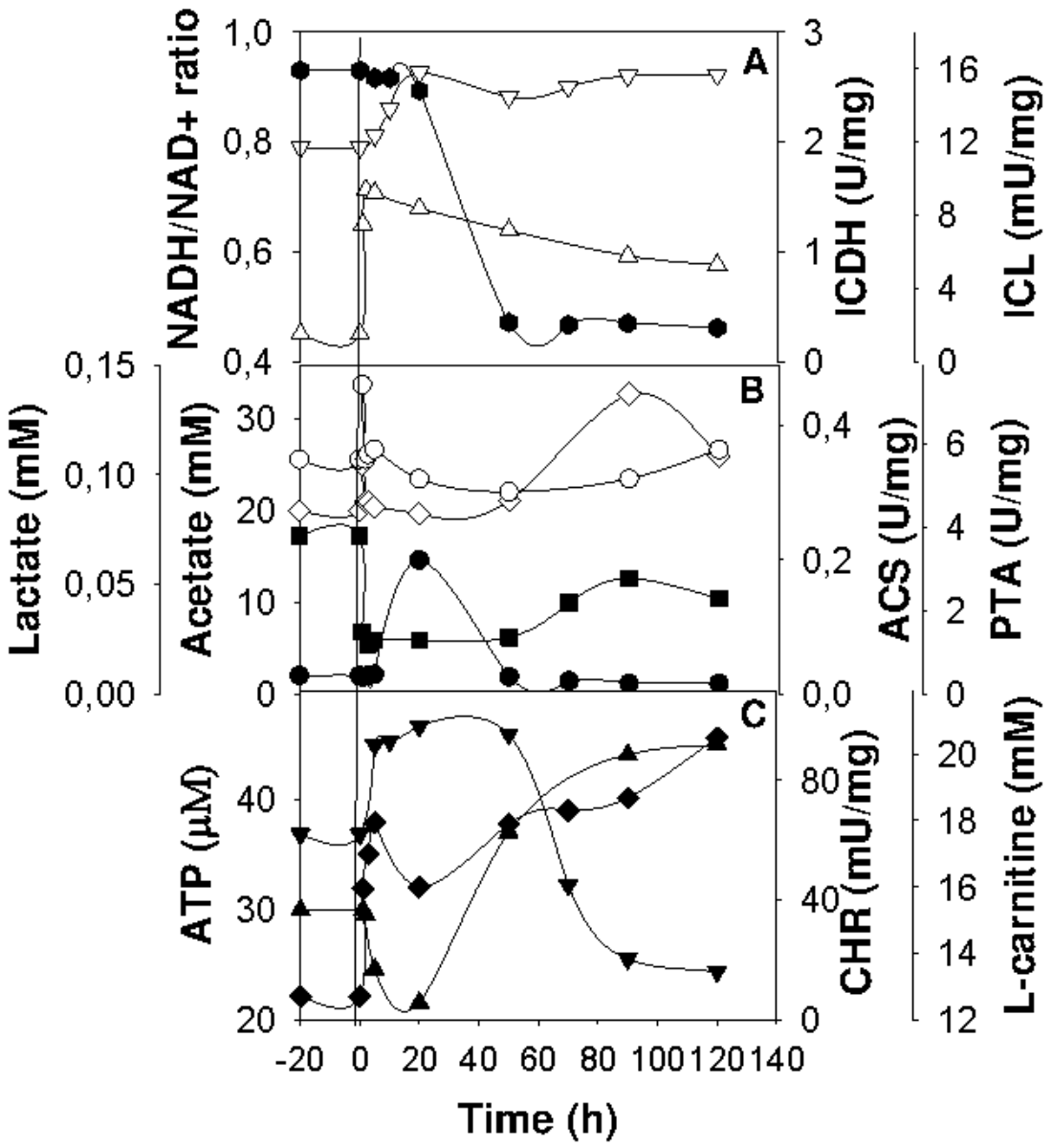


Figure 8. Evolution of *E. coli* O44K74 metabolism growing on a complex medium containing 75 mM of glycerol in a high density cell recycle membrane reactor system under anaerobic conditions and after a pulse of crotonobetaine five-fold that of the basal steady state at t=0 in the presence of NaCl. **A)** (△) NADH/NAD$^+$ ratio, (▽) ICDH activity, (●) ICL activity, **B)** (◇) acetate, (○) lactate, (■) PTA activity, (●) ACS activity and **C)** (▲) ATP, (▼) CHR activity and (◆) L(-)-carnitine.

DISCUSSION

It has been demonstrated that L(-)-carnitine and crotonobetaine are involved in the osmoprotection of *E. coli* both in aerobic and anaerobic growth conditions (Jung et al., 1990; Verheul et al., 1998). Besides, anaerobic L(-)-carnitine uptake by the carrier CaiT is not related to osmoprotection (Verheul et al., 1998) and thus its metabolization by *cai* operon-coded protein products must be considered mainly as an alternative anaerobic respiration pathway, which would be physiologically important in intestinal populations of enterobacteria. Consistent with this double role as osmoprotectant and electron acceptor, crotonobetaine always increased the final biomass growth of *E. coli* in the presence of NaCl with respect to control experiments in which crotonobetaine was not added. Further, when no salt was added to the reactor, crotonobetaine itself increased the final biomass growth (Fig. 2), which can be explained by its energetic role in anaerobic respiration.

On the other hand, the growth of cells under salt stress provoked a decrease in the biotransformation capacity of resting cell processes carried out both with and without NaCl addition (Fig.3A). This might mean that growth on NaCl affects the expression of the carnitine metabolism, carnitine transporter activity (CaiT) or even whole cell membrane activity, finally leading to a loss in viability (Table 2). However, cells which had been grown without NaCl, showed an increased L(-)-carnitine yield (Fig. 3A) if subjected to salt shock only during the biotransformation process in resting with respect to unshocked cells. Therefore, the difference between growth with and without salt stress and resting in the presence of salt stress must have been due to the effect on the cell metabolism and/or physiological state.

No simple answer can be proposed to explain this empiric observation. The facts seem to point to the activation of betaine transport by long term exposure to salt or some beneficial effect on mass-transfer from the medium, through modification of the structure of the *E. coli* cell outer membrane (OM), cell wall or plasmatic membrane, which would facilitate the interchange of extracellular compounds with the cytoplasmic compartment. In fact, the biotransformation of crotonobetaine into L(-)-carnitine by resting cells in the presence of 0.5 M NaCl was almost two-fold that of the control. Moreover, it was observed that during salt stress-associated betaine-transporter activation (i.e. salt induced transporters, ProU and ProP), CaiT was affected and this slightly reduced the uptake of L(-)-carnitine (Cánovas et al., 2003c). Therefore, when crotonobetaine is biotransformed into L(-)-carnitine under salt stress, the carriers ProU and ProP, which have both been shown to be stimulated or/and induced under salt stress (Verheul et al., 1998), and

the antiporter CaiT (Jung et al., 2002; Cánovas et al., 2003c) coded by the ***cai*** operon, would work together. However, since ProU and ProP are irreversible transporters, they might have achieved kinetic equilibrium by adjusting the trimethylammonium compounds uptake/efflux ratio, and thus CaiT, which is an antiporter and thus reversible, would be the most active during biotransformation, while the osmotically-induced transporters would increase the concentration of intracellular betaines against the transmembrane gradient. The ProP transporter is a compatible solute/H^+ antiporter (Macmillan et al., 1999), while the ProU system belongs to the ATP binding cassette transport systems (Csonka, 1991), and so, directly or indirectly, both transporters are ATP-dependent and require energy (Fig. 4B).

Metabolic studies performed in batch with resting cells (crotonobetaine only in the buffer biotransformation medium) underlined the importance of the glyoxylate shunt, since the ICL activity was double that of the control (Fig.4A), indicating that the cell stored material was being mobilized for maintenance, since no carbon source was used. In anaerobic batch growing cells, Cánovas et al. (2003a) also observed the importance of the glyoxylate shunt compared with the Krebs cycle during the bioprocess, since both compete for the same substrate. Competition was already mentioned by Cronan and La Porte (1996), who described the existence of the ***aceBAK*** operon, codifying for the glyoxylate enzymes (ICL: ***aceA*** and MS: ***aceB***) and a third enzyme (ICDH-kinase/phosphatase: ***aceK***), which performs a post-transcriptional modification of ICDH, which inactivates it and improves opportunities for ICL. Another fact to bear in mind is that ICDH activity remained higher than control levels, indicating a certain TCA cycle activity under salt stress, corroborating, as mentioned above, the higher demand for energy and metabolic intermediates in the presence of salt stress. Despite this, the cell energetic status, as revealed by the ATP level, decreased during the biotransformation by resting cells, since ATP was being used in cell maintenance and in the biotransformation (Cánovas et al., 2003a), this decrease being faster than in the absence of salt stress (Fig. 4B). Varela et al. (2004) also observed and determined that the maintenance coefficients for cellular integrity decreased with the medium osmolality when working with *Corynebacterium glutamicum*. Moreover, in this work, the ICDH/ICL ratio ranged from 5 at the beginning to 2 at the end of the process, suggesting the importance of this ratio to gain energy and intermediaries. Higher ratios have been found for growing cells in cell recycle reactors, indicating that the functioning of the glyoxylate cycle is less important compared to TCA during exponential cell growth (Cánovas et al., 2006).

Besides, the PTA and ACS activities decreased throughout the biotransformation process both in control and NaCl treated cells (Fig. 5), partly due to the lack of carbon source and partly because these enzymes belong to the *E. coli* anaerobic metabolism and the biotransformation with resting cells was performed in the absence of aeration. Cánovas et al. (2003a), using batch growing biotransformation systems, suggested that acetyl-CoA was transformed into CoA and acetyl-phosphate, this pathway rendering acetate and ATP by the acetate kinase enzyme (ACK) (Kleman and Strohl, 1994) (Fig. 1). In fact, in batch cell growth biotransformations, acetate can also be used by *E. coli* to produce acetyl-CoA through ACS (high affinity, working at low concentrations) or through ACK-PTA (low affinity and reversible, uptaking acetate at high concentrations); however, in resting cells only traces of acetate were detected. This work also shows that in resting cell systems using control and NaCl treated cells and microaerophilic conditions (absence of aeration), PDH activity slightly increased and remained constant during the experiment, rendering pyruvate and acetyl-CoA. Furthermore, the NADH/NAD^+ ratio (Fig 4B) regulates the PDH (Fig. 4A) and PFL enzymes, a low ratio implying low levels of reducing power and higher PDH enzyme activity, while high ratios inactivate the PDH and activate PFL (De Graef et al., 1999). Correspondingly, this study reveals that in the biotransformation by resting cells, the NADH/NAD^+ ratio was higher in the presence of NaCl than in the control, and that the increase in the PDH activity was lower than that observed for the control (Fig. 4A). Therefore, the results demonstrate the relationship between the central carbon and the carnitine metabolism both in the presence and the absence of NaCl, and confirm the importance of the TCA and glyoxylate shunt during the biotransformation. Moreover, the increase in ICL and ICDH activity in the presence of NaCl (Fig. 4) indicates that the stress imposed by the high salt concentration affected the cells at cell metabolism level, as reflected in the NADH/NAD^+ ratio and by the evolution of the intracellular ATP pool, the cell metabolism being addressed towards energy production and the synthesis of intermediates to maintain the cell structure. Further, though it has been described that IclR is involved in regulating the coordinated expression of the glyoxylate bypass and the ACS pathway to allow growth on acetate (Cortay et al., 1991, Shin et al., 1997), under resting cell conditions the glyoxylate bypass is probably mainly devoted to the mobilization of stored material.

Cánovas et al. (2003a) stated that ATP and acetyl-CoA/CoA pools were the points of connection between carnitine and central carbon metabolisms (Fig.1). Furthermore, the importance of the ATP pool was highlighted, since the decrease of

its level coincided with the cessation of L(-)-carnitine production and a sharp decrease in CHR activity (CaiD:CaiB, Figs. 1, 4B and 6B) at 15-18 h, as well as with the decrease in the transport rate throughout the biotransformation process (Fig. 4B and 5C). In fact, Cánovas et al., (2003a) showed that during batch processes the best biotransformation conditions were associated with the growth phase, in which higher production of energy from the reducing power and higher levels of acetyl-CoA/CoA were evident. Furthermore, ATP is necessary for the transport of trimethylammonium compounds (Cánovas et al., 2003a) and since energy is required for the action of other ligases (Vessey and Kelley, 2001), energy would also be necessary for the action of the putative crotonobetaine/butyrobetaine:CoA ligase (CaiC) that has been proposed to synthesize the crotonobetainyl-CoA or γ-butyrobetainyl-CoA required for the activation of the trimethylammonium compounds (Elssner et al., 2000) (see Fig. 1). In this work, the results related with the biotransformation enzyme activities in resting followed the same behaviour both in the presence and absence of NaCl. The decrease in CHR activity may be due to the fact that if ATP decreases, crotonobetaine/butyrobetaine:CoA ligase (CaiC) cannot work, lowering the level of Crot-CoA required as a co-substrate for the CHR activity (CaiB:CaiD). Furthermore, during resting cell processes, the importance of the cell stored material was obvious in both the presence and the absence of NaCl, since the activities of ICL, ICDH and PDH remained high (Fig. 4A, 4C and 6). This suggests the synthesis of cell-maintenance compounds and energy through the mobilization of energetic stored material. Besides, cell metabolism was not devoted to the production of new cells (in Fig. 6B, the biomass decreased slightly) and the cell ATP levels fell rapidly from the start. These facts provide a possible explanation for the high yield of L(-)-carnitine since, rather than being devoted to growth during the first 22 h (Fig. 6B), the energy and material stored within the cell was devoted to the biotransformation and cell maintenance in the resting state. This decrease in the ATP level may thus be the possible limiting step of the biotransformation, since ATP might be necessary for transport and activation prior to further biotransformation (Fig. 1).

In order to unravel the underlying mechanisms responsible for the distinct effects on growing and resting cells (Figs. 2 and 3), experiments were also planned on growing cells. High density cell recycle membrane reactors were chosen as a model system since they have previously been characterized from the metabolic and productive points of view (Obón et al., 1999; Cánovas et al., 2003a). Pulses of NaCl (to generate a sudden osmotic stress response in steady state growing

biotransforming cells) and biotransformation substrate in the presence of a steady state level of NaCl (to make osmotically stressed cells in the steady state respond to a higher biocatalytical pressure) were carried out and the effects on the central and carnitine metabolism were followed. Fast responses in the intracellular coenzyme pools were ascertained after the NaCl pulse, since the NADH/NAD^+ ratio decreased parallel to an increase in the ATP content. It has previously been reported that in the first minutes after an osmotic upshift several membrane-dependent functions stop, ATPase activities are inhibited and respiration temporarily ceases (Wood, 1999). However, steep increases in intracellular ATP due to substrate level phosphorylations have also been described (Ohwada and Sagisaka, 1987). Moreover, the effect on the profiles of fermentation end-products was represented by a decrease in lactate and ethanol levels and an increase in acetate synthesis (Fig. 7B), since the cell rearranged its metabolism to obtain ATP (Fig 1). The PTA and ACS enzyme profiles correlated well with this latter observation, also responding to the up-regulation of RpoS by inhibition of ACS and activation of the PTA expression (Fig. 7B), as previously reported (Shin et al., 1997, Kumari et al., 2000b). Considering the low energetic yield that is associated with the production of acetate, this pathway has usually been considered as an overflow metabolism under aerobic conditions (Chang et al., 1999). It works to provide additional energy when the respiration capacity of the cell is saturated. Thus, the increase in acetate production which follows the NaCl pulse can be considered as a consequence of the transient inhibition of anaerobic respiration (Wood, 1999) and may explain the parallel ATP production.

Although the ATP level increased at the beginning, it soon fell since it was used by the carnitine metabolism (transport and activation) as well as other central metabolic pathways. At the beginning too, both ICDH and ICL enzymes were probably inhibited and/or a sort of regulation occurred, the ICDH/ICL ratio ranging from 0.13 to 0.24, although both recovered their initial levels afterwards. In a previous work, ICL activity was seen to increase during the entry into the steady state of a continuous reactor, indicating the need for energy production and intermediaries through anaplerotic reactions (Cánovas et al., 2003a). After the NaCl pulse, the NADH/NAD^+ ratio fell since the cell addressed the metabolic fluxes through other pathways to produce acetate and synthesise ATP (Fig.1 and 7A) and produce ethanol (which levels decreased from 60 to 37 mM 20 min after the pulse). After quantifying the increase in the maintenance coefficient for ATP (mATP), cell integrity and cell productivity with medium osmolality for *Corynebacterium*

glutamicum, other authors (Varela et al., 2004) have also shown that flexible nodes alter flux distribution in order to allow a reordering of the central metabolism and the covering of cell ATP necessities under these conditions. Similarly, in our work, CHR activity and L(-)-carnitine production increased after the NaCl pulse. The rapid increase seen in the intracellular ATP content, favouring substrate activation through the putative CoA-ligase CaiC (Eichler et al., 1994a) and probably substrate and product transport (Jung et al., 2002; Cánovas et al., 2003a) might explain this fast response. Further, the previously reported activating effect of NaCl on *E. coli* betaine transport (Cánovas et al., 2003c) could also reduce the transport limitation, which has already been suggested as a feasible biotransformation bottleneck (Sevilla et al., 2005). Nevertheless, osmotically stressed growing cells have been seen to express lower biotransforming activity (Fig. 3A), probably due to the up-regulation of RpoS, which acts as an inhibitor of *cai* and *fix* operons (Eichler et al., 1996). However, even though *cai* operon might have been repressed under the salt stress conditions (the turnover of the proteins coded is sufficiently low and not limiting), the mentioned effects of NaCl could be taken as a sort of activation of its expression in short-term experiments (two hours lasting experimental window).

After the pulse of crotonobetaine in the presence of 0.3 M bulk reactor NaCl, the NADH/NAD^+ ratio increased. To ascertain whether this response depended on the presence of salts, a control experiment in the absence of salt was performed and the same behaviour was found (data not shown). Thus, either the NAD^+ regeneration rate was lowered or NADH generation was increased after crotonobetaine pulse. Crotonobetaine can be used by *E. coli* as an electron acceptor, as confirmed by the generation of γ-butyrobetaine in the absence of alternative electron acceptors in the media, such as oxygen (Cánovas et al., 2002) or fumarate (Castellar et al., 1998). Under anaerobiosis, and considering the probable transient inhibition of respiration of bacteria, the production of acetate rather than lactate and ethanol was required in order to obtain "fast" ATP. The last two routes are only used to re-generate NADH, as is depicted in Figure 1, and the level of lactate, after a peak devoted to generating NAD^+, diminished since the salt stressed cell was now principally devoted to obtaining energy (Fig. 1 and 8B). Also as a consequence of the osmotic stress, there was an increase in crotonobetaine uptake into the cell to counteract stress, which also helps to explain the increase in L(-)-carnitine production (Fig. 8C). However, after the first few minutes, acetate recovered its basal steady state level and so the ATP cell content kept increasing, matching its behaviour. Moreover, it was obvious that there was an increase in the

acetate level parallel to the increase in PTA activity and the decrease in ACS activity, as depicted in Figure 8B. This represented a sort of regulation to generate ATP since the PTA/ACS ratio ranged from 10 to 30 at the end of the study (need for ATP). The ACS pathway is considered to be repressed by catabolites and induced by acetate, acting at low concentrations of acetate (Brown et al., 1977). Further, during cell growth the enzyme ACS is positively regulated (thus generating higher acetyl-CoA equivalents) by CRP (in the presence of high levels of cAMP) and FNR (at low oxygen partial pressure) (Kumari et al, 2000a). Furthermore, its transcription has been determined to be primarily dependent on the σ^{70} subunit of RNA polymerase, while it is negatively affected by the σ^{s} factor (RpoS protein) (Kumari et al., 2000b), which is responsible for the stress response after osmotic upshift (Hengge-Aronis, 2002). It should be remarked that the steady state activities of ICL and ICDH in the high density cell recycle reactor in the presence of NaCl were four-fold the steady state levels in the absence of NaCl, thus demonstrating the effect of a steady state salt stress on the central metabolism of the cell (Figs. 7A and 8A), at the level of the cellular need for anaplerotic reactions and energy. Higher fluxes in the central energy-producing and anaplerotic pathways have also been found when *Corynebacterium glutamicum* was exposed to increased osmolality (Varela et al, 2003, 2004). Thus, the results obtained after the pulse of crotonobetaine in steady state salt stress conditions might indicate crucial changes around isocitrate, since the glyoxylate shunt was nearly inhibited and/or regulated (Fig. 8A), while the tricarboxylic acids cycle (TCA) was more active since the ICDH activity increased and the ICL activity decreased up to the level of the steady state of the reactor prior to the NaCl pulse (Fig. 7A). Therefore, the ICDH/ICL ratio ranged from 0.13 to 1.30 at the end of the study. When the crotonobetaine pulse was performed in the absence of salt stress (results not shown), the response was the contrary, that is, decreased ICDH and increased ICL. Although in the absence of salt stress the NADH/NAD^+ ratio followed a similar trend, the time profile of intracellular ATP showed a decreasing trend, probably as a result of the totally different response around the TCA/glyoxylate cycle node. The greater need for energy to maintain the cellular functions of cells exposed to salt stress together with the increase of the biotransformation rate after the pulse would explain this. Furthermore, the expression of ATP-driven transporters for compatible solutes, such as ProU and ProP, which might favour the biotransformation, could also respond for these energy-depleting conditions.

Around 50 and 60 min after the crotonobetaine pulse, the level of CHR activity (twenty-times the steady state level) diminished until it reached the steady state levels. On the other hand, the levels of L(-)-carnitine kept increasing as a consequence of the high levels of ATP generated, confirming that the production of L(-)-carnitine depends on the levels of ATP and that as a consequence of a salt stress, its level was higher. Therefore, after the crotonobetaine pulse, the L(-)-carnitine level increased principally due to the following causes: a) The reactor bulk contained five-fold the standard NaCl concentration, and b) There was an increase in available crotonobetaine, leading to increased intracellular crotonobetaine levels and, as a result, L(-)-carnitine production. Moreover, as a result of the crotonobetaine pulse the PTA/ACS ratio controlled the production of ATP coupled to acetate production and ICDH activity just as the lactate and ethanol production was controlled by the NADH/NAD^+ ratio. Finally the perturbation allowed a substantial increase in the level of L(-)-carnitine in the reactor, showing the possibilities of new strategies for process improvement.

On the other hand, during the biotransformation with resting cells, the BOX-stained cell population (depolarised cells) grew with the incubation time, implying the existence of intact cells which were probably viable but non-culturable. This fact indicates that, as cells become stressed, the metabolic pumps will be inactivated and the cytoplasmic membrane will eventually depolarise, permeabilizing before cell death. In the presence of NaCl (0.5 M) in the biotransformation media, a higher BOX staining percentage than that without NaCl was ascertained due to the osmotic stress. It has been observed that NaCl produces a degree of cell permeabilization due to osmotic stress (Nebe-Von Caron et al., 1998). These findings are very important since for the first time it can be demonstrated that biotransformation with resting cells is also performed by damaged cells. This is also important since the state of each cell contributes to the overall rate and efficiency of the metabolic activity of the cell population as a whole, and the quantification of heterogeneity during biotransformation constitutes an important achievement for process control and optimization. However, the most important fact is that NaCl provoked a permeabilizing effect, probably at the level of the OM. Detergents and organic solvents cause cell permeabilization at the level of the OM, even though the mechanism is not totally clear (Cánovas et al., 2005). Therefore, in this study NaCl permeabilization probably affected the OM so that crotonobetaine would enter the cell more readily and L(-)-carnitine would leave more rapidly by carrier-mediated

transport, an effect, together with the metabolic effects observed, which would justify the improvement in the biotransformation yield.

Thus, from the metabolic point of view the increase in L(-)-carnitine production in the presence of salt may be the result of an increase in the ICDH/ICL, PTA/ACS and $NADH/NAD^+$ ratios, which would increase the activity of ATP-dependent transporters (ProU and ProP) and carnitine metabolism enzymes, together with the possible permeability effect caused by NaCl at long times. Furthermore, when the pulse of crotonobetaine was made in the presence of NaCl, the cell required so much ATP that PTA/ACS increased and also ICDH generated NADH in the TCA cycle for ATP production.

The metabolic enzyme activities measured herein are in fact the combined result of the whole metabolic function involving, for instance, the presence of positive and negative allosteric effectors. Wittman et al. (2005) showed that the tight correlation of many metabolites throughout the entire oscillation cycle of ***S. cerevisiae*** could be an indication that these pools are efficiently equilibrated by the corresponding enzymes, i.e. the corresponding enzyme is operating near equilibrium. Finally, it must be remarked that stress response in ***E. coli*** is triggered by the up-regulation of RpoS (Hengge-Aronis, 2002; Mandel and Silhavy, 2005), which is being displayed not only in the presence of salt and starvation (our study with resting cells), but also due to other environmental stresses, all of them affecting the central metabolism of the cell.

CONCLUSIONS

This study shows, for the first time, that as a result of osmotic stress *E. coli* resting cells were affected at the level of glyoxylate shunt/TCA cycle, indicating the important role played by gluconeogenesis and the consumption of cell stored material, as well as the Krebs cycle activation for intermediates, ATP and reducing power. However, the effect of subjecting the cells to a pulse of osmotic stress in the continuous reactor increased the concentration of ATP and decreased the levels of NADH and, as a result, there was a higher synthesis of L(-)-carnitine. Moreover, after the pulse of crotonobetaine in the presence of NaCl, it was observed that the higher ATP availability stemmed from the osmotic stress to which the cells were subjected, allowing a higher production of L(-)-carnitine, without letting the levels of ATP diminish as dramatically as in its absence. Furthermore, the cellular energetic levels were regulated by the ICDH/ICL and PTA/ACS ratios, which indicate the participation of the glyoxylate cycle (need for energy and cellular intermediates), the tricarboxylic acid cycle (needs for intermediates and to lower the reducing power) and the metabolism of the acetyl-CoA/acetate in the cell. The increment in L(-)-carnitine obtained in the presence of NaCl might be also due to the effect of salt on the OM, permeabilizing the cell, as was shown by staining with PI and BOX. However, flux analysis with respect to the central carbon and the carnitine metabolisms, as well as a study of the still kinetically unknown enzymes (CaiC and CaiB) and the RpoS regulatory network under the different production environments should be carried out. This work is being undertaken by our group to fully understand biotransformation by the complete *E. coli* metabolism.

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NOMENCLATURE

aceBAK: Operon for the glyoxylate shunt cycle.

ACK: Acetate kinase.

ACS: Acetyl-CoA synthetase.

ArcAB: Regulator of the anaerobic/aerobic metabolism that in anaerobic inhibits TCA, ETC, and PDH complex, while activates PFL,

cAMP: Cyclic AMP.

CHR: Crotonobetaine hydration reaction (CaiD:CaiB).

CRP: Catabolic repression via protein receptor AMPc.

CRR: Crotonobetaine reduction reaction (CaiA:CaiB).

CS: Citrate synthetase.

ETC: Electron transport chain.

fixABCX: Operon necessary for crotonobetaine reduction in *Escherichia coli.*

FNR: Transcriptional regulator of the *cai* operon under anaerobiosis.

H-NS: Histone protein.

ICDH: Isocitrate dehydrogenase.

IclR: Represor of ICL and activator of ACS.

ICL: Isocitrate lyase.

LDH: Lactate dehydrogenase.

MS: Malate synthetase.

PEP: Phosphoenolpyruvate.

PEPCK: PEP carboxykinase.

PEPCX: PEP carboxylase.

PFL: Pyruvate formate lyase.

PK: Pyruvate kinase.

PTA: Phosphotransacetylase.

TCA: Tricarboxylic acids cycle.

σ^S or RpoS: Sigma subunit of RNA polymerase, upregulated upon entry into stationary phase, and in response to various stresses, inhibiting carnitine metabolism,

***caiTABCDE* : Carnitine operon.**

CaiT: D,L-carnitine/crotonobetaine/γ-butyrobetaine transporter.

CaiA: Crotonobetaine reductase.

CaiB: CoA-transferase.

CaiC: Putative crotonobetaine/D,L-carnitine/γ-butyrobetaine:CoA ligase.

CaiD: Enoyl-CoA hydratase.

CaiE: Protein of unknown function.

CaiF: Activator of ***cai*** operon, active in presence of D,L-carnitine mixture or crotonobetaine.

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Capítulo 6

Role of betaine:CoA ligase (CaiC) in the activation of betaines and the transference of coenzyme A in the production of L(-)-carnitine in *Escherichia coli*.

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ABSTRACT

In order to determine its role in the metabolism of trimethylammonium compounds, carnitine:CoA ligase (CaiC) was cloned and overexpressed in *Escherichia coli*. Betaine:CoA ligase activity was detected in cell free extracts and the products were analyzed by electrospray-mass-spectrometry (ESI-MS). The substrate specificity of the enzyme was high, reflecting the high specialization of the carnitine pathway. A CoA-transferase activity was also detected *in vitro*, though the most feasible role of CaiC is probably in the synthesis of betainyl-CoAs. The overexpression of CaiC enhanced the biotransformation of crotonobetaine and D(+)-carnitine into L(-)-carnitine. In fact, the yield from crotonobetaine was improved nearly ten-fold, reaching up to 42%. Higher yields were obtained using resting cells, even when D(+)-carnitine was used as substrate.

INTRODUCTION

The metabolism of L(-)-carnitine (R(-)-3-hydroxy-4-trimethylaminobutyrate) in *E. coli* has been widely studied because of its implication in stress survival and anaerobic respiration, although its role is not totally understood (Kleber, 1997; Elssner et al., 2001; Engemann et al., 2005). Increasing demand for this compound has led to greater efforts to develop new production processes. Whole cell biotransformation by Enterobacteria has a great potential for the recycling of waste products such as D(+)-carnitine or crotonobetaine (Obón et al., 1999; Cánovas et al., 2002). The secondary metabolism of *E. coli* has been used for L(-)-carnitine production with both growing and resting cells (Castellar et al., 1998; Obón et al., 1999; Cánovas et al., 2002).

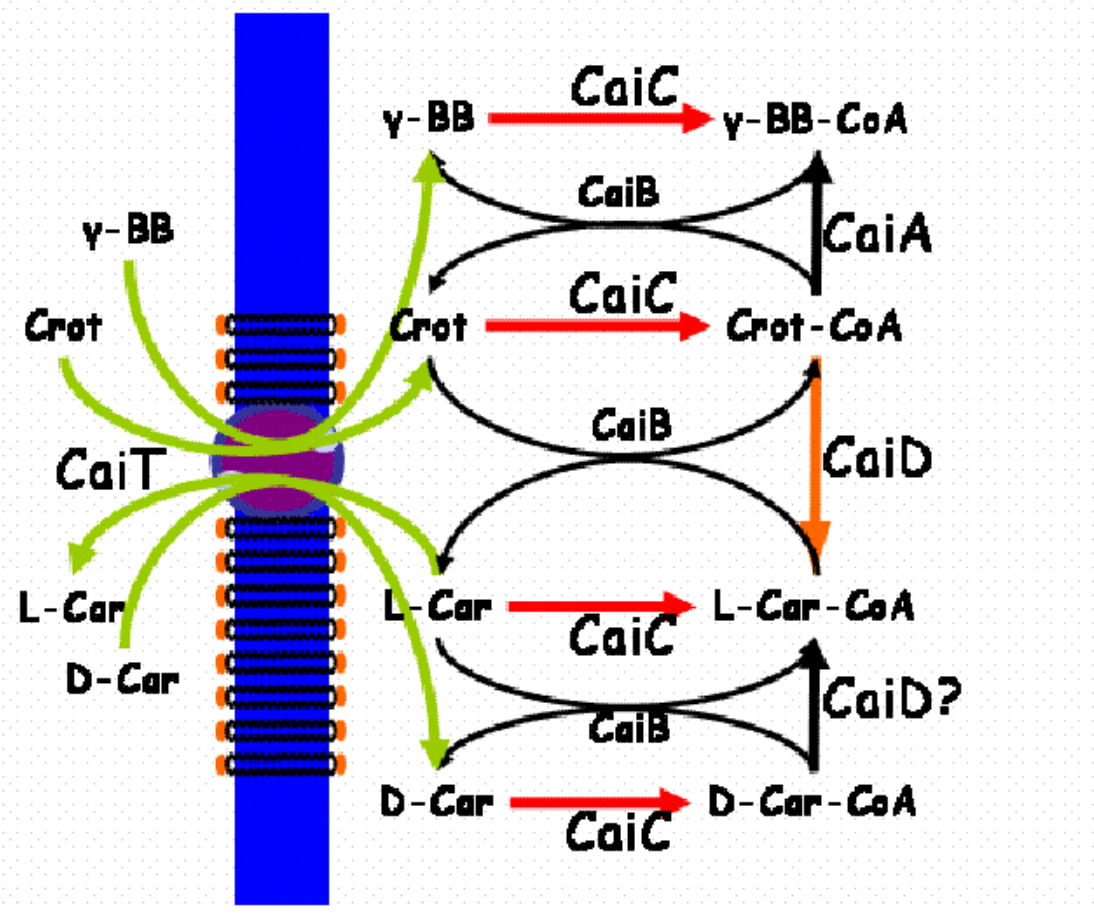

Figure 1. Schematic representation of the metabolism of trimethylammonium compounds in *E. coli* strains. CaiA: crotonobetainyl-CoA reductase; CaiB: CoA transferase; CaiC: betaine:CoA ligase; CaiD: enoyl-CoA hydratase; CaiT: carnitine/crotonobetaine/γ-butyrobetaine antiporter. L-car: L(-)-carnitine; Crot: crotonobetaine; γ-BB: γ-butyrobetaine. The carnitine racemase activity is probably due to CaiD.

Though L(-)-carnitine is a secondary metabolite of *E. coli*, biotransformation occurs at the coenzyme-A (CoA) level (Elssner et al., 2000; Cánovas et al., 2003). In brief, crotonobetaine is transformed into L(-)-carnitine through the involvement of two enzymes, an enoyl-CoA hydratase (CaiD) and a CoA-transferase (CaiB), which are induced anaerobically in the presence of D,L-carnitine or crotonobetaine (Elssner et al., 2001). Besides, reduction into γ-butyrobetaine is carried out by crotonobetainyl-CoA reductase (CaiA) (Preusser et al., 1999; Elssner et al., 2001), which is only active on the CoA derivatives (Fig. 1). Genetic studies have shown that the *cai* operon is responsible for expression of the transporter and carnitine metabolism enzymes (Eichler et al., 1994a).

Elssner et al. (2000) determined the nature of the cofactor necessary for L(-)-carnitine metabolization, which allowed them to propose a mechanism for the biotransformation process (Elssner et al., 2001). In this model, the transfer of CoA played a central role in the biotransformation. However, a CoA-ligase activity to activate the substrate should be essential (Fig. 1). Though CaiC was initially proposed to be a betaine:CoA ligase (Eichler et al., 1994a), its actual role has not been characterized to date.

The role of CaiB and CaiC in maintaining the cellular betainyl-CoA pool and in the biotransformation is also insufficiently understood. In this work, CaiC has been cloned and overexpressed for the first time and its enzyme activity and role in the biotransformation analyzed.

MATERIALS AND METHODS

Strains and plasmids

E. coli O44K74 (DSM 8828; Kleber, 1997) overexpresses carnitine metabolism. *E. coli* LMG194 (ATCC 47090; Guzmán et al., 1995) [F⁻ *ΔlacX74 galE galK thi, rpsL ΔphoA (PvuII) Δara714 leu::Tn10*] is defective in L-arabinose metabolism (Guzmán et al., 1995) and was used as expression host. *E. coli* BW25113 (Baba et al., 2006) and its *caiB* and *caiC* KO-derivatives were kindly supplied by Prof. H. Mori (Keio University, Japan). All the strains containing the complete divergent structural *cai* operon express carnitine racemase (CRac) and carnitine dehydratase (CDH) activities. The strains were stored on culture medium containing glycerol (20%) at –20ºC.

Plasmid construction was performed using standard molecular biology techniques (Sambrook et al., 2001). 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 (Guzmán et al., 1995). The oligonucleotides employed were: CaiC-forward: 5'-GGTGGT*TCTAGA*AATGGATAGAGGTGCAATGGAT-3'; and CaiC-reverse: 5'-GGTGGT*CTGCAG*TTATTTCAGATTCTTTCTAATTATTTT-3'. Construction was verified through sequencing.

Batch cultures

Cells were grown using LB-broth. The pH was adjusted to 7.5 with KOH and ampicillin (100 µg/mL) was added after autoclaving. For the biotransformation experiments, the media were supplemented with 50 mM crotonobetaine or D(+)-carnitine as substrates. Anaerobic conditions were maintained to induce the enzymes involved in the carnitine metabolism, while D,L-carnitine or crotonobetaine were supplied as inducers of the *cai* operon. L-arabinose 0.15% was used as the inducer of the cloned genes.

Anaerobic batch experiments were performed under nitrogen atmosphere in Biostat B (Braun, Germany) reactors, using a 0.5 L working volume.

Resting experiments

For the resting cell experiments, anaerobic cultures were harvested at the end of the exponential growth phase, centrifuged at 16,000 x g for 10 min and washed twice with 67 mM phosphate buffer, pH 7.5. Finally, the cells were re-suspended in 50 mM crotonobetaine or D(+)-carnitine in 50 m*M* phosphate buffer, pH 7.5 at 37ºC

and left in the reactor system. All the experiments were performed at least in triplicate and under sterile conditions. The values reported are the means of the assays performed.

Assays

Cell growth was followed as absorbance (A) at 600 nm with a spectrophotometer (Novaspec II, Pharmacia-LKB, Sweden) and correlated with cell dry weight. The analytical methods used for carnitine, crotonobetaine and γ-butyrobetaine have already been described (Cánovas et al., 2003).

CaiC enzyme activity detection

CaiC enzyme activity was assayed following a modification of the method described by Vessey and Kelley (2001). The reaction mixture (1 mL) contained 50 mM sodium phosphate buffer (pH 7.5), 3 mM ATP, 10 mM $MgCl_2$, 0.5 mM CoA and an appropriate amount of cell free extract. After 5 min incubation at 37ºC, 5 mM substrate (L(-)-carnitine unless otherwise stated in the text) was added and the reaction was started. The reaction was stopped with 15% (w/v) trichloroacetic acid (TCA). After centrifugation (10 min at 19,000 x g), the supernatant was neutralised with KOH. The reaction was monitored by HPLC.

HPLC: detection of CoA derivatives

CoA derivatives were analyzed in an HPLC system (Shimadzu, Kyoto, Japan) equipped with a µ-Bondapak™ C_{18} (Millipore) column (4.5 mm x 25 cm) with UV detection at 254 nm. Two mobile phases were used: A (0.2 M sodium phosphate pH 5 buffer) and B (80% 0.25 M sodium phosphate pH 5 buffer, 20% acetonitrile) at a flow rate of 1.0 mL/min (Boynton et al., 1994). The gradient profile was: B, 3% at 0.0 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, 3% at 45 min. The compounds were identified on the basis of their retention times.

HPLC: detection of ATP, ADP and AMP

A modification of the method by Manfredi et al. (2002) was followed. The above mentioned HPLC system and column were used. Samples of CaiC enzyme activity were compared with standards of ATP, ADP or AMP. The mobile phases were: A (25 mM NaH_2PO_4, 100 mg/L tetrabutylammonium, pH 5) and B (10% acetonitrile, 200 mM NaH_2PO_4, 100 mg/L tetrabutylammonium, pH 4). Flow rate was adjusted to 1 mL/min. 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, 0% at 30 min. Samples were UV monitored at 254 nm.

Mass Spectrometry

The ESI-MS spectra were recorded using an AGILENT VL HPLC/MS system. Samples were solid phase extracted using Sep-Pack$^{(R)}$ Cartridges Plus C_{18} (Waters, Milford, USA). Aqueous samples were applied to the cartridges and the CoA derivatives were eluted with 40% acetonitrile.

The partially purified samples were injected into 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 the acquisition of the spectra, Octopole RF amplitude was 150 Vpp. Capillary Exit was set at -154.6 V and Capillary Exit Offset at -90.4 V. The dry gas flow rate was 9 L/min and the pressure of the nebulizer was set at 60 psi. Dry temperature was set at 350 °C.

RESULTS

Cloning and expression of CaiC.

CaiC was originally proposed to be a betaine:CoA ligase on the basis of sequence similarities (Eichler et al., 1994a). Nevertheless, no experimental observation supports this statement to date. A database search revealed the existence of several bacterial sequences very similar to CaiC (EcoGen reference: EG11558), most of them belonging to the group of Enterobacteria (*Escherichia*, *Shigella*, *Salmonella*, *Proteus*) and annotated as probable crotonobetaine/carnitine:CoA ligase because of their homology with the gene of *E. coli* O44K74. However, none of them has been functionally characterized yet. In addition, a high degree of homology was observed with other bacterial and eukaryotic proteins. In all cases the most remarking structural features of these sequences are the presence of specific AMP and CoA binding sites, which would be necessary for the putative ligase activity.

In order to explore the enzymatic role of CaiC in the metabolism of trimethylammonium compounds in *E. coli*, cloning and overexpression experiments were conducted. Specific primers were used to amplify *caiC* by PCR and this was cloned into the expression vector pBAD24 (Guzmán et al., 1995). The correct construction of the plasmid pBADcaiC was confirmed by sequencing.

To verify the correct expression of the protein, an enzyme assay for the CoA:carnitine ligase activity was optimized. A modification of the method described by Vessey and Kelley (2001) was set up. Monitoring of the reaction by HPLC revealed the presence of a new compound, while the peak of CoA was observed to fade away (Fig. 2A). Control assays lacking ATP demonstrated this compound to be indispensable for the enzyme activity. The formation of AMP in the reaction medium was confirmed by using a modification of the method by Manfredi et al. (2002) (data not shown).

CaiC was demonstrated to be the sole protein able to catalyze the formation of the compound detected, since, when the assay was performed using protein extracts of *E. coli* BW25113 *ΔcaiC* (Baba et al., 2006), the only peak observed was that of CoA. When the mutant strain was transformed with the pBADcaiC plasmid, the ability to form the product was restored.

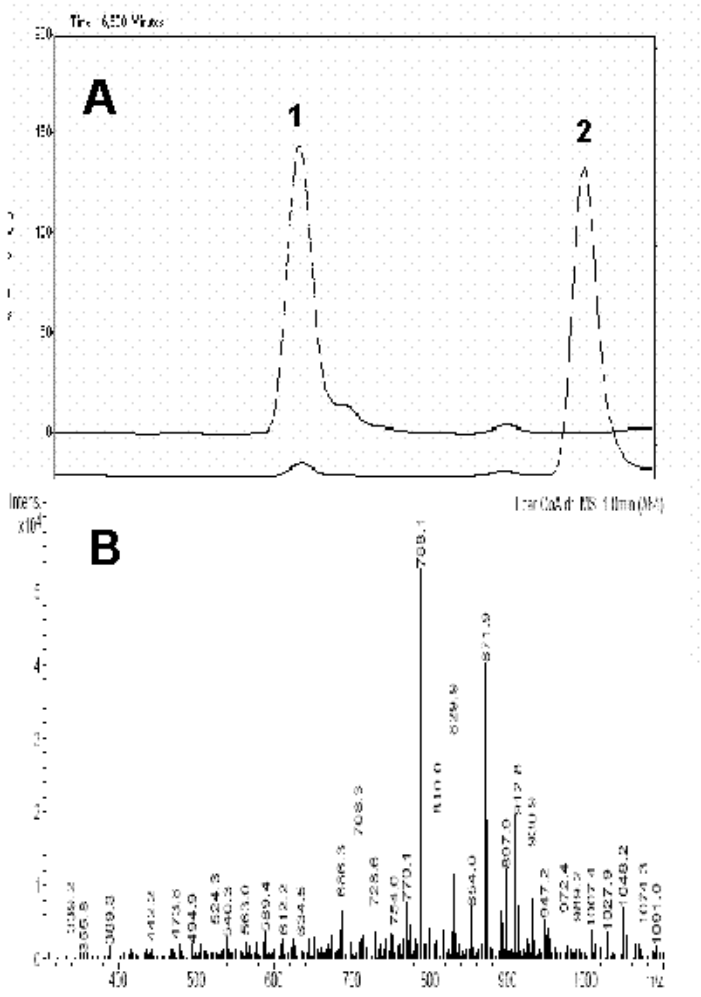

Figure 2. Betaine:CoA ligase enzyme activity assay. (A) HPLC profiles of enzyme activity assays using cell free extracts of *E. coli ΔcaiC* (1) and *E. coli* pBADcaiC (2). Peaks shown correspond to (1) free coenzyme A (retention time: 10.3 min; confirmed by comparison with a commercial standard) and (2) L(-)-carnitinyl-CoA (retention time: 13.6 min; confirmed by ESI-MS). (B) Negative mode ESI-MS spectrum of (2). The assays were performed as stated in Materials and Methods.

Substrate specificity of CaiC.

The substrate specificity of CaiC was checked in order to determine its catalytical flexibility. CoA-ligase assays were performed using the substrates summarized in Table 1. The reaction was monitored by reversed-phase HPLC. Product formation was only observed in the case of compounds very closely related to L(-)-carnitine, such as its isomer (D(+)-carnitine), its dehydratation product (crotonobetaine) and the reduction product of this latter (γ-butyrobetaine). The

retention times reported are in good agreement with increasing hydrophobicity of the products. The osmoprotectant glycinebetaine, which only differs from γ-butyrobetaine in one methylene group, was not a suitable substrate for CaiC. Furthermore, the trimethylamino group was seen to be critical for the activity, since no product formation was assessed on butyric, crotonic, γ-amino-butyric or γ-amino-3-hydroxibutyric acids. These observations confirm that CaiC is a highly specific betaine:CoA ligase.

Table 1. Substrate specificity of CaiC. Activity assays were performed using the standard protocol described for CaiC in Materials and Methods. The betaines assayed were added at 5 mM to start the reaction. The reaction was HPLC monitored for the formation of betainyl-CoAs. An enzyme activity assayed was considered negative when CoA was not consumed and/or no other peak was detected. Under the analytical conditions, the detection limit determined for the betainyl-CoAs detected was below 1 µM.

Substrate	Activity	Retention time (min)
L(-)-carnitine	+	13.6
D(+)-carnitine	+	13.6
crotonobetaine	+	13.7
γ-butyrobetaine	+	16.2
butyric acid	-	---
crotonic acid	-	---
4-amino-butyric acid	-	---
D,L-4-amino-3-hydroxybutyric acid	-	---
glycinebetaine	-	---

Product characterization by ESI-MS

To determine the identity of the four compounds generated by the CaiC activity, the products were further analyzed by Electrospray Mass Spectrometry (ESI-MS). The positive mode spectra of these compounds showed multiple peaks because of the formation of multiple positively-charged adducts with the ions in solution, which complicated their identification. On the other hand, the negative mode spectra were simpler and easier to interpret (Fig. 2B). In all cases, the peak of the mono-negatively charged specie was detected. Other peaks were assigned to

ion pairs formed between polyanionic species and cations from the solution. The results best fitted the possibility of ion pairs with Na^+ or Mg^{2+}, probably the latter, since it is well known that multiple negative charges from nucleotides are stabilized by the formation of Mg^{2+}-chelates. The expected and experimental molecular masses of the species detected are presented in Table 2. Other secondary peaks were due to the neutral loss of trymethylamine (m/z: MW-59). In addition, a peak corresponding to free CoA-Mg^{2+} was also found though, since no free CoA was observed by reversed-phase HPLC, this was considered to be formed through fragmentation of the compound.

Reversibility of CaiC activity: CoA-transferase like activity of CaiC

Until now, all assays for the detection of CaiC activity have been performed in the direction of synthesis of the CoA-derivatives of betaines. The synthesis reaction is coupled to the consumption of one molecule of ATP, forming AMP and pyrophosphate. We decided to determine the degree of reversibility of the reaction mixture. For that purpose, an enzyme activity assay using L(-)-carnitine, crotonobetaine or γ-butyrobetaine was carried out as usual. Following the incubation, an aliquot was analyzed in order to determine whether the reaction had taken place and was complete. Once that the first betainyl-CoA was formed, it was used as the substrate for a CoA-transferase (betaine exchange) activity assay. For that, a second betaine and ATP were added to the mixture of the first reaction. Following incubation, the reaction mixture was analyzed by HPLC as before. With the pairs L(-)-carnitine/γ-butyrobetaine and crotonobetaine/γ-butyrobetaine, whose derivatives have sufficiently different retention times, two peaks were observed. This indicated that CaiC was able to exhibit CoA-transferase like activity, similar to that described for CaiB.

Role of CaiB in the metabolism of L(-)-carnitine in *E. coli*.

This transferase-like activity exhibited by CaiC led us to hypothesize that CaiB may be redundant. To verify this hypothesis, L(-)-carnitine production assays were performed with *caiB* and *caiC* KO-mutants. No L(-)-carnitine was produced with either of the strains. Moreover, the transformation of the *caiB* mutant with pBADcaiC did not increase productivity in this strain.

Table 2. Electrospray-Mass Spectra of the betainyl-CoAs detected.

Assignation of peaks in ESI-MS of betainyl-CoAs			
L(-)-carnitinyl-CoA			
Peak	**Expected Mass**	**m/z**	**Specie**
1	788	788	$[CoA\text{-}3H\cdot Mg]^-$
2	850	850	$[LCarCoA\text{-}2H\text{-}Me_3N]^-$
3	872	870	$[LCarCoA\text{-}5H\cdot Mg\text{-}Me_3N]^-$
4	909	912	$[LCarCoA\text{-}2H]^-$
5	931	930	$[LCarCoA\text{-}5H\cdot Mg]^-$
D(+)-carnitinyl-CoA			
Peak	**Expected Mass**	**m/z**	**Specie**
1	788	788	$[CoA\text{-}3H\cdot Mg]^-$
2	850	850	$[DCarCoA\text{-}2H\text{-}Me_3N]^-$
3	872	870	$[DCarCoA\text{-}4H\cdot Mg\text{-}Me_3N]^-$
4	909	908	$[DCarCoA\text{-}2H]^-$
5	931	930	$[DCarCoA\text{-}4H\cdot Mg]^-$
γ-butyrobetainyl-CoA			
Peak	**Expected Mass**	**m/z**	**Specie**
1	834	834	$[\gamma BBCoA\text{-}2H\text{-}Me_3N]^-$
2	856	856	$[\gamma BBCoA\text{-}4H\cdot Mg\text{-}Me_3N]^-$
3	893	893	$[\gamma BBCoA\text{-}2H]^-$
4	915	914	$[\gamma BBCoA\text{-}4H\cdot Mg]^-$
Crotonobetainyl-CoA			
Peak	**Expected Mass**	**m/z**	**Specie**
1	788	788	$[CoA\text{-}3H\cdot Mg]^-$
2	832	829	$[CBCoA\text{-}2H\text{-}Me_3N]$
3	854	850	$[CBCoA\text{-}4H\cdot Mg\text{-}Me_3N]$
4	891	890	$[CBCoA\text{-}2H]^-$
5	913	908	$[CBCoA\text{-}4H\cdot Mg]^-$

Role of CaiC in the metabolism of trimethylammonium compounds: production of L(-)-carnitine with growing and resting cells.

Finally, biotransformation experiments were conducted in order to test the effect of the overexpression of CaiC in the production of L(-)-carnitine. Maximum production with *E. coli* LMG194 pBADcaiC was 16 mM (32% yield) (Fig. 3). These

results are much higher than those obtained with *E. coli* LMG194 and *E. coli* O44K74 strains (Table 3). Thus, CaiC overexpression almost doubles the production of *E. coli* O44K74 strain, which is considered to be an overproducer (Kleber, 1997).

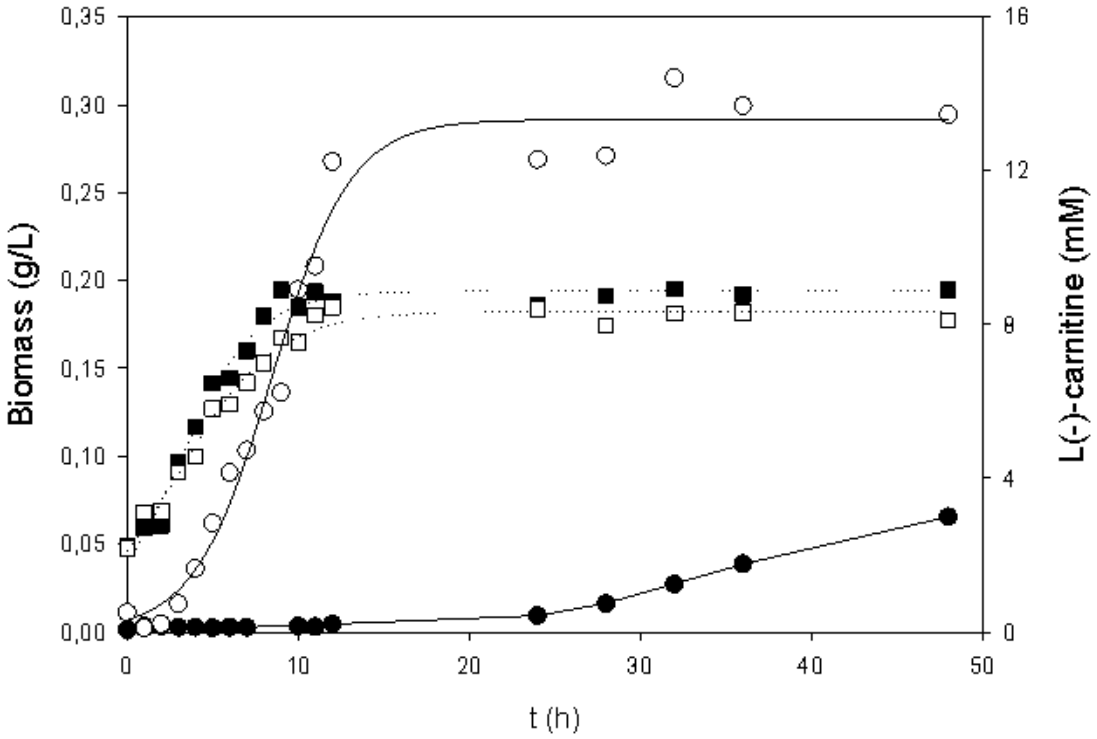

Figure 3. Evolution of L(-)-carnitine (circles) and biomass (squares) during biotransformation by growing wild-type (black symbols) and pBADcaiC-transformed (white symbols) *E. coli* LMG194 strains. Experiments were performed under anaerobiosis in LB-Broth as stated in the Materials and Methods section.

Further experiments were performed to determine whether *E. coli* LMG194 pBADcaiC was able to racemize D(+)-carnitine into L(-)-carnitine (Fig. 1), as already reported for *E. coli* O44K74 and *E. coli* K38 pT7-5KE32 (Castellar et al., 1998; Cánovas et al., 2003). When biotransformation experiments were performed using 50 mM D(+)-carnitine as substrate for growing cells, L(-)-carnitine was below 1 mM and no biotransformation capacity was detected. Further experiments were performed using resting cells (see Materials and Methods section). Control experiments using the *w.t. E. coli* LMG194 strain showed yields lower than 8% using crotonobetaine as substrate. However, conversions up to 60% and 40%, using crotonobetaine and D(+)-carnitine as substrates were obtained with the transformed

E. coli LMG194 pBADcaiC (Table 3). Similar yields were obtained with the overproducing strain *E. coli* O44K74. Thus, it seems likely that the Carnitine Racemase (CRac) activity of *E. coli* is limited by the activation of substrate (D(+)-carnitine) into its CoA derivative although, racemization does not seem to occur in growing cells.

Table 3. Production of L(-)-carnitine with growing and resting *E. coli* cells. 50 mM crotonobetaine or D(+)-carnitine were used as substrates for the biotransformation. Experiments were performed in triplicate (see the Materials and Methods section for details).

	Growing Cells				**Resting Cells**			
	Crot		**D-Car**		**Crot**		**D-Car**	
	Prod. (mM)	Yield (%)	Prod. (mM)	Yield (%)	Prod. (mM)	Yield (%)	Prod. (mM)	Yield (%)
E. coli LMG194	0.7	1.4	0.2	0.5	4.2	8.4	N.D.*	
E. coli LMG194 pBADcaiC	14.5	29.0	0.3	0.6	30.5	60.9	10.5	21.0
E. coli O44K74	8.5	17.0	0.3	0.6	22.6	45.2	11.8	23.6

** N.D.: not determined.*

DISCUSSION

In this work, the betaine:CoA ligase activity of CaiC is confirmed for the first time. The enzyme function of CaiC had previously been proposed on the basis of sequence similarities (Eichler et al., 1994a). Furthermore, several other putative bacterial betaine:CoA ligases have been cloned in the last decade, although none has been characterized to date. On the other hand, CaiB was the first enzyme in the carnitine metabolism of *E. coli* O44K74 to be characterized (Eichler et al., 1994b). Its identification and cloning was the milestone which allowed the complete sequencing and characterization of the *cai* operon (Eichler et al., 1994a). Recently, its role as a CoA-transferase has been suggested (Elssner et al., 2001), whereby it cycles the CoA moiety from substrates to products, thus allowing the biotransformation to proceed in an energetically inexpensive way.

The step of substrate activation catalyzed by CaiC (Fig. 1) is a requirement for the biotransformation. The overexpression of CaiC in *E. coli* LMG194 converted this poor-producer strain into an overproducing strain, indicating that this is a limiting factor for the production of L(-)-carnitine. Moreover, it should be noted that yields and specific productivities were higher than for the overproducing strain *E. coli* O44K74 (Kleber, 1997) under all tested conditions. It seems likely that post-translational effects can account for the sub-optimal *in vivo* activity of CaiC. In fact, the Shine-Dalgarno (ribosome-binding) sequence of *caiC* (3 nucleotides at -5 from start ATG) is the shortest of the genes in the *cai* operon (Table 4), which may explain the low expression level of this enzyme.

Table 4. Shine-Dalgarno (SD) sequences and distance to initial codon of the 6 ORFs of the *cai* operon (Sequence Accession Number: X73904)

Gene	SD sequence	Distance (bp)
caiT	GGAA	7
caiA	AAGAGG	7
caiB	AGGAG	6
caiC	GAA	5
caiD	GAAAGAA	10
caiE	AGAAG	11

Analysis of the substrate specificity of the enzyme allowed us to confirm the high specificity of CaiC. Product formation was only assessed in the case of compounds very closely related to L(-)-carnitine. Glycinebetaine, a not only structurally but also functionally related compound, was not accepted as substrate by CaiC, despite the fact that the only difference with γ-butyrobetaine lies in the lengh of the carbon backbone. Furthermore, the importance of the trimethylamino-group for activity was underlined. The high substrate specificity of CaiC underlines the degree of specialization of the carnitine pathway.

Moreover, a betaine exchange (CoA-transferase) activity, which had not been described before, was observed. However, this might not be relevant for the biotransformation *in vivo*, since a CaiB mutant strain was not able to produce L(-)-carnitine. Thus enzyme redundancy does not occur *in vivo*, and CaiC activity is probably mainly devoted to the activation of trimethylammonium compounds, while CaiB would transfer the CoA moiety. Moreover, CaiC activity has been demonstrated to be dependent on ATP consumption. On the other hand, CaiB activity allows the biotransformation to proceed in an energetically inexpensive way once it has started. Despite these energetic considerations, the overexpression of CaiC led to the higher production of L(-)-carnitine. In addition, the overexpression of CaiC did not allow L(-)-carnitine production in a *ΔcaiB* mutant, meaning that the joint action of these two proteins is necessary for the biotransformation. Both the *caiB* and *caiC* KO-mutants showed their inability to biotransform crotonobetaine into L(-)-carnitine, confirming that both activities are required for proper functioning.

Finally, despite the fact that CaiC was active on D(+)-carnitine and γ-butyrobetaine, the activation of these compounds was not a sufficient condition for them to be considered acceptable substrates in the biotransformation process with growing *E. coli* LMG194 cells. However, racemization of D(+)-carnitine was possible using resting cells.

CONCLUSIONS

A bacterial betaine:CoA ligase has been characterized for the first time. CaiC has resulted to be a highly specific CoA ligase, which also exhibited *in vitro* CoA-transferase activity. However the *in vivo* activity of CaiC is restricted to the synthesis of betainyl-CoAs. Finally, the importance of CaiC as a feasible bottleneck in the L(-)-carnitine biotransformation process is highlighted, and its overexpression opens up new perspectives in the field of catalyst optimization and metabolic engineering.

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Capítulo 7

Cofactor engineering for the improvement of L(-)-carnitine production in *Escherichia coli*.

Los contenidos de este capítulo han sido enviados para publicar como:

Bernal V, Masdemont B, Arense P, Cánovas M, Iborra JL. Cofactor engineering for the improvement of L(-)-carnitine production in *Escherichia coli*. J Biotechnol. (submitted).

ABSTRACT

Cofactor engineering, defined as the purposeful modification of the pool of intracellular cofactors, was demonstrated to be a very suitable strategy for the improvement of L(-)-carnitine production in *Escherichia coli* strains. Overexpression of CaiB (CoA-transferase) and CaiC (CoA-ligase), both enzymes involved in coenzyme A transfer and substrate activation during the bioprocess, led to an increase in L(-)-carnitine production. Under optimal concentrations of inducer and fumarate, used as electron acceptor, yields reached two- and ten-fold, respectively, that for the wild type strain. In addition, the levels of coenzyme A limited the activity of these two enzymes since the addition of pantothenate increased production. Growth on substrates whose assimilation yields acetyl-CoA (such as acetate or pyruvate) further inhibited L(-)-carnitine production. Interestingly, feasible limiting steps in the metabolism of acetyl-CoA of *E. coli* were detected. The glyoxylate shunt and anaplerotic pathways are bottlenecks of the bioprocess since strains carrying deletions of isocitrate lyase and isocitrate dehydrogenase phosphatase/kinase yielded 20-25% more L(-)-carnitine than the control. On the other hand, the deletion of phosphotransacetylase strongly inhibited the bioprocess, suggesting that the adequate flux of acetyl-CoA and connection of the phosphoenolpyruvate-glyoxylate cycle together with the acetate metabolism are crucial for the biotransformation.

INTRODUCTION

Several clinical applications have been identified for L(-)-carnitine (R(-)-3-hydroxy-4-trimethylaminobutyrate) a compound which is essential for the metabolization of fatty acids in mitochondria and peroxisomes. Because of its increasing worldwide demand, several biotechnological processes have been developed for its production, whole-cell based methods being among the most promising. Enterobacteria are able to racemize D(+)-carnitine or biotransform crotonobetaine, both of which are waste products from the chemical synthesis process and which represent an environmental problem (Kleber, 1997; Obón et al., 1999; Cánovas et al., 2003).

In *E. coli,* trimethylammonium compound metabolism has been studied, because of its implication in stress survival and anaerobic respiration, although its role is not totally understood (Eichler et al., 1994; Kleber, 1997; Elssner et al., 2001). Genetic studies led to the description of the structural *cai* operon which is responsible for the expression of a betaine transporter, CaiT, and the carnitine metabolism enzymes (crotonobetainil-CoA reductase, CaiA, coenzyme A transferase, CaiB, coenzyme A ligase, CaiC, enoyl-CoA hydratase, CaiD) (Eichler et al., 1994). All the enzymes involved in the biotransformation are induced in anaerobiosis in the presence of D,L-carnitine and/or crotonobetaine. The biotransformation of trimethylammonium compounds occurs at the level of CoA-thioester derivatives in *Escherichia coli* (Elssner et al., 2000). Interestingly, these trimethylammonium compounds are activated by an uncharacterized betainyl-CoA ligase (CaiC). On the other hand, CoA-transfer between substrates and products is performed by a transferase (CaiB). Furthermore, the pivotal role of CaiB, cycling coenzyme A, and the activating role of CaiC (Fig. 1) make both enzymes crucial in the biotransformation (Cánovas et al., 2003).

In previous works, the expression of certain central metabolic pathways, such the Krebs cycle, the glyoxylate shunt and acetate metabolism (Fig. 1) was analyzed both in batch and continuous reactors under standard (Cánovas et al., 2003) and salt stress (Cánovas et al., 2006a) conditions. It has been observed that the link between the central and carnitine metabolism occurs in the pool of ATP and the acetyl-CoA/CoA ratio (Cánovas et al., 2003) and that the energetic cofactor pool re-arranges after metabolic pulses (Cánovas et al., 2006b). However, the limitations imposed by the composition of the cellular CoA-thioester pool and the enzymes involved in the metabolic link in the biotransformation still remain unclear.

Furthermore, for optimization of the bioprocesses based on secondary metabolism, the role of the central pathways also needs to be determined.

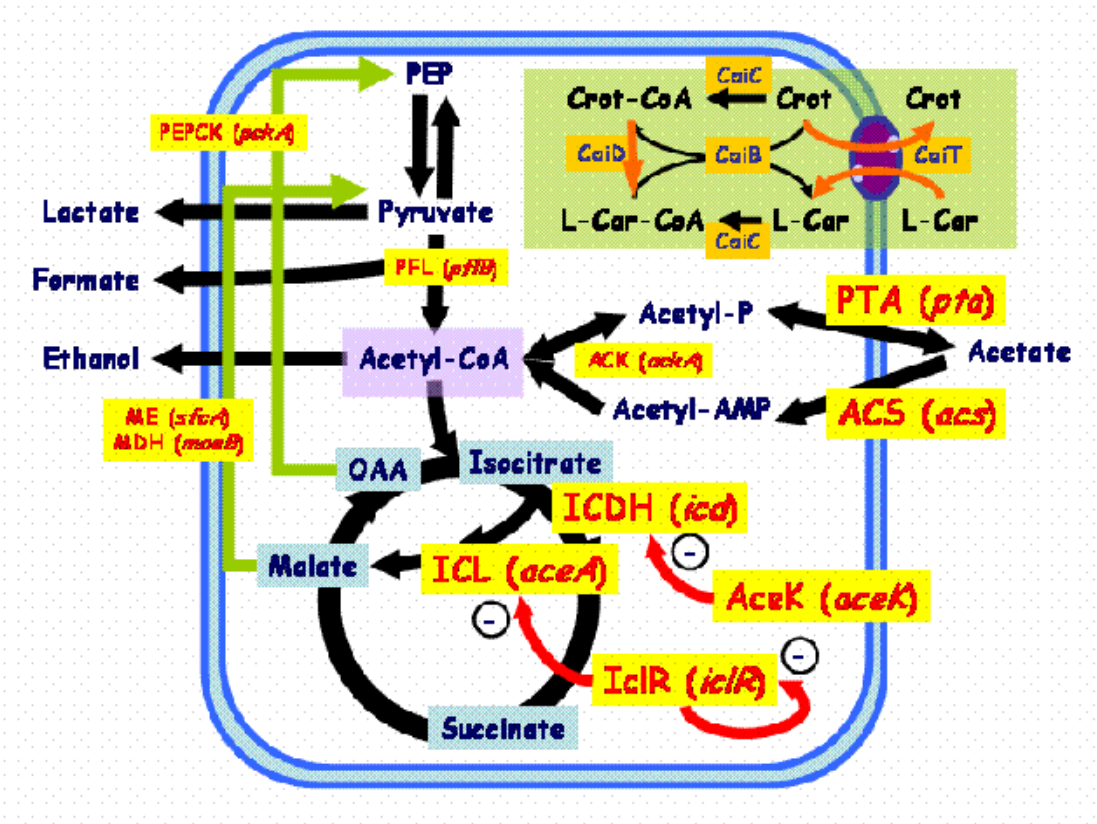

Figure 1. Simplified model for the link between the central metabolic network of *Escherichia coli* and carnitine metabolism in anaerobiosis. Enzymes involved (and their codifying genes) are shown in the figure. Data were obtained from: Ecocyc-Metacyc (Keseler et al., 2005) and Cánovas et al. (2003).

In this context, cofactor level is one of the control parameters which the cell utilizes to regulate fluxes through various metabolic pathways, since it not only affects enzyme activity but also regulates gene expression. Cofactor level reflects the physiological state of the cell and can suffer adaptations to deal with the various metabolic situations that cells can face (Cánovas et al., 2006b). The perturbation of coenzyme pools, also known as cofactor engineering (CE), is an emerging strategy for redirecting metabolic fluxes and shows a high potential for metabolic engineering (San et al., 2002; Vadali et al., 2004). Coenzyme A and its thioester derivatives are recognized as central cofactors (Chohnan, 1997); coenzyme A regulates the central and intermediary metabolism and acetyl-CoA has a common metabolite linking glycolysis, the Krebs cycle, glyoxylate shunt and acetate metabolism. In the

secondary metabolism, CoA-derivatives are involved in the synthesis of products such as PHAs, terpenoids, polyketides and flavonoids.

In this work, the effect of the overexpresssion of carnitine:coenzyme A ligase (CaiC) and carnitine:crotonobetaine:CoA-transferase (CaiB) in *E. coli* LMG194 is analyzed. In addition, the effects of mutations in the glyoxylate shunt and acetate metabolism, both of which affect the metabolism of acetyl-CoA, are studied in order to unravel their involvement in the biotransformation process. Finally, the limitations imposed by alterations in the acetyl-CoA/CoA ratio and the effect of different carbon sources on enzymes and the CoA-thioesters pool of the central metabolic pathways were analyzed. Interrelation between the metabolisms of L(-)-carnitine and coenzyme A is discussed.

MATERIALS AND METHODS

Strains and plasmids

E. coli O44K74 (DSM 8828; Kleber, 1997), *E. coli* LMG194 (ATCC 47090; Guzmán et al., 1995) and *E. coli* BW25113 (wild-type and *aceA*, *aceK*, *iclR*, *acs* and *pta* deletion mutants) were used throughout this study (Table 1). The *E. coli* BW25113 derivatives belong to the KO-collection (http://www.ecoli.aist-nara.ac.jp/; Baba et al., 2006) and were kindly provided by Prof. H. Mori from Keio University (Japan). *E. coli* O44K74 has been isolated as an overexpressing strain for carnitine metabolism (Kleber, 1997; Obón et al., 1999). *E. coli* LMG194, which is defective in L-arabinose metabolism (Guzmán et al., 1995), was used as expression host.

PCR primers were designed in accordance with the database sequences of *caiB* and *caiC* genes (Accession Number: X73904), and XbaI and PstI sites were included at the 5' and 3' ends of the genes to be used in direct cloning (CaiBfwd: GGTGGT*TCTAGA*AATGGATCATCTACCCATGCCG; CaiBrev: GGTGGT*CTGCAG* TTAGTCCTCAACTTTGGCCAGA; CaiCfwd: GGTGGT*TCTAGA*AATGGATAGAGG TGCAATGGAT; CaiCrev: GGTGGT*CTGCAG*TTATTTCAGATTCTTTCTAATTATT TT). Both genes were PCR-amplified and cloned downstream of the multicloning site of the arabinose inducible vector pBAD24. Constructions were verified through sequencing. Standard molecular biology techniques were employed (Sambrook et al., 2001).

Batch cultures

Cells were grown using Miller's LB medium. The final pH of the medium was adjusted to 7.5 with KOH. Antibiotics were added whenever necessary (ampicillin, 100 µg/mL; kanamycin, 30 µg/mL). For the biotransformation experiments, 50 mM crotonobetaine was added prior to autoclaving. Whenever stated in the text, fumarate, pyruvate or acetate were added to the growth media. Anaerobic conditions were maintained to induce the metabolism of L(-)-carnitine, while D,L-carnitine mixture or crotonobetaine were supplied as inducers of *cai* operon. L-arabinose was used as inducer of the cloned genes, at the different concentrations stated in the text. Batch experiments in anaerobic assays (under nitrogen atmosphere) were performed in reactors equipped with temperature, pH, oxygen and pumps controllers (Biostat B, Braun, Melsungen, Germany). A 1 L culture vessel with 0.5-0.8 L working volume was used.

Table 1. Strains used in this work.

Strain	Reference	Genotype	Antibiotics
Escherichia coli O44K74	Kleber (1997)	Unknown	None
Escherichia coli LMG194	Guzmán et al. (1995)	F⁻ *ΔlacX74 galE galK thi rpsL ΔphoA* (PvuII) *Δara714 leu::Tn10*	None
Escherichia coli LMG194 pBADcaiB	This study	[LMG194]	Ampicillin
Escherichia coli LMG194 pBADcaiC	This study	[LMG194]	Ampicillin
Escherichia coli BW25113	Baba et al. (2006)	*rrnB3 ΔlacZ4787 hsdR514Δ(araBAD)567 Δ(rhaBAD)568 rph-1*	None
Escherichia coli BW25113 *ΔaceA*	Baba et al. (2006)	[BW25113] *ΔaceA*	Kanamycin
Escherichia coli BW25113 *ΔaceK*	Baba et al. (2006)	[BW25113] *ΔaceK*	Kanamycin
Escherichia coli BW25113 *Δacs*	Baba et al. (2006)	[BW25113] *Δacs*	Kanamycin
Escherichia coli BW25113 *ΔiclR*	Baba et al. (2006)	[BW25113] *ΔiclR*	Kanamycin
Escherichia coli BW25113 *Δpta*	Baba et al. (2006)	[BW25113] *Δpta*	Kanamycin

Assays

Sample absorbance (A) was followed at 600 nm with a spectrophotometer (Novaspec II, Pharmacia-LKB, Uppsala, Sweden) as a measure of cell concentration. L(-)-carnitine concentration was determined by an enzymatic test (Cánovas et al., 2003), while D,L-carnitine, crotonobetaine and γ-butyrobetaine were determined by HPLC (Obón et al., 1999).

Samples were withdrawn from the reactor. The bacteria were pelleted in a bench-top centrifuge and the supernatant was filtered through 0.22 µm pore size PVDF filters. A cation exchange Aminex HPX-87H column supplied by BioRad Labs (Hercules, CA, USA) was used with a HPLC system from Shimadzu (Kyoto, Japan). The isocratic mobile phase was 5 mM H_2SO_4 at a flow rate of 0.5 mL/min. The effluent was monitored using a refractive index detector (Shimadzu, Kyoto, Japan).

RESULTS

Cloning and overexpression of coenzyme A transferase (CaiB) and betaine:CoA ligase (CaiC)

In order to determine the effect of the pool of coenzyme A derivatives in the biotransformation of L(-)-carnitine, CaiB and CaiC were overexpressed in *E. coli*. These enzymes were selected for overexpression because of their implication in coenzyme A transfer between substrate and products and in the synthesis of CoA-derivatives of trimethylammonium compounds. Briefly, genes were PCR amplified using specific primers, cloned into the arabinose-inducible pBAD24 vector and introduced in *E. coli* LMG194 as expession host.

In order to set optimized levels of expression of CaiB and CaiC proteins, the effect of the concentration of inducer (0.0001 to 1% w/v) on growth and biotransformation was studied. The results showed that optimal concentration of L-arabinose was 0.15% in the case of CaiB, while 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 in both strains, compared with the wild type host strain, though productivity was much higher with the strain overexpressing CaiC (14.5 mM, 29% yield). Further, the production of L(-)-carnitine by the wild-type overproducing strain, *E. coli* O44K74, under the same experimental conditions was lower (8.5 mM, 17% yield) than in the CaiC overexpressing strain.

Since the presence of alternative electron sinks can inhibit the formation of the by-product γ-butyrobetaine (Kleber, 1997; Cánovas et al., 2003), the effect of media supplementation with fumarate was studied. The addition of 0-4 g/L of fumarate to the growth medium produced an enhancement in L(-)-carnitine production for all the strains assayed. Maximum production was assessed at 2 g/L. Yields increased by two- and ten-fold, in the CaiB and CaiC overexpressing strains (10.7 and 41.2%, respectively), in contrast to the low yield obtained (3.8%) with the control *E. coli* LMG194 strain.

Pantothenate addition to the media.

In order to determine whether intracellular levels of coenzyme A were limiting for the activation of trimethylammonium compounds, a saturating concentration of pantothenate (a precursor of coenzyme A biosynthesis) to the culture media and growth and L(-)-carnitine production were analyzed. The results showed that the

presence of a higher amount of available coenzyme A increased productivity (Fig. 2). In fact, productivity rose from 1.8 to 5.3 (mmol/gDCW) in the control strain, from 7.4 to 17.5 (mmol/gDCW) in the CaiB overexpressing strain and from 85.2 to 97.9 (mmol/gDCW) in the CaiC overexpressing strain. In the case of the wild type overproducing strain *E. coli* O44K74 (DSM 8288), L(-)-carnitine production also increased, although the specific productivity was lower (from 37.5 to 32.7 mmol/gDCW). In addition, the final yield and specific productivities were much lower than those obtained using the strain overexpressing CaiC. Moreover, the specific production of acetate decreased in all the strains (in mmol/g, from 47.8 to 37.6 in *E. coli* LMG194, from 45.9 to 41.7 in *E. coli* O44K74 and from 83.6 to 74.7 in *E. coli* LMG194 pBADcaiC), perhaps reflecting the distinct composition in the intracellular acetyl-CoA/CoA ratio under these conditions.

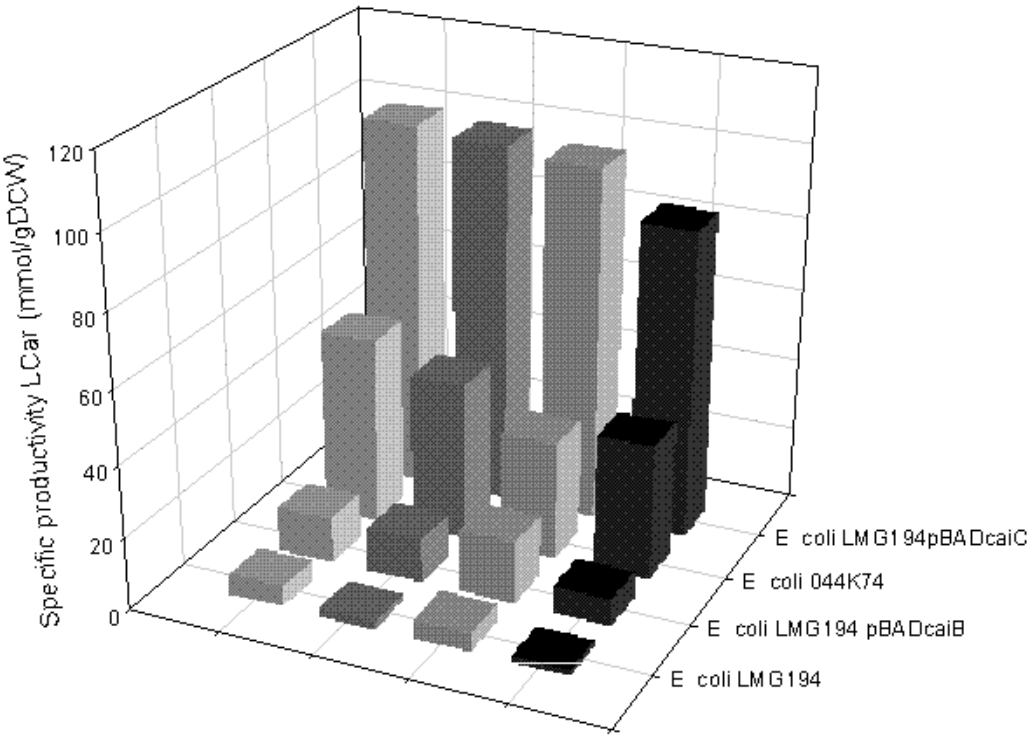

Figure 2.

Effect of fumarate and pantothenate in the production of L(-)-carnitine by wild-type and transformed *E. coli* strains. Cells were anaerobically grown in the presence of 50 mM crotonobetaine as stated in the text. L(-)-carnitine data refer to 24 h of incubation, when the biotransformation had already finished. The media assayed were (A), LB-Broth, (B), LB-Broth with 5 mM pantothenate, (C) LB-Broth with 12.5 mM fumarate and (D) LB-Broth with 5 mM pantothenate and 12.5 mM fumarate. The strains assayed were: (I) *E. coli* LMG194 (w.t.), (II) *E. coli* LMG194 pBADcaiB, (III) *E. coli* O44K74, (IV) *E. coli* LMG194 pBADcaiC.

Effect of acetate and pyruvate.

Experiments were carried out to alter 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 transformed *E. coli* LMG194 pBADcaiC strains (Fig. 1). In the case of *E. coli* LMG194 pBADcaiC 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). Analysis of the metabolites in the supernatants 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.

Effect of deletions of acetyl-CoA metabolism genes on L(-)-carnitine production.

With the aim of engineering L(-)-carnitine production in *Escherichia coli,* the effect of modifying central metabolic pathways indirectly linked to this production metabolism was assessed. Target genes selected for this work were related to the metabolism of acetate (*pta* and *acs*, coding for phosphotransacetylase and acetyl-CoA synthetase, respectively) and glyoxylate shunt and its main regulators (*aceA*, isocitrate lyase; *aceK* isocitrate dehydrogenase phosphatase/kinase; *iclR*, repressor of glyoxylate shunt enzymes) (Table 1). All these deletions were expected to modify the metabolism of acetyl-CoA and so their effect on L(-)-carnitine production was analyzed.

The productivity results are summarized in Figures 3A and 3B (control and fumarate grown cells, respectively). As shown, the deletion of *aceA* and *aceK* led to a higher production of L(-)-carnitine, especially in the presence of fumarate. The production of L(-)-carnitine by the *aceK* mutant was higher during the exponential growth phase, but decreased once that the strain had entered the exponential growth phase (data not shown). In addition, when *pta* was deleted, the growth of the

E. coli strain was lower than in the wild type strain and the lowest production was observed in this strain. The effect of the deletions of *iclR* and *acs* was not so relevant in the experimental conditions. The *pta* mutant presented a higher L(-)-carnitine production in the presence of fumarate. It should be noted that under the conditions of the experiments, the glyoxylate shunt and acetate metabolism were the main pathways using acetyl-CoA as substrate.

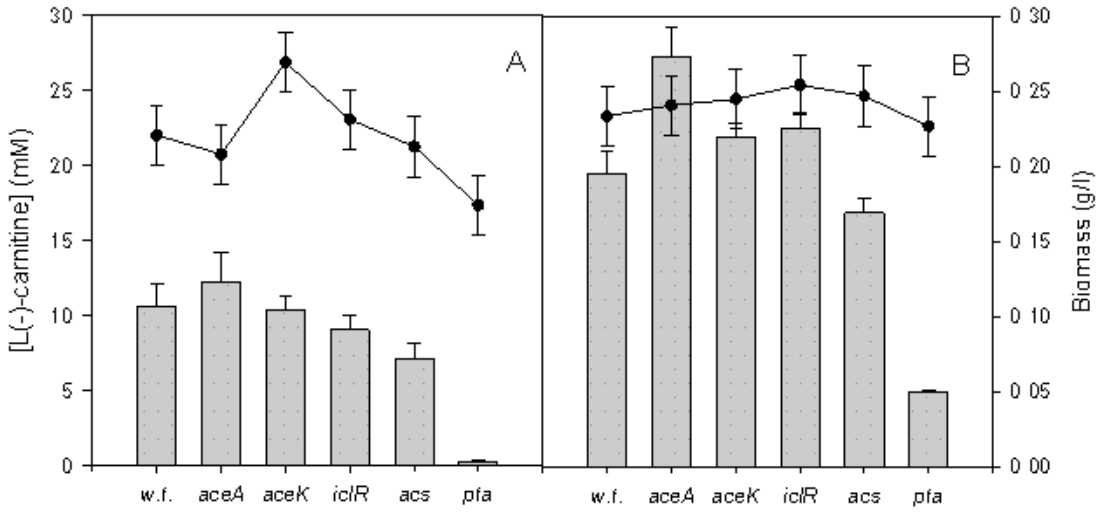

Figure 3. Effect of deleting genes of the acetyl-CoA metabolism on the production of L(-)-carnitine. Biotransformation experiments were performed under the conditions stated in the text in LB-Broth (A) and LB-Broth supplemented with 12.5 mM fumarate (B). Bars represent the L(-)-carnitine and dots biomass levels after 24 h incubation (when the biotransformation had already finished).

Effect of the deletions: growth on pyruvate and acetate.

To gain further insight into the differential response of *E. coli* to the deletion of these genes, cells were grown in LB-broth supplemented with 2 g/L pyruvate or acetate as carbon sources and the production of L(-)-carnitine was analyzed. In all cases, production decreased when the cells were grown in the presence of acetate (Fig. 4A). In fact, the differences between the strains were much reduced and the production of L(-)-carnitine was almost the same in all cases. L(-)-carnitine production was also reduced when the cells were grown in the presence of pyruvate (Fig. 4B), although, in this case, the extent of inhibition differed between the strains assayed. While both the control and the *ΔiclR* strains produced similar amounts of

L(-)-carnitine, inhibition was lower in the case of *Δacs* and, especially, with *ΔaceA* and *ΔaceK* strains.

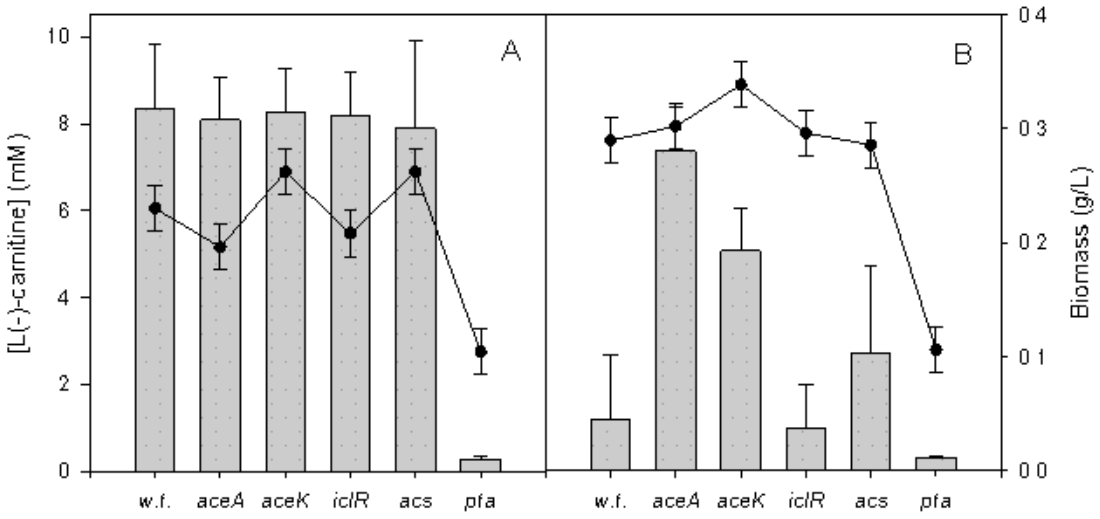

Figure 4.

Effect of deleting genes of the acetyl-CoA metabolism on the production of L(-)-carnitine. Biotransformation experiments were performed under the conditions stated in the text in LB-Broth supplemented with 2 g/L acetate (A) or pyruvate (B). Bars represent the L(-)-carnitine and dots biomass levels after 24 h incubation (when the biotransformation had already finished).

DISCUSSION

Conceived as the rational modification of intracellular coenzyme pools, CE has revealed itself to be a very promising strategy for the modification of metabolic fluxes (López de Felipe et al., 1998; San et al., 2002). The main novelty of CE is that it directly affects the coenzyme pools, which are common to a great number of enzymes, belonging to different pathways and thus a more global response is expected. The first works published on CE focused on the alteration of the redox balance as a parameter for controlling the metabolism (López de Felipe et al., 1998; Roca et al., 2003). San and co-workers devoted their efforts to the engineering of the central metabolism of *E. coli*, altering both the cell redox state and the acetyl-CoA/CoA ratio (San et al., 2002; Vadali et al., 2004). In this work, we have attempted for the first time to improve a bioprocess based on the cell 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 (Elssner et al., 2001). Furthermore, it has been established that the link between central carbon and carnitine metabolisms resides in the ATP and acetyl-CoA levels (Cánovas et al., 2003), while strong *in vivo* alteration of the energetic cofactor pools has been observed in pulse experiments (Cánovas et al., 2006b).

Two approaches were undertaken. In the first part of the study, CaiB and CaiC, which control activation of the substrates -crucial for the biotransformation to occur- were overexpressed. In the second part, the acetyl-CoA/CoA ratio was altered by deletion of the genes belonging to the central metabolic network. Selected target genes belong to pathways directly linked to acetyl-CoA and are involved in the biotransformation process (Cánovas et al., 2003).

It has been proposed that CaiC is a highly specific carnitine/crotonobetaine/γ-butyrobetaine:CoA ligase (Eichler et al., 1994), and is responsible for activating L(-)-carnitine and its derivatives. Further, the action of CaiB, which cycles the CoA moiety between substrates and products, allows the biotransformation to proceed in an energetically inexpensive way. Despite this energetic consideration, the enhancement of L(-)-carnitine production with growing *E. coli* cells was much greater after overexpression of the ATP-dependent CaiC (Fig. 1).

The addition of fumarate to the growth media allowed us to further increase production. Previous results in our group pointed to an activation of the glyoxylate shunt and an increase in the production of acetate after increasing the levels of

fumarate within the reactor (Cánovas et al., 2006b). 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 both CaiB and 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 (Vallari and Jackowski, 1988), while acetyl-CoA levels are a function of the cellular metabolic state (Vadali et al., 2004). Pantothenate kinase (PanK) is the rate-controlling step in the biosynthesis of coenzyme A (Rock et al., 2000; Song and Jackowski, 1992; Vallari and Jackowski, 1988), though supplementation with pantothenate has been shown to be necessary for increasing the coenzyme A cell content, especially upon overexpression of PanK (Vadali et al., 2004).

During the biotransformation of trimethylammonium compounds, a higher demand for cofactor is to be expected. The addition of pantothenate to the *E. coli* cultures under biotransformation conditions increased L(-)-carnitine specific productivity by 137% and 15% in the CaiB and CaiC overexpressing strains, respectively. Bacterial PanK has been reported to be more effectively inhibited by CoA than by its thioester derivatives (Vallari et al., 1987) and thus, the build-up of betainyl-CoAs, together with other derivatives, would not inhibit the biosynthesis of CoA. Thus, it is possible to engineer the total concentration of CoAs, provided that free CoA levels remain sufficiently low, independently of the overexpression of PanK.

While pyruvate is a highly energetic and readily assimilable carbon source, the metabolization of acetate only occurs in stationary phase cells. Acetate is produced by exponentially growing cells and only consumed once the other substrates are exhausted. At high concentrations, acetate is taken up by the reversible and low-affinity PTA-ACK pathway, while at lower concentrations it is scavenged from the growth medium by the high-affinity irreversible ACS pathway (Brown et al., 1977). Regardless of the pathway followed, the acetate taken up enters the central metabolism as acetyl-CoA. 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. Growth and biotransformation experiments in the presence of pyruvate and acetate illustrated the importance of a functionally active glyoxylate shunt and the role of the acetyl-CoA/CoA ratio (Cánovas et al., 2003). With both substrates, L(-)-carnitine production was largely inhibited. In

addition, in the case of pyruvate-grown cells, higher formate and lactate levels were detected and acetate production was also increased, probably reflecting the build up of a large intracellular pool of acetyl-CoA. This would reduce the availability of free-coenzyme A for the biotransformation, thus explaining the inhibitory effect observed. Another observation supporting this hypothesis is the already mentioned fact that the inhibition of L(-)-carnitine production coincided with the consumption of pyruvate and acetate as carbon source.

Experiments with strains in which genes related to the glyoxylate shunt and acetate metabolism had been deleted (Fig. 1) further corroborated the dependence of L(-)-carnitine production on the metabolic fate of acetyl-CoA. These pathways are the main routes using acetyl-CoA as substrate. Increased production was observed in the *aceA* and *aceK* mutants (Fig. 3), in which decreased flux through glyoxylate shunt is to be expected (and also through anabolic pathways, mainly gluconeogenesis). Furthermore, the linking of the glyoxylate shunt and the PEP to acetyl-CoA pathways through pyruvate carboxykinase (Pck) has recently been recognized as a novel metabolic cycle. In addition to the generally described function in gluconeogenesis and anaplerosis, the PEP-glyoxylate cycle is able to catalyze the complete oxidation of PEP to CO_2, a function that was considered exclusive of the TCA cycle (Fischer and Sauer, 2003). The PEP-glyoxylate cycle, it has been suggested, functions in redox-cofactor balancing, especially under hunger or starvation conditions, but also during conditions leading to a higher formation than consumption of NADPH, such as growth on certain substrates or when little biomass is synthesized. However, the reasons behind why this decreased flux through the PEP-glyoxylate cycle causes such an increase in the biotransformation need to be further studied.

Moreover, L(-)-carnitine production fell after the deletion of *pta* (Fig. 3), while the deletion of *acs* had a much less pronounced effect. The Pta-AckA pathway, which is responsible for acetate production (Fig. 1), is reversible and constitutively expressed (Brown et al., 1977; Kumari et al., 1995). Mutants lacking *pta* have already been described as excreting unusual by-products such as pyruvate, lactate and glutamate rather than acetate, and the perturbation of acetyl-CoA flux causes defective growth and starvation survival (Chang et al., 1999; Zhu and Shimizu, 2005). The production of acetate is not suppressed in this mutant due to the existence of alternative routes, such as that of pyruvate oxidase (PoxB) (Chang et al., 1994), or even to the degradation of acetyl-phosphate by AckA (Brown et al., 1977). In addition, increased synthesis of poly-hydroxybutyrate (PHB) in a *pta*

mutant indicates that this strain accumulates acetyl-CoA (Chang et al., 1999). In our work, the observed profile of metabolite production pointed to the accumulation of pyruvate and acetyl-CoA, this latter explaining the inhibition of L(-)-carnitine production.

The TCA cycle, the glyoxylate shunt and the acetate metabolism are subjected to coordinated regulation. Briefly, at the onset of the stationary phase, primary carbon source is fully consumed and the decrease in the growth rate is paralleled by the up-regulation of the glyoxylate shunt and the reversible inactivation of ICDH by AceK. The flux through ICDH has been shown to be essential for the interconversion of the enzymatic machinery necessary for the synthesis and assimilation of acetate (Aoshima et al., 2003; Phue et al., 2005) or acetate switch (Wolfe, 2005; El-Mansi et al., 2006). The use of mutant strains devoid of isocitrate lyase and pyruvate dehydrogenase has revealed that the signal which triggers the reversible inactivation of ICDH is not directly related to acetate itself, but rather to the need to maintain high intracellular levels of isocitrate and free coenzyme A to ensure flux through the glyoxylate shunt (El-Mansi, 1998), since the anaplerotic sequence of the glyoxylate bypass is required for the oxidation of the acetate taken up. Moreover, PEP has been demonstrated to severely impair the binding of the repressor IclR to the promoter region of *aceBAK*, acting as an inducer of the acetate switch (Cortay et al., 1991). More recently, it has been hypothesized that direct competition of PTA and α-KGDH for their common cofactor is crucial for growth on acetate. Further, under anaerobiosis, the TCA cycle does not function as an energy-generating cycle, but almost exclusively to provide precursor metabolites, namely, oxaloacetate, α-ketoglutarate and succinyl-CoA (Chang et al., 1999).

Finally, several studies have stated that differential regulation of the glyoxylate shunt, the TCA cycle and the acetate uptake alter the efficiency of acetate utilization. When acetate is supplied as carbon source, gluconeogenesis and glycogen synthesis help ACS and the glyoxylate shunt in the efficient utilization of acetate (Oh et al., 2002; Phue et al., 2005). In addition, the conversion of pyruvate to acetate by PoxB and the conversion of malate to pyruvate by NAD-linked malate dehydrogenase (MDH) would represent a futile cycle which might also impede the assimilation of acetate. These observations are in good agreement with the results observed in this work, since acetate was not fully consumed as carbon source, further supporting the hypothesis of active PTA-AckA, ACS and even PoxB pathways taking up acetate and maintaining high acetyl-CoA levels which would not

be assimilated through the glyoxylate bypass, thus, inhibiting L(-)-carnitine production.

Nevertheless, the reason why the down-regulation of glyoxylate shunt increases the yield of L(-)-carnitine remains unclear and further work has to be carried out to unravel the interacting network which underlies the observed adaptations.

CONCLUSIONS

The availability of free coenzyme A affects L(-)-carnitine metabolism. Not only the enzymes involved in the activation of trimethylammonium compounds (CaiB and CaiC) are crucial in the biotransformation, but also the central metabolism, regulating the levels of coenzyme A and, especially, the acetyl-CoA/CoA ratio. Taken together, these data suggest the activation of trimethylammonium compounds into CoA derivatives as the main bottleneck 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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DISCUSIÓN

Este trabajo se ha centrado en la optimización de un bioproceso desde un punto de vista multidisciplinar, ahondando en la aplicabilidad de la ingeniería metabólica para el desarrollo de bioprocesos. Para ello se ha tomado como modelo de estudio la producción del fármaco L(-)-carnitina por cepas de *Escherichia coli* y se han tenido en cuenta tanto el sistema de reactor como los factores metabólicos.

En trabajos previos, se ha caracterizado la ruta de biotransformación de compuestos de trimetilamonio de *Escherichia coli* (Eichler et al., 1994; Kleber, 1997; Elssner et al., 2001). Este metabolismo secundario se puede utilizar para la producción de L(-)-carnitina empleando como sustratos sustancias de desecho tales como crotonobetaína y D(+)-carnitina (Jung et al., 1993; Naidu et al., 2000). Hasta la fecha se han empleado diversos sistemas biotecnológicos para la producción de L(-)-carnitina. De hecho, se ha comprobado que la biotransformación no sólo la pueden realizar las células en crecimiento, sino también las células en reposo o células durmientes, con productividades elevadas (Castellar et al., 1998; Cánovas et al., 2003b). Además, también se han estudiado sistemas en continuo (Obón et al., 1997 y 1999).

La ingeniería metabólica se define como la *"mejora de las actividades celulares mediante la manipulación del transporte enzimático y de las actividades reguladoras de la célula mediante el empleo de la tecnología del DNA recombinante"* (Bailey, 1991) o, más concretamente, como la *"modificación intencionada del metabolismo intermediario usando las técnicas de DNA recombinante"* (Cameron and Tong, 1993). La característica más sobresaliente de la ingeniería metabólica es la elección racional de dianas para la modificación genética. Al igual que otros campos de la ciencia y la ingeniería, la ingeniería metabólica consiste en dos pasos: análisis y síntesis (Stephanopoulos, 1994). Para la optimización de un bioproceso se deben considerar varios puntos de vista. En primer lugar, se debe poseer un conocimiento de la fisiología celular para la optimización inicial de la expresión del metabolismo bacteriano que se va a explotar (Eichler et al., 1994; Kleber, 1997; Elssner et al., 2001). En segundo lugar, la optimización del bioproceso en sí mismo pasa por una elección del sistema de reactor, que debe suplir las necesidades celulares en términos de fisiología y metabolismo (Cánovas e Iborra, 2006). El funcionamiento de las redes metabólicas en un punto importante y, para determinar las condiciones óptimas, son necesarios un buen análisis de las variables del proceso y el establecimiento de modelos. Todos estos factores se han tenido en cuenta en el trabajo desarrollado en esta

Tesis Doctoral y se ha realizado conjuntamente, el estudio (y mejora) de las condiciones de operación del bioproceso y la optimización del estado celular a través de ingeniería metabólica. La información obtenida previamente en estudios *in vivo* e *in silico* en nuestro grupo de investigación fue el punto de partida que nos permitió el establecimiento de dianas para la mejora (Cánovas et al., 2002; Álvarez-Vásquez et al., 2002, Cánovas et al., 2003b; Sevilla et al., 2005).

Empezamos llevando a cabo un análisis sistemático de los principales factores que afectan a la producción de L(-)-carnitina mediante cepas de *Escherichia coli* (Capítulo 1). Se compararon sistemas discontinuos y continuos, crotonobetaína y D(+)-carnitina como sustratos y condiciones aerobias y anaerobias. Además, se emplearon dos cepas: la cepa salvaje superproductora *E. coli* O44K74 y la modificada genéticamente *E. coli* K38 pT7-5KE32. La cepa modificada genéticamente permitió alcanzar productividades mayores, incluso permitiendo llevar a cabo el proceso en condiciones aerobias. De hecho, la disponibilidad de oxígeno se identificó como un factor crucial que afecta al conjunto de reacciones, especialmente en el caso de la racemización de D(+)-carnitina. En anaerobiosis o microaerobiosis se observó un efecto similar, puesto que la adición de fumarato, un compuesto que puede emplearse como aceptor de electrones, incrementó el rendimiento del bioproceso. Los reactores continuos fueron ideales para la producción de L(-)-carnitina con cepas salvajes, permitiendo incluso la realización de experimentos dinámicos. El empleo de cartuchos de fibra hueca permitió la retención de mayores niveles de biomasa, aunque los mayores niveles de actividad L(-)-carnitina deshidratasa (CDH) correspondieron a los experimentos con retención celular a través de un módulo de microfiltración. Por otro lado, la cepa de *E. coli* modificada con el sistema de dos plásmidos resultó genéticamente inestable en cultivos continuos con células en suspensión, tanto en sistemas de alta como de baja densidad celular, por lo que fue necesario recurrir a la inmovilización para conseguir mantener las células transformadas en el reactor.

Una vez que se determinó cómo la configuración del reactor determinaba el rendimiento del bioproceso (escala macroscópica), se llevó a cabo un análisis del efecto sobre las células (escala microscópica). La estabilidad del biocatalizador es de gran importancia cuando se consideran sistemas de producción con reutilización de biomasa (en aplicaciones en discontinuo) o incluso en la operación en continuo con dispositivos de retención celular (Cánovas e Iborra, 2006). El modo de operación del reactor afecta no sólo al funcionamiento celular, a la morfología y viabilidad (Capítulo 2), sino también a la expresión metabólica y al rendimiento del

proceso, que dependerá de la adecuada selección de medios y condiciones (Capítulos 4 y 5). Además, también era necesario un análisis de la inestabilidad segregacional de los plásmidos durante la operación en continuo (Capítulo 3).

La aplicación de la citometría de flujo como técnica analítica nos permitió determinar cómo los diferentes sistemas de reactor (continuo y discontinuo) así como modos de operación (células en crecimiento y células durmientes) afectaban al microorganismo. Los niveles intracelulares de DNA, RNA y proteínas mostraron respuestas interesantes, fundamentalmente en relación al consumo (limitación) de fuente de carbono (fase estacionaria de crecimiento) o incluso a la ausencia de esta (células durmientes). Los ciclos de replicación del DNA ocurren independientemente de la división celular, mientras que los niveles de RNA y proteínas reflejan las situaciones de estrés. Además, la integración de estos datos con la cuantificación de la heterogeneidad de las poblaciones celulares supuso una nueva manera de analizar el modo en que la configuración del reactor afecta a la fisiología celular. A pesar de que la función de la membrana celular depende de la actividad metabólica celular, se observó que la síntesis de RNA y proteínas prosigue incluso en células dañadas. De hecho, en sistemas con células durmientes, la actividad celular continuó a pesar del gran descenso en viabilidad provocado por la ausencia de nutrientes. En sistemas continuos de reciclado celular el tanto por ciento de viabilidad celular fue elevado a pesar de las limitaciones por carbono y/o nitrógeno. Finalmente se empleó una metodología para la reutilización celular, alternando ciclos de reposo/biotransformación y crecimiento/re-energización (Capítulo 2).

Como ya se ha determinado (Capítulo 1), la inestabilidad genética dificulta la aplicación de células modificadas genéticamente en el bioproceso. A pesar de que este es un inconveniente generalmente observado para la aplicación de células modificadas en procesos continuos, la inmovilización celular es una técnica general y sencilla que permite la estabilización de la cepa (Kumar et al., 1991). Se estudiaron los factores determinantes de la estabilización genética de *E. coli* empleando citometría de flujo (Capítulo 3). Los experimentos iniciales demostraron que la sensibilidad de la técnica era suficiente para distinguir entre células crecidas en discontinuo con y sin plásmidos en términos del contenido en DNA y proteínas. En cultivo continuo, los distintos ambientes del reactor afectaron a la fisiología celular, especialmente en lo referente a la viabilidad celular y al contenido en DNA. En sistemas continuos se observó un contenido inferior en DNA, tanto en células en suspensión como en células atrapadas en el gel, probablemente debido a un

descenso en el número de copias del plásmido en sistemas en crecimiento estacionario. A pesar de esto, la menor tasa de crecimiento de las células inmovilizadas supuso un descenso en la carga metabólica impuesta por la replicación del plásmido y la síntesis de proteínas (Jones y Keasling, 1998; Flores et al., 2004). Además, los niveles de RNA estuvieron fuertemente controlados por la maquinaria celular, aunque tanto los niveles de RNA como los de proteínas aumentaron en cultivos continuos. El mayor grado de protección física de las células atrapadas en el gel permitió la acumulación de una población de células catabólicamente activas que, sin embargo, tenía sus membranas despolarizadas. Estas células fueron capaces de recuperar su potencial de membrana cuando se añadió medio fresco al cultivo, apoyando la idea de que las células energéticamente agotadas pueden, al menos parcialmente, recuperar sus funciones (Nebe von Caron et al., 2000) lo que abre nuevas vías para el uso de bacterias como catalizadores para biotransformaciones industriales.

En la optimización de bioprocesos en los que está implicada una ruta perteneciente al metabolismo secundario del microorganismo, la aproximación más común ha sido la optimización de su expresión independientemente de la fisiología celular y del metabolismo primario. Desde un punto de vista bioquímico clásico, se puede definir en metabolismo central como el conjunto de las rutas metabólicas celulares que proveen a la célula de energía y moléculas sillares, siendo el metabolismo secundario el conjunto de todo el resto de reacciones. Sin embargo, el metabolismo secundario no se puede considerar como una entidad totalmente independiente de las rutas metabólicas centrales de la célula. En muchos casos la expresión de ambos metabolismos está coordinada, siendo controlada por el mismo conjunto de proteínas reguladoras, mientras que los sustratos y productos de ambos metabolismos pueden ser comunes. Esto permite a la célula administrar los recursos metabólicos de manera óptima, especialmente bajo condiciones de estrés. Lo que es más importante, si los intermedios metabólicos o los cofactores de esta red están compartidos, la evolución del *pool* de éstos determinará en gran medida la unión o integración de ambos metabolismos (López de Felipe et al., 1998; San et al., 2002; Cánovas et al., 2003a; Vadali et al., 2004).

Puesto que la comprensión de la red metabólica implicada en la biotransformación es de importancia primordial, en el Capítulo 4 se llevó a cabo un análisis en profundidad de las adaptaciones metabólicas sufridas en condiciones de biotransformación. Así, se estableció que la unión o integración del metabolismo primario y el metabolismo de compuestos de trimetilamonio se produce a través de

los cofactores, especialmente el ATP y la relación acetil-CoA/CoA. Además, la expresión del ciclo de los ácidos tricarboxílicos, el ciclo del glioxilato y el metabolismo del acetato está relacionada con la biotransformación, limitando los flujos máximos y la productividad de L(-)-carnitina. El estado energético de la célula se correlacionó con la regulación coordinada del ciclo del glioxilato y el ciclo de Krebs, de tal manera que la relación ICDH/ICL respondió a la ausencia o limitación de sustrato y al crecimiento celular rápido. En el caso de las células durmientes, se observaron modificaciones metabólicas debidas a la adaptación de las células a las condiciones microaeróbicas y a la utilización del material almacenado por la célula, lo que favoreció la biotransformación.

Además, L(-)-carnitina es un osmoprotector, de modo que la presencia de sal en el medio de cultivo afecta al rendimiento del bioproceso y al nivel de expresión. Se estudió el efecto del estrés salino sobre la biotransformación (Capítulo 5) observándose un aumento en la productividad como consecuencia de la combinación de una serie de factores: la permeabilización celular y la activación de rutas metabólicas para la generación de energía y precursores. El análisis metabólico de ***E. coli*** en condiciones de estrés osmótico reveló que el aumento en la productividad estaba relacionado con la modificación del estado del metabolismo central, corroborando así las restricciones metabólicas determinadas previamente.

En condiciones de agotamiento o limitación de sustrato se producen importantes cambios metabólicos y fisiológicos que activan los mecanismos celulares de mantenimiento y supervivencia (disparados por la activación de RpoS), provocando una disminución del rendimiento del metabolismo secundario. Los estudios metabólicos y fisiológicos que hemos llevado a cabo apoyan la importante influencia que el estado energético celular tiene en la expresión y rendimiento del metabolismo de compuestos de trimetilamonio. Se sabe que el transporte y la activación de sustrato son los pasos clave dependientes de ATP que pueden explicar esta observación. Además, durante la optimización del bioproceso se determinó que los niveles de oxígeno y fumarato son factores cruciales, no sólo como consecuencia de la inhibición de la actividad crotonobetaína reductasa (CRR), sino también debido a las consideraciones energéticas anteriores. La adición de fumarato aumentó la producción en anaerobiosis, pero también en procesos con células durmientes realizados en condiciones microaerobias, en las que se pueden producir limitaciones en la tasa de transporte de oxígeno. Aunque también se puede producir L(-)-carnitina en aerobiosis, la presión parcial de oxígeno tiene que controlarse muy cuidadosamente, puesto que a niveles óptimos conduce

a un compromiso entre la inhibición de la reacción lateral de reducción de crotonobetaína y la represión de la expresión del operón *cai* (Castellar et al., 1999; Cánovas et al., 2002). Las variables metabólicas y fisiológicas celulares están estrechamente ligadas a la capacidad de biotransformación. De hecho, el estado fisiológico de *Escherichia coli* determina en gran medida el rendimiento del proceso. La máxima productividad específica se observó en células en crecimiento exponencial, aunque la gran cantidad de biomasa en los sistemas continuos de reciclado celular y en los sistemas discontinuos con células durmientes hace posible las elevadas producciones obtenidas con estos sistemas. En continuo, las células en estado pre-estacionario son biocatalíticamente más activas, lo que coincide con una menor acumulación de células despolarizadas y muertas, mientras que en los sistemas con células durmientes el gran deterioro fisiológico celular está acompañado con una disminución de la productividad. La entrada en estado estacionario está acompañada de un importante deterioro de la viabilidad celular (Capítulo 2) y de importantes alteraciones metabólicas (Capítulo 4).

El análisis metabólico realizado en condiciones estándar y de estrés salino (Capítulos 4 y 5) nos permitió detallar la unión o integración entre la biotransformación y las rutas metabólicas centrales de *Escherichia coli* determinando así dianas para la optimización. El análisis de la evolución de actividades enzimáticas seleccionadas reveló adaptaciones tanto en el estado de crecimiento como en el estado durmiente. De hecho, los puntos de control más probables son el estado energético celular y el *pool* de acetil-CoA/CoA. Con el conocimiento actual, se sabe que el ATP es necesario en el metabolismo de compuestos de trimetilamonio para el transporte y la activación. ProU y ProP son dos sistemas de transporte dependientes de ATP dedicados a la acumulación de compuestos osmoprotectores bajo condiciones de estrés. Por otro lado, a pesar de que CaiT se ha descrito como intercambiador de betaínas (Jung et al., 2002; Vinothkumar et al., 2006), las células de *E. coli* desenergizadas exhiben tasas de incorporación de L(-)-carnitina menores (Cánovas et al., 2003a; Cánovas et al., 2003b). Parece factible que los niveles de ATP afecten indirectamente al proceso de transporte, muy probablemente a nivel de la conversión de los compuestos de trimetilamonio en sus derivados de CoA mediante la ligasa dependiente de ATP (Capítulo 6), que es un paso imprescindible para la biotransformación (Elssner et al., 2000).

Además de la actividad ligasa de CoA dependiente de ATP de CaiC, la actividad transferasa de CoA de CaiB recicla el grupo CoA entre sustratos y

productos, permitiendo que la biotransformación transcurra a través de una vía independiente de energía (Elssner et al., 2001). Es por esto que se propone que el papel conjunto de las actividades CaiB and CaiC en la activación de sustrato, junto con el metabolismo de acetil-CoA/CoA, que controla los flujos a través de rutas de producción de ATP (metabolismo del acetato) y anapleróticas (ciclo de los ácidos tricarboxílicos y ciclo del glioxilato) constituyen los principales puntos de control en la biotransformación (Capítulo 7). Además, hemos detectado *in vitro* una actividad transferasa de CoA para CaiC, si bien los experimentos realizados apuntan a que el papel más relevante *in vivo* es la actividad ligasa. Además, el análisis de los mutantes *knock-out* de *caiB* y *caiC,* así como su sobreexpresión nos permitieron determinar el papel de estas enzimas en la biotransformación (Capítulo 6), puesto que, a pesar de que CaiB es necesaria para la producción de L(-)-carnitina, su sobreexpresión no condujo a una mejora del rendimiento de la biotransformación en comparación con la cepa superproductora *E. coli* O44K74 (Capítulo 7).

Finalmente, la información obtenida en los estudios metabólicos nos permitió modificar genéticamente *E. coli* con objeto de mejorar la producción de L(-)-carnitina (Capítulo 7). La relación existente entre las rutas expresadas nos ha indicado que la disponibilidad de CoA libre para la activación de sustrato es limitante de la biotransformación, por lo que tenía que considerarse como posible cuello de botella del proceso. Puesto que, además, se ha demostrado la importancia de los cofactores celulares en la biotransformación (Capítulos 4 y 5), hemos llevado a cabo la modificación de sus niveles, lo que se conoce, en general, como ingeniería de cofactor. Con ese propósito se realizaron sobreexpresiones y deleciones de genes determinados. Las dianas seleccionadas fueron la activación de los compuestos de trimetilamonio y la relación acetil-CoA/CoA, que se modificaron mediante la expresión de las actividades CaiB y CaiC y mediante la adición de un precursor de la síntesis de CoA, el ácido pantoténico, al medio de cultivo. La sobreexpresión de CaiC permitió incrementar el rendimiento de la biotransformación. Además, a pesar de que no se consiguió mejorar la racemización de D(+)-carnitina en cultivos anaerobios discontinuos, se consiguió en experimentos microaeróbicos con células durmientes, subrayando, nuevamente, la importancia de los niveles de oxígeno para este proceso (Capítulos 1 y 6). El estudio de deleción de genes permitió determinar el papel limitante que la expresión de ciertas actividades del metabolismo central de *E. coli* tienen en la biotransformación. De hecho, se vio que la ruta de fosfotransacetilasa/acetato-quinasa (PTA-ACK) es esencial debido a su función en la síntesis de ATP en

anaerobiosis y a la necesidad de evitar la acumulación de acetil-CoA hasta niveles inhibitorios. Por otro lado, un descenso del flujo a través del ciclo del glioxilato aumentó la productividad, especialmente en células en crecimiento exponencial (Capítulo 7). El ciclo del fosfoenolpiruvato-glioxilato, recientemente descrito (Fischer y Sauer, 2003), se ha implicado en la limitación de la acumulación de cofactores redox reducidos, impidiendo así la producción de un exceso de NADH/NADPH, que es especialmente importante en las condiciones de crecimiento lento o limitado, como ocurre en el caso de células durmientes o en cultivos continuos de alta densidad.

A pesar de que la función del metabolismo de L(-)-carnitina en *E. coli* sigue sin conocerse, se ha propuesto anteriormente una posible función como ruta de respiración anaerobia (Kleber, 1997; Elssner et al., 1999). Además, se ha demostrado que la acumulación intracelular de betaínas y compuestos osmoprotectores relacionados es una estrategia general para la supervivencia celular en condiciones de estrés, no sólo en medios hiperosmóticos, sino también en otras condiciones. En determinadas especies, tales como *Listeria monocitogenes*, también es ha establecido una conexión con la capacidad infectiva *in vivo* (Sleator et al., 2003). En *E. coli* se ha propuesto la implicación de dos sistemas de transporte (ProU y ProP) en la incorporación de osmoprotectores (Csonka 1991; Verheul et al., 1998; Mcmillan et al., 1999), mientras que el intercambiador CaiT sólo funcionaría ligado a la biotransformación (Verheul et al., 1998; Jung et al., 2002). De hecho, en presencia de concentraciones elevadas de sal, la expresión del metabolismo de carnitina se reprime (Cánovas et al., 2003b), lo que apoyaría el hecho de que este metabolismo no supone una ventaja en osmoprotección. Los resultados aquí presentados apoyan esta función del metabolismo de L(-)-carnitina en *E. coli*. Además, tanto la respiración aerobia como anaerobia (con oxígeno o fumarato como aceptores electrónicos finales) aumentaron la producción de L(-)-carnitina, inhibiendo la "respiración de crotonobetaína" a nivel de crotonobetaína reductasa (CRR, catalizada por CaiA/CaiB). Por otro lado, a pesar de que el metabolismo de L(-)-carnitina se inhibe a concentraciones altas de sal, se observó un incremento en la producción en determinadas condiciones experimentales, favoreciéndose la biotransformación a raíz de las alteraciones metabólicas observadas en estrés salino.

Tanto el metabolismo como la fisiología celular están íntimamente ligados con el modo de operación del reactor, contribuyendo conjuntamente a determinar el rendimiento del bioproceso. Además, la configuración del reactor debe

seleccionarse en base al rendimiento del bioproceso en las condiciones seleccionadas, asegurando una expresión óptima y mínimas limitaciones por gradientes físicos y químicos. En este trabajo se ha subrayado la importancia de la elección del reactor debido a su efecto en la fisiología celular, que debería considerarse como un criterio importante, puesto que la máxima producción de L(-)-carnitina corresponde a células en crecimiento exponencial. En sistemas con células durmientes, el estado celular tiene una importancia crucial. De hecho, para asegurar rendimientos elevados se deberían alternar ciclos de biotransformación y re-energización en un proceso integrado. En condiciones de bioproceso, la fisiología celular se correlaciona en gran extensión con el estado intracelular, de modo que, alterando la configuración del reactor, el modo de operación o, incluso, las condiciones de estrés, se puede afectar la expresión y el rendimiento del bioproceso.

El escenario más realista para la interacción entre el metabolismo central y secundario durante la producción de L(-)-carnitina con *Escherichia coli* está determinado por la disponibilidad de cofactores. La evolución del nivel de ATP y de las relaciones NADH/NAD^+ y acetil-CoA/CoA está ligada a la producción (Capítulo 4) y también se ve afectada por el estrés salino (Cánovas et al., 2003b y Capítulo 5). La activación y el reciclado de compuestos de de trimetilamonio en *E. coli* y el destino metabólico de acetil-CoA son cuellos de botella en la biotransformación. Además, el estado energético de la célula no sólo afecta a la biotransformación, sino también al estado fisiológico de la bacteria. Recapitulando, en este trabajo se muestra la necesidad de una estrategia global de optimización de bioprocesos, considerando los niveles del reactor (macroscópico) y del microorganismo (microscópico). Además, para la mejora de cualquier proceso de biotransformación, tanto el metabolismo primario como el secundario deberían ser tenidos en cuenta, y el análisis de las adaptaciones metabólicas que ocurren en condiciones estándar y en presencia de estrés es un requisito previo para la selección de dianas de optimización. El análisis de las adaptaciones fisiológicas sufridas debería realizarse para asegurar la máxima expresión y rendimiento.

Aunque aún queda trabajo por delante, de los resultados aquí presentados puede concluirse que la interfase bioquímica-microbiológica-técnica debería ser el *leit motif* para la optimización de un bioproceso, puesto que la adecuada selección de una configuración de biorreactor y del modo de operación afecta al estado metabólico celular. La optimización de bioprocesos se encuentra en el punto de unión entre la química, la biología, la ingeniería y la informática, lo que hace precisa

una aproximación multidisciplinar. Además, la aplicación de técnicas analíticas para determinar la evolución de las poblaciones celulares, así como las adaptaciones metabólicas que estas sufren en conjunto (fundamentalmente enzimas, cofactores y metabolitos) permitirá el desarrollo racional de estrategias para la mejora de cepas. Además, la aplicación de técnicas novedosas para el análisis de la evolución del transcriptoma, proteoma y metaboloma en diferentes condiciones permitirá ahondar en la complejidad de las redes metabólicas.

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CONCLUSIONES

1. Se ha estudiado la biotransformación de compuestos de trimetilamonio en L(-)-carnitina:

   - El rendimiento con cepas salvajes es satisfactorio en los sistemas discontinuos y continuos estudiados, mientras que la inestabilidad segregacional de la cepa portadora de plásmidos es el principal inconveniente para su aplicación en la producción de L(-)-carnitina en continuo.

   - Crotonobetaína es mejor sustrato que D(+)-carnitina debido a que el nivel de expresión de la actividad enzimática carnitina racemasa es menor que el de la L(-)-carnitina deshidratasa.

   - La respiración bacteriana aumenta la producción de L(-)-carnitina en *E. coli* O44K74 y *E. coli* K38 pT7-5KE32 mediante (a) un aumento del nivel energético celular y (b) una inhibición de la actividad crotonobetainil-CoA reductasa (CaiA) tanto en sistemas continuos como discontinuos.

2. *Escherichia coli* O44K74 sufre importantes cambios fisiológicos y metabólicos como consecuencia de la adaptación a la ausencia o limitación por nutrientes.

   - En sistemas discontinuos con células durmientes y continuos de recirculación celular se detectan distintas poblaciones bacterianas mediante citometría de flujo multi-tinción, estando ligado el fin de la biotransformación de compuestos de trimetilamonio a la acumulación de células dañadas en el reactor.

   - La evolución del nivel de macromoléculas intracelulares refleja la tasa de división y el estado metabólico celular, especialmente en el caso del DNA·célula^{-1}, que aumenta en condiciones de baja tasa de división y que disminuye en sistemas con células durmientes como consecuencia de la ausencia de nutrientes.

3. La inestabilidad genética de *E. coli* K38 pT7-5KE32 en sistemas de cultivo continuo con células en suspensión es debida a la combinación del descenso del número de copias del plásmido y a la alta tasa de división celular. La menos tasa de división en sistemas continuos con células inmovilizadas supone un descenso de la carga metabólica, permitiendo la segregación correcta de los plásmidos entre las células hijas y contribuyendo a la estabilización genética de la cepa. En el estado inmovilizado, las células

experimentan un aumento en el nivel de proteínas intracelulares (40% aprox.), mientras que la concentración de RNA está fuertemente controlada por la maquinaria celular, permaneciendo prácticamente constante durante los procesos en continuo.

4. El metabolismo secundario de compuestos de trimetilamonio en *Escherichia coli* O44K74 está conectado con el metabolismo central (o de carbono) a nivel de cofactores, especialmente la necesidad de ATP para el transporte y activación de sustratos y de coenzima A libre. De hecho:

   - El estado energético de la célula se correlaciona con la activación del ciclo del glioxilato (isocitrato liasa) bajo condiciones de limitación/ausencia de sustrato (células durmientes y estado estacionario de reactores continuos), y del ciclo de Krebs (isocitrato deshidrogenasa) en condiciones de crecimiento celular rápido (células en crecimiento exponencial y puesta en marcha de reactores continuos), marcando las limitaciones metabólicas a la producción de L(-)-carnitina.
   - La expresión del metabolismo del acetato (fosfotransacetilasa y acetil-CoA sintetasa), que controla la relación de acetil-CoA/CoA, es superior en sistemas anaeróbicos en continuo.

5. Aunque el metabolismo de carnitina de *Escherichia coli* O44K74 no está relacionado con la osmoprotección y su expresión se reprime a concentraciones altas de sal,

   - El estrés salino incrementa la producción de L(-)-carnitina debido a la combinación de la permeabilización de la membrana celular y la activación de rutas metabólicas para la generación de energía y precursores (ciclo del glioxilato, ciclo de Krebs y metabolismo del acetato) tanto en sistemas discontinuos con células durmientes como en sistemas continuos en crecimiento.
   - La realización de pulsos en biorreactores es una estrategia muy adecuada para el estudio dinámico del metabolismo y la fisiología celulares.

6. El cuarto gen del operón *cai*, CaiC, es una ligasa de CoA dependiente de ATP, que es activa sólo sobre D,L-carnitina, crotonobetaína y γ-butirobetaína. Además, se ha observado que:

- A pesar de que en extractos libres de células se ha detectado una actividad betaína:coenzima A transferasa *in vitro*, los datos *in vivo* apoyan que esta no es su función principal.
- Tanto la actividad carnitina:CoA transferasa (CaiB) como la carnitina:CoA ligasa (CaiC) son necesarias para la producción de L(-)-carnitina por *Escherichia coli*.
- La sobreexpressión de CaiC provocó un incremento en la producción de L(-)-carnitina tanto por células en crecimiento como durmientes, a partir de crotonobetaína y D(+)-carnitina, indicando que la activación de betaínas es un paso limitante en la biotransformación.

7. Finalmente, la disponibilidad de coenzima A libre afecta al rendimiento de la biotransformación. Este trabajo ha mostrado que:
   - La sobreexpresión de las enzimas implicadas en la activación del sustrato del *Escherichia coli* (CaiB y CaiC) permite modificar el *pool* celular de betainil-CoAs, provocando un aumento de dos a diez veces en el rendimiento de la biotransformación.
   - La expresión de las rutas de glioxilato y acetato son parámetros de control para el metabolismo de L(-)-carnitina debido a su papel en el destino metabólico de acetil-CoA y en el mantenimiento de la relación acetil-CoA/CoA.
   - La deleción de genes del ciclo del glioxilato aumentó la producción de L(-)-carnitina en un 20-25%, mientras que la supresión de fosfotransacetilasa suprimió prácticamente en su totalidad la biotransformación.

ENGLISH VERSION

ABSTRACT

In this PhD Thesis, the optimization of L(-)-carnitine production using the trimethylammonium compound metabolism of *Escherichia coli* has been tackled from the bioprocess and metabolic points of view.

A systematic analysis of the main factors which affect L(-)-carnitine production using *Escherichia coli* strains has been undertaken, and the effect of the availability of electron sinks (such as oxygen or fumarate) in both batch and continuous operation conditions have been studied. An analysis of the effect on the cells using flow cytometry, allowed us to determine the responses in intracellular levels of DNA, RNA and proteins ant their integration with the heterogeneity of cell population. This allowed a novel way for analyzing how reactor configuration affects the physiology of cells. Further, the DNA content was studied in transformed *E. coli* cells in continuous culture, allowing to determine the factors affecting the genetic stabilization of the immobilized strain. Further, the expression of secondary metabolism was coordinated with central pathways by general regulatory proteins, the evolution of the pools of cofactors and metabolites largely ensuring the linking of both metabolisms. Metabolic analysis under stress conditions revealed an enhancement in productivity due to cell permeabilization and the activation of metabolic pathways for the generation of energy and precursors. CaiC protein was cloned, overexpressed and partially characterized, revealing to be a highly specific CoA ligase. L(-)-carnitine production was engineered in CaiB (CoA transferase) and CaiC (CoA ligase) overexpression and deletion strains, underlining the importance of substrate activation. Finally, the TCA cycle, the glyoxylate shunt and the acetate metabolism, which expression is related to the biotransformation, limit maximum productivity, and novel engineering strategies were developed and experimentally assessed for strain improvement.

Taking all together, not only metabolism, but also cell physiology are in deep relation with the mode of reactor operation and jointly contribute to determine the performance of the bioprocess. The role of the cell metabolic state as a determinant in L(-)-carnitine production has been further clarified, specially at the cofactor level (ATP and acetyl-CoA/CoA). Furthermore, bioreactor operation, determining the physiological state of *Escherichia coli* cells has been shown to limit to a great extent the performance of the bioprocess. In addition, the function of CaiC has been determined and, more importantly, the importance of the joint consideration of central and secondary metabolisms in the improvement of strains for biotechnological ends has been stated.

INTRODUCTION

A PERSPECTIVE ON THE HISTORY OF L(-)-CARNITINE

L(-)-carnitine (R(-)-3-hydroxy-4-trimethylaminobutyrate) is an ubiquitous compound, found in animal and vegetal tissues, as well as in microorganisms. In the early 20th century, L(-)-carnitine was first found in muscle extracts, thus being named from the Latin ***caro, carnis*** (meaning flesh or meat), almost simultaneously by Gulewitch and Krimberg in Russia and Kutscher in Germany. For years, its function and structure remained unknown. In 1927, Tomita and Sendju confirmed its chemical structure and during the 1930s, first physiological investigations on L(-)-carnitine functions were performed by Strack in the University of Leipzig. Fraenkel and collaborators discovered that in mealworms (*Tenebrio molitor*) L(-)-carnitine was an essential growth factor in addition to folic acid and the other B vitamins; L(-)-carnitine was thus called vitamin B_T. In 1955, Fritz found that the rate of fat burning at mitochondrial level depended on L(-)-carnitine levels, establishing its fundamental role in fat oxidation for the first time. Basic research in the metabolic role of L(-)-carnitine as well as in its applications continued throughout the second half of the 20th century. In the 1980's, L(-)-carnitine became commercially available and in 1993 it was established to be Generally Recognized As Safe (GRAS) as a dietary supplement, by an independent expert committee of scientists.

PHYSIOLOGICAL ROLE OF L(-)-CARNITINE IN HUMANS

L(-)-carnitine plays a significant role at various stages of the intermediary metabolism: for example, in the β-oxidation of medium and long chain fatty acids in the mitochondria, in the oxidation of fatty acids in the peroxisomes, in the exchange of acyl and acetyl groups with CoA in the mitochondria (thus altering the acyl-CoA/CoA and acyl-carnitine/carnitine ratios) and in the production of the ketone bodies. Acetyl-L(-)-carnitine may be seen as a second form of activated acetic acid, an acetyl buffer or a depot of acetyl groups (Löster, 2003). Hence, L(-)-carnitine is considered an essential and integral part of fatty acid catabolism in the mitochondria and is closely related to acetyl-CoA in various cell organelles, playing a fundamental role in energy metabolism. The metabolization of lipids provides energy for longer periods and more prolonged load than carbohydrates, and L(-)-carnitine is typically used in all bodily functions that have a high energy demand. This role is especially relevant in the physiology of the heart which, because of its higher energy demand, mainly depends on the metabolization of lipids and fatty acids (Löster, 2003). In addition, acetyl-L(-)-carnitine

participates in neuronal metabolism as acetyl-donor in the biosynthesis of acetyl-choline. Many other functions of L(-)-carnitine have been established: its implication in the heart, liver, muscle, brain and lipid metabolisms has been proved, as well as a certain role in sperm maturation, immune system and connecting tissue (Löster, 2003).

L(-)-carnitine is present to varying extents in foods of animal origin, while fruits and vegetables contain very little, if any. A small amount of L(-)-carnitine is naturally produced within the human body and high concentrations are present in the human heart and skeletal muscle, where it helps support physiological activity. Adults store about 20 g of L(-)-carnitine, primarily in skeletal muscle, in the liver and in the heart. The major sites for L(-)-carnitine biosynthesis are the liver and kidney (Löster, 2003) and this involves a series of steps, requiring two essential amino acids, lysine and methionine, as substrates. Vitamin C, iron, vitamin B_6 and niacin are needed as cofactors and malnutrition exerts a highly negative effect on its biosynthesis. Approximately 20 mg of L(-)-carnitine are produced per day, which represents around 10% of the daily necessities. A well balanced diet can additionally supply 100-300 mg of L(-)-carnitine to the body each day. Interestingly, for infants, L(-)-carnitine is essential because of limited endogenous synthesis during the first months of life.

Summarizing, the L(-)-carnitine requirements of the organism can be met by endogenous synthesis and exogenously supplied food. In the healthy organism there is also no notable catabolism but L(-)-carnitine is excreted in the urine, mostly as esters. Healthy adults store adequate quantities of L(-)carnitine and do not need additional contribution from food, although an exogenous supply can be of importance in various disorders, in pregnancy and old age, as well as in the case of deficiencies (Borum, 1991). L(-)-carnitine can therefore be considered an essential nutrient. Deficiency can be defined as an intracellular deficit of L(-)-carnitine, with free concentrations of less than 20 µmol/L and tissue concentrations of less than 10-20% of normal values. This deficit leads to an accumulation of acyl-CoA esters and an inhibition of the transport of acyl groups through the inner mitochondrial membrane. L(-)-carnitine deficiencies have been subdivided into primary (due to a basic defect in carnitine metabolism) and secondary (as a result of other diseases and conditions). Primary carnitine deficiencies are characterized by myopathy, episodes of hypoketotic hypoglycemia, hyperammonemia, failure to grow and cardiomyopathy. Secondary deficiencies are associated to other metabolic abnormalities which are characterized by heterogeneous clinical symptoms with excessive lipids and low carnitine concentrations in muscle. On other occasions, deficiency can be due to acquired disturbances, such as haemodialysis, parenteral nutrition, valproic acid therapy, hepatic cirrhosis with

cachexia, Reye-like syndrome, various chronic muscular diseases, endocrine disorders, acquired immune deficiency syndrome (AIDS), kwashiorkor and cardiomyopathy after diphtheria.

APPLICATIONS OF L(-)-CARNITINE

Multiple applications have been found for L(-)-carnitine. First of all, it is administered in the treatment of the specific deficiencies summarized above. In addition, L(-)-carnitine supports cardiovascular system, increasing heart muscle viability and the maintenance of a healthy heartbeat, and promotes a healthier weight. Because of its fundamental role in fat metabolism, L(-)-carnitine also has multiple benefits for the optimization of sports performance, delaying the onset of fatigue, and improving recovery processes. Other clinical research data also indicate its role in maintaining cholesterol and triglyceride levels, improving weight management and muscle deposition. Recent works have shown that oral L(-)-carnitine supplementation improves long chain fatty acid oxidation in healthy persons (Muller et al., 2002) and in slightly overweight adults (Wutzke and Lorentz, 2004). Used in conjunction with a reduced calorie diet and moderate exercise, L(-)-carnitine supplementation resulted in a much greater loss in body weight in obese people, also regulating low density lipoprotein (LDL) and sugar levels in blood and reducing blood pressure.

As regards the brain, acetyl-L(-)-carnitine has restorative or even protective properties against aging processes and neurodegeneration, helping in the maintenance of brain function. L(-)-carnitine dietary deficiencies can occur in newborn babies, vegetarians and high performance athletes. L(-)-carnitine is also recommended to overcome deficiencies in sperm quality, number, motility or shape. Finally, a decrease in L(-)-carnitine content has been described during aging, mainly due to lower energy demand and changing dietary habits, together with a decreased endogenous synthesis. For a comprehensive review of L(-)-carnitine role in human health, see Löster (2003).

THE PRODUCTION OF L(-)-CARNITINE

CHEMICAL vs. BIOTECHNOLOGICAL METHODS

As a result of the many possible applications of L(-)-carnitine and the increasing demand for this product, much research effort has been focused on the development of

methods for its industrial scale production. Numerous chemical procedures for L(-)-carnitine production have been reported in the literature involving asymmetric synthesis (Kitamura et al., 1988; Kolb et al., 1993); resolution through diasteroisomeric derivatives (Cavazza, 1981; Voeffray et al., 1987); microbiological or enzymatic techniques (Kasai and Sagaguchi, 1992; Hashiguchi et al., 1992; Jung et al., 1993) and the use of chiral starting materials (Takano et al., 1987; Bellamy et al., 1990; Bols et al., 1992). For instance, the method developed by Bellamy et al., consisted of 6-steps which, using as starting material (R)- and (S)-malic acid, respectively, specifically obtained both enantiomers. More recently, Marzi et al., (2000) from the Department of Chemical Research of Sigma-Tau described an enantioselective synthesis of L(-)-carnitine using achiral glycerol as starting material and a chiral auxiliary. However, few of these chemical procedures are of practical use on an industrial scale. The potential advantages of biotechnological methods, employing both enzymes and microorganisms have motivated extensive research into the microbial metabolism of L(-)-carnitine and its derivatives (Kulla, 1991, Jung et al., 1993, Kleber, 1997, Naidu et al., 2000).

USE OF BACTERIA FOR THE PRODUCTION OF L(-)-CARNITINE

The biotechnological procedures for L(-)-carnitine production have advantages over the chemical processes: 50% less total organic waste, 25% less waste water and 90% less waste for incineration. The most commonly used starting materials for the production of L(-)-carnitine are achiral precursors (mostly crotonobetaine, γ-butyrobetaine and 3-dehydrocarnitine) or racemic mixtures (such as D,L-acyl-carnitine, D,L-carnitinamide and D,L-carnitine) (Jung et al., 1993; Naidu et al., 2000). The microorganisms used for these biotransformations are summarized in Table 1.

Since the early 1980s, many companies worldwide have patented bioprocesses for L(-)-carnitine production (Seitetsu, Kyowa Hakko, Chou Kaseihih, Toyo Jozo, Ajinomoto, Sigma Tau, Lonza, Nippon Pet Food, Yakult Honsha, Elf Aquitaine, Sanofi) (Naidu et al., 2000). As an example, while bioprocesses developed for commercial production of L(-)-carnitine by Sigma Tau (Italy) are based on the biotransformation of crotonobetaine by *Escherichia coli* and *Proteus mirabilis* strains, Lonza (Switzerland) has been using γ-butyrobetaine as starting material and a derivative of the HK4 strain. This latter strain was isolated from a soil sample and its carnitine pathway resembled that found in a strain situated between *Agrobacterium* and *Rhizobium.* This strain was able to grow on L(-)-carnitine as the sole source of carbon and nitrogen under aerobic conditions. In the production strain, the degradation of L(-)-carnitine was blocked by frameshift mutagenesis, giving rise to a derivative strain, HK13, lacking L-carnitine

dehydrogenase (Kulla and Lehky, 1985). This pathway from γ-butyrobetaine to L(-)-carnitine is analogous but not identical to fatty acid degradation (Kulla, 1991). In a similar manner, several Cai proteins of *Escherichia coli* show homologies with enzymes involved in fatty acid degradation, such as acyl-CoA dehydrogenase and CaiA, acetate-CoA ligase and CaiC, and enoyl-CoA hydratase and CaiD (Eichler et al., 1994a).

Table 1. Precursors for the production of L(-)-carnitine using microorganisms. (Adapted from Naidu et al., 2000)

	Substrates	**Strains**
Achiral precursors	Crotonobetaine	*Escherichia coli, Proteus mirabilis, Acinetobacter lwoffi, Achromobacter xylosoxydans*
	γ-butyrobetaine	HK4, HK13, HK1349, *S. cerevisiae, Penicillium, Rhizopus, Mucor, Actinomuchor, Neurospora, Aspergillus, Achromobacter, Pseudomonas, Nocardia crassa*
	3-dehydrocarnitine	*Agrobacterium, Pseudomonas*
Racemic mixtures	D,L-carnitinenitrile	*Corynebacterium sp.*
	D,L-acyl-carnitine	*Fusarium oxysporum sp. lini, Corynebacterium, Bacillus, Pseudomonas*
	D,L-carnitineamide	*Pseudomonas sp.,* DSM 6320 *(Agrobacterium or Sphingomonas sp.)*
	D,L-carnitine	*Acinetobacter calcoaceticus* and *Acinetobacter lwoffi* (assimilation of D-isomer), *Pseudomonas sp.* and *Escherichia coli* (racemization of D-isomer)

THE METABOLISM OF L(-)-CARNITINE IN BACTERIA

Although the role of L(-)-carnitine is well established in eukaryotic cells, it is not so clear in bacteria (Kleber, 1997). The existence of uptake systems with different degrees of specificity in very different bacteria has been connected with its protective properties (Jung et al., 1990; Verheul et al., 1998). Moreover, in some species, such as *Listeria monocitogenes*, the existence of betaine uptake systems has been related to its ability to grow and survive in foods and to provoke infections *in vivo* (Sleator et al., 2003).

In addition to the protective roles exhibited after accumulating betaines, some bacterial species are also able to metabolize these trimethylammonium compounds under different conditions. Depending on the species and cultivation conditions (carbon and nitrogen sources, aerobic or anaerobic conditions), different pathways are involved in L(-)-carnitine catabolism (Fig. 1). The initial enzymes of the various catabolic pathways are induced by L(-)-carnitine, but also partly by other trimethylammonium compounds. Different genera are able to degrade L(-)-carnitine under aerobic conditions. Some *Pseudomonas* species (like *Pseudomonas aeruginosa* A7244 and *Pseudomonas* sp. AK1) are able to grow aerobically on L(-)-carnitine as the sole source of carbon and nitrogen. In these species, L(-)-carnitine degradation starts by oxidation of the hydroxyl group with the concomitant formation of 3-dehydrocarnitine by a L(-)-carnitine dehydrogenase (Aurich et al., 1967). *Pseudomonas* sp. AK1 is also able to grow on γ-butyrobetaine, which is an intermediate in the degradation pathway (Lindstedt et al., 1977). This pathway has similarities with the biosynthetic pathway of L(-)-carnitine in eukaryotes. Furthermore, some species like *Acinetobacter calcoaceticus* 69/V are not able to assimilate nitrogen from the L(-)-carnitine skeleton and degradation occurs with the stoichiometrical formation of trimethylamine (Miura-Fraboni et al., 1982). This bacteria is able to metabolize L(-)-carnitine, L-O-acylcarnitines and γ-butyrobetaine as sole carbon sources. D(+)-carnitine can also be metabolized but only in the presence of L(-)-carnitine to act as an inducer (Miura-Fraboni et al., 1982). The stereoselectivity assessed in *Acinetobacter* metabolism could be a result of the existence of two separate transport systems for D- and L- isomers, since the wild-type strain *A. calcoaceticus* ATCC 39647 exhibited enantiomer discrimination due to the differential cell membrane permeability (Ditullio et al., 1994).

On the other hand, Enterobacteriaceae, such as *Escherichia coli*, *Salmonella typhimurium*, *Proteus vulgaris* and *Proteus mirabilis,* do not assimilate the carbon and nitrogen skeleton of trimethylammonium compounds, but are able to metabolize carnitine, *via* crotonobetaine, to γ-butyrobetaine (Kleber, 1997). The presence of adequate carbon and nitrogen sources during anaerobic (and in some cases also

aerobic) growth is necessary for proper expression of the biotransformation machinery. Further, the biotransformation can also occur in the absence of nutrients, as shown in studies with resting cells (Castellar et al., 1998).

In contrast to the ubiquitously occurring L(-)-carnitine, the D-enantiomer does not exist in nature. However, it is produced as a waste product in some chemical procedures for L(-)-carnitine production based on the resolution of racemic carnitine or its precursors through the formation of their diastereoisomers by means of optically active acids. Nevertheless, various bacteria are able to catabolize D(+)-carnitine (Kleber, 1997).

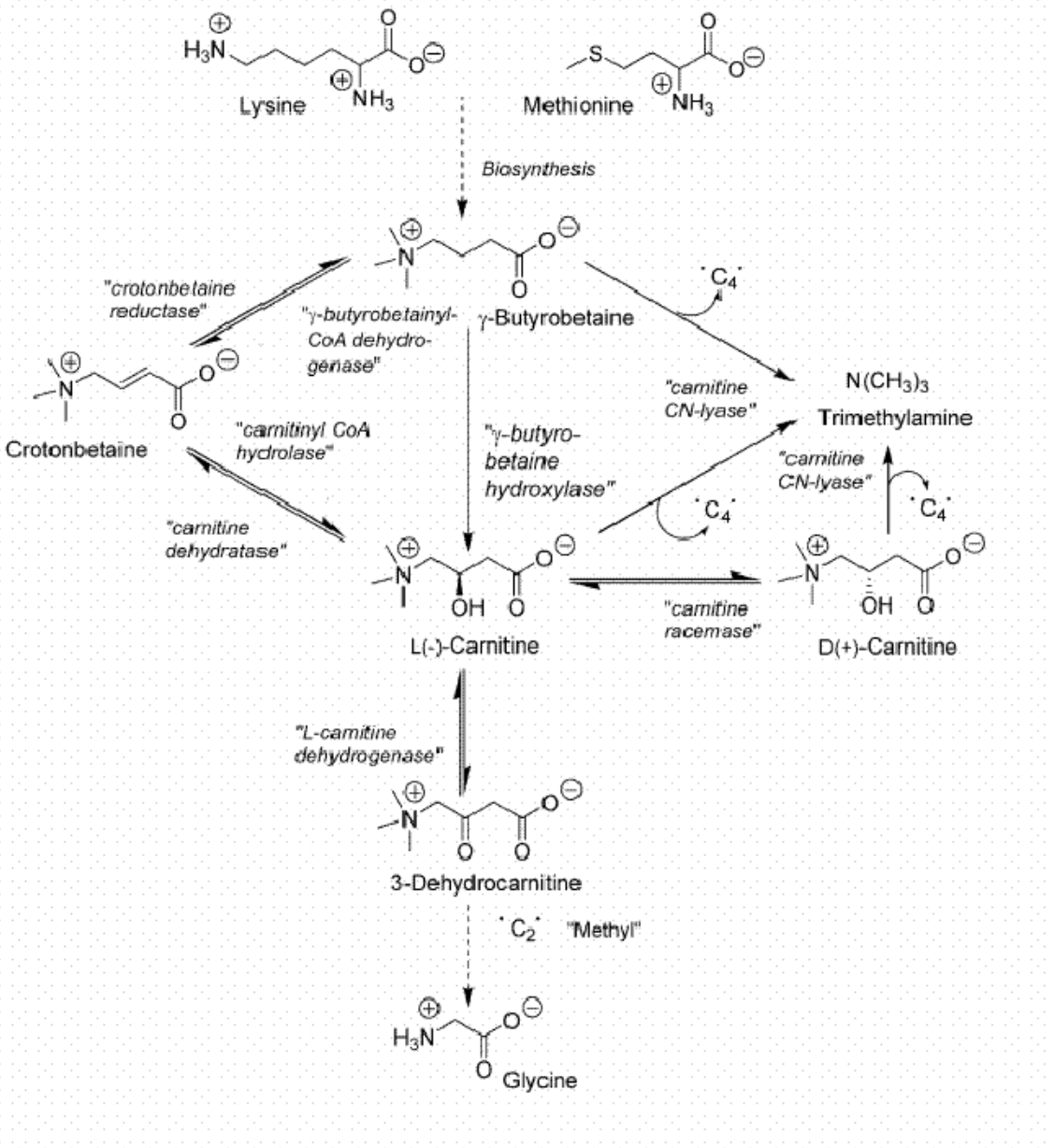

Figure 1.. Overview of microbial metabolism of L(-)-carnitine. [Adapted from Kamm et al., (2005)].

METABOLISM OF L(-)-CARNITINE IN ENTEROBACTERIA: *Escherichia coli* AND *Proteus sp.*

Initially, a two-step pathway was proposed for L(-)-carnitine metabolization in *Escherichia coli* and *Proteus* sp. strains, including two enzyme activities: an L(-)-carnitine dehydratase (CDH) and a crotonobetaine reductase (CR) (Eichler et al., 1994b, Roth et al., 1994). Subsequently, carnitine racemase activity (CRac), interconverting the D- and L-isomers, was also described (Jung and Kleber, 1991). High regulation of this metabolism has been demonstrated in *Escherichia coli* and *Proteus sp.* strains. The cloning of the *cai* operon in *Escherichia coli* (Eichler et al., 1994a) showed that it was composed of six ORFs and functions were assigned on the basis of sequence homology (Table 2). More recent studies have stated that CaiT is a highly specific transporter working as antiporter, allowing substrate and product exchange (Jung et al., 2002, Vinothkumar et al., 2006). Further studies showed that the biotransformation occurs at the CoA level (Elssner et al., 2000). In addition, the initially described CDH and CR activities were shown to depend on two proteins (enoyl-CoA hydratase, CaiD, and crotonobetainyl-CoA reductase, CaiA, respectively) needing the joint action of a transferase (CaiB) to allow the CoA-cycling between products and substrates of the biotransformation (Elssner et al., 2001). CaiC (betainyl-CoA ligase) has been shown to catalyze the synthesis of the CoA-derivatives of trimethylammonium compounds, which are the substrates of both CaiD and CaiA activities (Bernal et al., 2006). The function of CaiE remains unconfirmed, though early overexpression experiments pointed to an activation of the CaiD/CaiB function (CDH activity), suggesting a role as cofactor for these enzymes (Eichler et al., 1994a). More recently, the cloning and sequencing of the *cai* operon in *Proteus sp.* showed a similar organization and a high level of homology between these two strains (Engemann et al., 2005) (Table 3).

The *cai* operon is located at the first minute of the chromosome of *Escherichia coli*. Its transcription is induced during anaerobic growth in the presence of L(-)-carnitine and occurs as a polycistronic mRNA. As regards the expression mechanism, the activator of carbon catabolic operons, CRP, is required for induction. In addition, the histone-like H-NS protein and the σ^S factor (RpoS), which is required for the activation of stationary-phase genes, exert a repressive effect on carnitine metabolism (Eichler et al., 1994a; Buchet et al., 1998; Buchet et al., 1999). A seventh ORF, the *caiF* gene, which is located in the 3' region of the *cai* operon and is transcribed in the opposite direction to this, has been cloned (Eichler et al., 1996) (Tables 2 and 3). The product is a transcriptional factor which is constitutively expressed under anaerobiosis

and which, in the presence of carnitine, is able to induce the expression of *cai* genes (Buchet et al., 1999).

Table 2 Properties of the *cai* genes and functions of the corresponding gene products in *E. coli* O44K74. (Eichler et al., 1994a; Ecocyc-Metacyc: Keseler et al., 2005)

Gene	Size (bp)	Function of gene product	Reference
caiT	1515	Transport protein	Jung et al., (2002)
caiA	1143	Crotonobetainyl-CoA reductase	Preusser et al., (1999)
caiB	1218	Betainyl-CoA transferase	Elssner et al., (2001)
caiC	1569	Betainyl-CoA ligase	Bernal et al., (2006)
caiD	894	Crotonobetainyl-CoA hydratase	Elssner et al., (2001)
caiE	591	Unknown	Eichler et al., (1994a)
caiF	396	Transcriptional regulator	Eichler et al., (1996)

Table 3 Properties and assigned functions of the *cai* genes and corresponding gene products in *Proteus sp.* (Adapted from Engemann et al., 2005)

Gene	Size (bp)	Function of gene product	Homology with *E. coli* (%)
caiT	1515	Transport protein*	88
caiA	1140	Crotonobetainyl-CoA reductase	92
caiB	1218	Betainyl-CoA transferase	85
caiC	1554	Betainyl-CoA ligase*	69
caiD	783	Crotonobetaynil-CoA hydratase	83
caiE	591	Unknown	77
caiF	390	Transcriptional regulator*	51

(*) Postulated function based on sequence similarities

Another operon composed of four ORFs was found in *E. coli* at the 5' end of the *cai* locus. It was shown to be co-transcribed from the same promoter/operator region (Eichler et al., 1995) and the corresponding proteins displayed significant sequence homology with polypeptides encoded by the *fix*ABCX operon from *Azorhizobium caulinodans* and *Rhizobium meliloti*. The four ORF were thus named *fix* and were proposed to be involved in electron transfer to crotonobetaine (Eichler et al., 1995), which was later confirmed (Walt and Kahn, 2002). Deletion studies have also shown that part of the *fix* sequence is necessary for the proper expression of *cai* operon (Buchet et al., 1998).

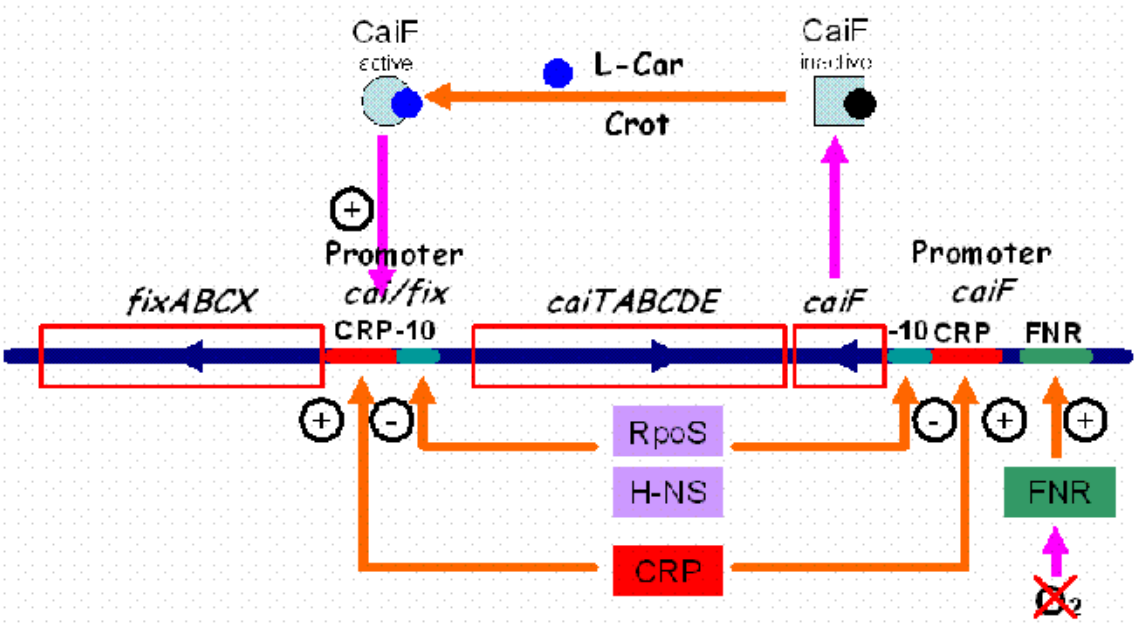

Figure 2. Schematic representation of the mechanism for the regulation of the expression of the *cai* and *fix* operons. RpoS: sigma subunit of RNApolymerase; CRP: cAMP receptor protein; H-NS: histone-like proteins. FNR: transcriptional activator for anaerobiosis. (Adapted from Eichler et al., 1994a).

Despite all this accumulated knowledge, the precise function of this reaction sequence in Enterobacteria remains unknown. Seim et al., (1982a) postulated that crotonobetaine serves as an external electron acceptor of anaerobic respiration similar to nitrate, fumarate and trimethylamine-N-oxide (Haddock and Jones, 1977). The stimulation of anaerobic growth in Enterobacteria by crotonobetaine and the suppression of this reaction by nitrate or glucose (Seim et al., 1982a, 1982b) certainly

support this hypothesis. The functional characterization of the transport system as an antiport (Jung et al., 2002) is also in accordance with this idea and would explain why this kind of transporter cannot be involved in osmoprotection (Verheul et al., 1998). However, the aerobic expression of carnitine metabolism in several Enterobacteriaceae (Elssner et al., 1999, Engemann and Kleber, 2001), including *Proteus* strains, suggests a possible loss of function due to mutations affecting the regulation of the expression of this pathway. In addition, some results show that microorganisms of the gastrointestinal tract may play a role in lowering the concentration of dietary L(-)-carnitine (Seim et al., 1982b; Kleber, 1997).

BIOTRANSFORMATIONS IN THE INDUSTRY

THE METABOLISM OF L(-)-CARNITINE MEETS BIOPROCESS AND METABOLIC ENGINEERING.

Despite the increasing demand for proteins for therapeutic application, the main focus of the pharmaceutical industry is still on small-molecules (MW<1000 Da). Moreover, increasing awareness of the variable activity of different optical isomers and the consequent increased regulatory pressure has accelerated the drive to manufacture and market only chirally pure therapeutic compounds (Persidis, 1997).

In nature, only L(-)-carnitine is present. Moreover, cellular uptake systems are not able to distinguish between the two enantiomers, even though D(+)-carnitine is not functionally active. Thus, the administration of racemic mixtures to humans would have the detrimental effect of decreasing total L(-)-carnitine cellular content. Pharmaceutical companies have been producing enantiomerically pure therapeutics for more than 30 years. The characteristic selectivity and soft reaction conditions of biocatalysts make them suitable candidates for use in the production of high added value compounds. Both isolated enzymes and whole cells systems can be applied. In general, isolated enzymes are used for simple reactions not requiring multiple enzymes or cofactors. On the other hand, whole cells are more appropriate when reactions involve multiple enzyme steps and/or cofactor recycling (Buckland et al., 2000).

Despite the efforts made to optimize L(-)-carnitine production using bacterial strains, most of the works found in the literature are devoted to the improvement of reactor performance. Very little is known about the characteristics that the bacterial metabolic landscape must fulfil to favour L(-)-carnitine production. Microbiologists and molecular biologists have thoroughly described the genetic constraints controlling the

expression of L(-)-carnitine metabolism in *Escherichia* and *Proteus* strains (Eichler et al., 1994a, 1996; Kleber, 1997; Elssner et al., 2001; Engemann et al., 2001 and 2005). This, together with the optimization studies performed by biochemical engineers has permitted the design of optimized media (Castellar et al., 1998) and the effect of environmental stress situations on the bioprocess to be assessed (Cánovas et al., 2003b). Industrially applicable and scalable biosynthetic methodologies for L(-)-carnitine production have been developed (Cánovas et al., 2002; Cánovas et al., 2003c; Giuliano et al., 2003). However, although the productivities attained are still far from what might be hoped, the yields obtained using wild type strains seem to have reached a plateau, and the way forward probably involve the application of new improvement methodologies to engineer bacterial metabolism and genetically altering the constraints of the system.

Metabolic engineering was first defined as the *"improvement of cellular activities by manipulation of enzymatic transport and regulatory functions of the cell with the use of recombinant DNA technology"* (Bailey, 1991). This definition was extended by Cameron and Tong (1993) who established metabolic engineering as the *"purposeful modification of intermediary metabolism using recombinant DNA techniques".*

The remarkable development of recombinant DNA techniques, which are now available for a wide range of organisms, has increased the range of opportunities in biocatalysis. The possibility of engineering cells by deleting or overexpressing whole pathways has opened up a new field in biotechnology: tailor-made microorganisms for bioprocesses. So far, very few works have tackled the use of genetically engineered strains for L(-)-carnitine production (Castellar et al., 2001; Cánovas et al., 2003b). Thus, the challenges for metabolic engineering are clear, since, most importantly, no rational or purposeful choice of target gene has been made. Furthermore, any optimization of bioprocesses involving secondary metabolism of the cell must not overlook the need to establish the optimal expression profile of primary metabolism. This is especially important when certain substrates or cofactors are shared by both metabolisms.

Today, there is growing awareness of the power of metabolic engineering approaches to design new biosynthetic pathways or to optimize existing ones. Recombinant DNA technology allows metabolic engineering for biotechnological ends, typically in metabolite overproduction. Improving metabolic pathways might involve modifying existing routes (altering expression and regulatory networks) as well as engineering new pathways by introducing foreign genes. The advantages of this

approach are clear compared with the main limitations of the classical random mutagenesis-driven strain improvement schemes.

The tools for Metabolic Engineers are experimental, theoretical and computational (Mendes and Kell, 1997). After completion of the genome sequences of many organisms, greater knowledge of the behavioural modes or algorithms of organisms is necessary. In this post-genomic era, the new challenge is to identify the function of all sequenced genes and, more importantly, to understand their concerted expression, defining the cellular phenotype. One important novel aspect of metabolic engineering is the emphasis placed on the integration of metabolic pathways as opposed to individual steps, considering the overall result from a network of reactions. Thus, it is fundamentally important to understand and control *in vivo* metabolic fluxes (Buckland et al., 2000) so that necessary strategies can be designed in a rational way.

In the field of natural products, a vast array of final compounds can be obtained by a combination of chemical synthesis and biosynthesis. This is the case with antibiotics such as penicillins, cephalosporins and macrolides. Knowledge of complex metabolic pathways at molecular level has blurred the traditional distinction between natural products and bioconversions. Microbial fermentation is now very well established as a reliable and cost-effective route for the synthesis of many organic compounds (e.g. citrate and glutamate), though yields of secondary metabolites are lower. Synthesis of organic compounds in the pharmaceutical industry can make use of bioprocess steps to make key intermediates, to implement biotransformations or to construct key natural product building blocks.

Biocatalysis in pharmaceutical applications often uses fed-batch processes in stirred tanks because these are better documented, process control is easier and the yields attained are high. In addition, stirred-tank bioreactors satisfy the aseptic requirements of biological processes. However, the many advantages of continuous processes make their design and development an interesting goal for many industrial scale applications. One of the main needs in the optimization of bioprocesses is the development of suitable analytical techniques, especially if these can sufficiently shorten the analysis time and allow more precise control of processes. In addition, techniques able to determine, and even quantify, the effect of reactor configuration on bacterial physiology are necessary, especially when genetic engineered strains are being employed.

Thus, although a lot of ground has been covered, further work on bioprocess development and optimization is still necessary, especially at the interface of biochemistry/biology and engineering.

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ORGANIZATION OF THIS PhD-THESIS

The optimization of bioprocesses is a complex issue which has to be tackled from a multidisciplinary point of view. In the field of biochemical engineering and biotechnology it is common to find joint efforts from microbiologists, engineers, chemists and biochemists, since the final performance of a bioprocess will depend on the knowledge of cell physiology, optimization of the functioning of biochemical networks of cells, optimization of bioprocesses, the correct choice of reactors, and an analysis of the variables of processes and models.

In the present PhD thesis, the production of the pharmaceutical L(-)-carnitine was taken as a model study system and a multiobjective approach was undertaken. As already mentioned, the existence of a biotransformation pathway in Enterobacteria can be used for the production of L(-)-carnitine. The *in vivo* and *in silico* data gained in our research group has allowed us to design new methodologies for the optimization of L(-)-carnitine production. The increasing complexity of the models developed has permitted the optimization of operation conditions of the bioreactors (Cánovas et al., 2002; Álvarez-Vásquez et al., 2002), a better understanding of the reactor-microorganism interface (Sevilla et al., 2005a) and even the deciphering of metabolic fluxes and optimization of the linking between central and carnitine metabolisms in terms of energy management and cofactor reutilization (Sevilla et al., 2005b). This work has permitted us to focus on targets for the improvement of bioprocess performance using metabolic engineering principles and tools.

In the first part of the work, a systematic analysis of the main factors which affect L(-)-carnitine production using *Escherichia coli* strains (both wild-type and transformed) is carried out. Batch and continuous systems are compared, and the effect of operation conditions on the genetic stability of the strain is studied.

In addition, flow cytometry is employed as an analytical technique to determine how the design of the process affects the microorganism. Thus, different reactor and operation configurations (growing and resting cells, batch and continuous systems) are compared. The effect of these variables on the physiology of *Escherichia coli* cells is also analyzed, with regard to both cell viability and macromolecules content.

Application of genetically engineered cells in bioprocesses is usually hampered by strain genetic stability. Strain stability is especially important in continuous processes with growing cells. Therefore, the stability of a transformed *E. coli* strain was analyzed in different operational conditions and a high degree of stabilization of genetically modified cells was obtained upon immobilization in κ-carrageenan gels.

Flow cytometry was employed to analyze physiological differences in the strain in the different reactor environments.

A thorough understanding of the metabolic network involved in the biotransformation is of prime importance. Thus, the link between the primary and the trimethylamonium compound metabolisms of *E. coli* was analyzed in different reactor environments. The link was established as occurring through cofactors and the expression of the tricarboxylic acid cycle, the glyoxylate shunt and the acetate metabolism are related to the biotransformation.

Moreover, since L(-)-carnitine is also an osmoprotectant, the presence of salt in the growth medium affects bioprocess performance. Therefore, metabolic analysis of *E. coli* in the presence and absence of salt stress is carried out. In fact, an increase in productivity can be obtained in certain conditions. The metabolic constraints related to this response in the biotransformation are characterized, allowing further detail of the link between the biotransformation and the central pathways in *Escherichia coli* to be obtained.

Further, the fourth ORF in the *cai* operon, which had previously been proposed to code for a carnitine:CoA ligase (CaiC) was cloned and preliminary characterization studies were performed. Its enzyme activity was assayed and its role in the biotransformation and in relation with the CoA transferase (CaiB) was more precisely established.

Finally, since application of molecular biology techniques allows the previously determined genetic constraints in the metabolism of L(-)-carnitine in *Escherichia coli* to be resolved, selected activities belonging to the *cai* operon were overexpressed and single gene deletion studies allowed us to determine the role of certain activities of the central pathways of *E. coli* in the biotransformation.

Below, we present an outline of this PhD Thesis:

1. To determine the main factors affecting L(-)-carnitine production by *Escherichia coli*, in batch and continuous systems, with wild type and genetically engineered strains and the presence or absence of electron acceptors such as fumarate or oxygen (Chapter 1).

2. To ascertain the effect of reactor configuration on the physiological state of *Escherichia coli* strains during L(-)-carnitine production, in the presence and absence of growth media (*growing* vs. *resting* cells), in batch systems and continuous cell-recycle membrane reactors. In addition, cell reuse strategies are studied (Chapter 2).

3. To establish the effects of reactor configuration and the cell immobilization on the physiological state and genetic stability of a plasmid-bearing genetically-engineered *Escherichia coli* strain during L(-)-carnitine production, in batch and continuous cultures (Chapter 3).

4. To determine the link between primary (carbon) and carnitine secondary metabolisms of *Escherichia coli* in production conditions, by reference to primary and secondary metabolite concentrations as well as enzyme activities, during process operation (Chapter 4).

5. To determine the link between primary (carbon) and carnitine secondary metabolisms of *Escherichia coli* in the presence of salt stress, by reference to primary and secondary metabolites concentrations as well as enzyme activities, during process operation and after salt and substrate pulses (Chapter 5).

6. To characterize CaiC protein in order to determine its actual enzyme activity and substrate specificity and to reveal the *in vivo* relevance of CaiC in the metabolism of trimethylammonium compounds, using different substrates and deletion mutant strains (Chapter 6).

7. To clone and overexpress *caiB* and *caiC* genes from *cai* operon in *Escherichia coli*, by molecular biology techniques in order to determine limiting factors in the production of L(-)-carnitine in *Escherichia coli* strains, and to assess the effect on central metabolism of the engineering of trimethylammonium:CoA derivatives. Further, the relation of the main pathways related to the metabolism of acetyl-CoA (glyoxylate shunt, acetate metabolism and Krebs cycle) with the production of L(-)-carnitine are analyzed (Chapter 7).

DISCUSSION

In this work, the optimization of a bioprocess has been tackled from a multidisciplinary point of view, furthering our knowledge on the application of metabolic engineering to bioprocess development. The production of the pharmaceutical L(-)-carnitine by *Escherichia coli* strains was taken as a model system and the bioreactor and metabolic factors were taken into consideration.

In previous works, the existing biotransformation pathway in *Escherichia coli* has been characterized (Eichler et al., 1994; Kleber, 1997; Elssner et al., 2001). This secondary metabolism can be utilized for L(-)-carnitine production using waste products such as crotonobetaine and D(+)-carnitine as substrates (Jung et al., 1993; Naidu et al., 2000). Various biotechnological systems have been employed to date for L(-)-carnitine production. In fact, not only growing but also resting cells can perform the biotransformation, with high degrees of productivity (Castellar et al., 1998; Cánovas et al., 2003b). In addition, continuous systems have also been studied (Obón et al., 1997 and 1999).

Metabolic engineering has been defined as the "*improvement of cellular activities by manipulation of enzymatic transport and regulatory activities of the cell with the use of recombinant DNA technology*" (Bailey, 1991) or, more concisely, as the "*purposeful modification of intermediary metabolism using recombinant DNA techniques*" (Cameron and Tong, 1993). The outstanding characteristic of the Metabolic Engineering approach is the rational choice of targets for genetic modification of the cells. As other fields of science and engineering, Metabolic Engineering consists of two steps: analysis and synthesis (Stephanopoulos, 1994). Various points of view have to be considered for the optimization of a bioprocess. First of all, a certain knowledge of cell physiology must be reached for the initial optimization of the expression and performance of the bacterial metabolism to be exploited (Eichler et al., 1994; Kleber, 1997; Elssner et al., 2001). Secondly, the optimization of the bioprocess itself must take into account the choice of reactor system, which has to meet the necessities of the cells in terms of physiology and cellular metabolism (Cánovas and Iborra, 2006). Functioning of the metabolic networks is an important issue, and proper analysis of the variables of the process and modelling are necessary to determine the optimal conditions. All these factors have been taken into consideration for the development of the work presented in this PhD thesis, and the study (and improvement) of bioprocess operation conditions (analysis) and cellular state optimization through metabolic engineering (synthesis) have been jointly undertaken. The *in vivo* and *in silico* data previously gained by our

research group were the starting point which permitted us to focus on improvement targets (Cánovas et al., 2002; Álvarez-Vásquez et al., 2002, Cánovas et al., 2003b; Sevilla et al., 2005).

Initially, a systematic analysis of the main factors which affect L(-)-carnitine production using *Escherichia coli* strains was carried out (Chapter 1). Batch and continuous systems, crotonobetaine and D(+)-carnitine as substrates and aerobic and anaerobic conditions were compared. In addition two strains were used: the wild type *E. coli* O44K74 and the genetically transformed *E. coli* K38 pT7-5KE32. This latter genetically modified strain showed higher productivities, and even pointed to the possibility of performing the process under aerobiosis. In fact, oxygen availability has been identified as a crucial factor influencing the set of reactions, especially in the case of the racemization of D(+)-carnitine. A similar effect was observed under anaerobiosis or microaerobiosis, since the addition of fumarate, acting as an alternative electron sink, further increased the yield. Continuous reactors were ideal for L(-)-carnitine production when using wild type strains, and showed their capacity to permit dynamic experiments. The use of hollow-fiber cartridges allowed higher retained biomass levels, though the best L(-)-carnitine dehydratase (CDH) activity levels corresponded to cells retained with a microfiltration device. On the other hand, the plasmid-bearing *E. coli* strain suffered from genetic instability both in freely suspended high- and low-cell density continuous systems, so that immobilization was necessary in order to maintain transformed cells in the reactor.

Once it had been seen how the reactor configuration determined the bioprocess performance (macroscopic scale), an analysis of the effect on the cells (microscopic scale) was undertaken. In fact, biocatalyst stability is an issue of general importance when considering biomass reutilization (in batch applications) or even continuous operation in reactors with cellular retention systems (Cánovas and Iborra, 2006). The operation mode of the reactor can affect not only cellular functioning, morphology and viability (Chapter 2), but also metabolic expression and performance, which will depend on the selection of media and conditions (Chapters 4 and 5). In addition, the occurrence of plasmid segregational instability also had to be explored under continuous operation (Chapter 3).

Application of flow cytometry as an analytical technique allowed us to determine how the different reactor systems (batch and continuous) and operation configurations (growing and resting cells) affect the microorganism. The intracellular levels of DNA, RNA and proteins showed interesting responses, mainly due to the consumption (limitation) of the carbon source (stationary phase of cell growth) or

even to the lack of this (resting cells). DNA replication cycles were shown to occur independently of the cell divisions, while RNA and protein levels reflected stress situations. Moreover, the integration of these data with the quantification of the heterogeneity of cell population meant a novel way for analyzing how reactor configuration affects the physiology of cells. Although membrane function was shown to be dependent on the metabolic activity of the cells, RNA and protein synthesis was observed even in damaged cells. In fact, in resting cell systems, cellular activity carried on despite the fast decay of viability occasioned by the absence of nutrients. Moreover, in continuous cell-recycle systems the degree of viability of cells was high, despite carbon/nitrogen limitations. Finally, a cell-reuse methodology was used, involving the alternation of resting/biotransformation and growth/re-energization cycles (Chapter 2).

As already ascertained (Chapter 1), the application of genetically engineered cells in the bioprocess was hampered by strain genetic stability. Though this is a commonly observed drawback for the application of genetically engineered cells in continuous processes, cell immobilization is a general and simple technique for allowing strain stabilization (Kumar et al., 1991). The factors determining the genetic stabilization of immobilized *E. coli* cells were studied employing flow cytometry (Chapter 3). Initial experiments demonstrated that the sensitivity of the technique was sufficient to distinguish between batch grown plasmid-bearing and plasmid-free cells in terms of DNA and protein cell content. During continuous cultivation, the physiology of the cells was affected by the different reactor environments, especially regarding cell viability and DNA content. A lower DNA content was observed in continuous systems, both with freely suspended and gel-entrapped cells, probably due to the decrease in plasmid copy number in steadily growing systems. Despite this, the lower growth rate of immobilized cells meant a decrease in the metabolic burden imposed by plasmid replication and protein synthesis (Jones and Keasling, 1998; Flores et al., 2004). Furthermore, RNA levels were shown to be tightly controlled by cell machinery and both RNA and protein composition increased upon continuous cultivation. Further, the higher physical protection of gel-entrapped cells permitted the build-up of a population of catalytically active cells with depolarized membranes. Moreover, cells were able to recover their membrane potential upon addition of fresh medium, further supporting the idea that energy-depleted cells can, at least partly, recover their functions (Nebe von Caron et al., 2000), opening new ways for the use of bacteria as catalysts for industrial biotransformations.

Further, when optimizing bioprocesses in which a pathway belonging to the secondary metabolism of the microorganism is involved, the most common approach has been to optimize its expression irrespectively of cellular physiology and primary metabolism. From a classical-biochemical point of view, central metabolism can be defined as all the cellular metabolic pathways providing the cell with energy and biosynthetic building blocks, secondary metabolism being the set of all other occurring reactions. Nevertheless, secondary metabolism can not be considered as an independent entity totally apart from the central metabolic pathways of the cell. In many cases the expression of both metabolisms is coordinated, and is controlled by the same set of regulatory proteins while substrates and products can be common to both metabolisms. This allows the cell to optimally manage metabolic resources, especially under stress conditions. More importantly, if metabolic intermediaries and/or cofactors are shared in the network, the evolution of the pools of cofactors and metabolites largely determines the linking or integration of both metabolisms (López de Felipe et al., 1998; San et al., 2002; Cánovas et al., 2003a; Vadali et al., 2004).

Since understanding the metabolic network involved in the biotransformation is of prime importance, a thorough analysis of metabolic adaptations under biotransformation conditions was performed in Chapter 4. Thus, it was established that the link between the primary and the trimethylammonium compound metabolisms of *E. coli* occurs through cofactors, especially ATP and the acetyl-CoA/CoA ratio. Further, the expression of the TCA cycle, the glyoxylate shunt and the acetate metabolism is related to the biotransformation, limiting maximum fluxes and L(-)-carnitine productivity. The energetic state of the cell correlated with the coordinated regulation of the glyoxylate shunt and the Krebs cycle, the ICDH/ICL ratio responding to substrate limitation/absence and cell fast growth conditions. In the resting cell state, metabolic re-arrangements occurred to adapt to microaerobic conditions and to permit the utilization of cell-stored material, all these features favouring the biotransformation.

Moreover, L(-)-carnitine is also an osmoprotectant, and the presence of salt in the growth medium affects bioprocess performance at the expression level. The effect of salt stress on biotransformation was studied (Chapter 5) and an enhancement in productivity was assessed by the combination of cell permeabilization and the activation of metabolic pathways for the generation of energy and precursors. Metabolic analysis of *E. coli* under stress conditions revealed that the increase in productivity was related to the modification of the

central metabolic state, further corroborating the metabolic constraints previously determined.

Important metabolic and physiological switches occur around substrate limitation/depletion events, meaning that the activation of cellular strategies for survival and/or maintenance (triggered by the up-regulation of RpoS), involves a decrease in the performance of the secondary metabolism. Both metabolic and physiological studies supported the important influence of cellular energetic state in the expression (and performance) of the biotransformation pathway. The transport and the activation of substrates are key ATP-dependent steps explaining this observation. In addition, oxygen and fumarate were important factors in the optimization of the biotransformation, due to the inhibition of the crotonobetaine reductase activity (CRR), further supporting these energetic considerations. Fumarate addition increased production not only in anaerobiosis, but also in resting cell processes performed under microaerobic conditions, in which oxygen transfer limitations can arise. Though L(-)-carnitine can also be produced in aerobiosis, oxygen partial pressure must be carefully controlled: the optimization of oxygen levels leads to a compromise between the inhibition of side reactions of crotonobetaine and the repression of ***cai*** operon expression (Castellar et al., 1999; Cánovas et al., 2002). Both the metabolic and physiological variables were closely related with the biotransformation capacity. In fact, the physiological state of *Escherichia coli* cells was shown to largely determine the performance of the bioprocess to a great extent. Maximum specific productivities were observed for exponentially growing cells, though the high amount of biomass in continuous cell-recycle and batch resting systems was responsible for the high productivities attained. In continuous systems, pre-steady state cells are more biocatalytically active, coinciding with the lowest accumulation of depolarized and dead cells, whereas in resting cell systems, the severe physiological decline of cells is accompanied by a decrease in productivity. Important cell viability decay (Chapter 2) and metabolic switches (Chapter 4) occur around the entrance into the steady state of the reactor.

Metabolic analysis performed under standard and stress conditions (Chapters 4 and 5) allowed us to further detail the linking between the biotransformation and the central pathways in *Escherichia coli* and to determine feasible optimization targets. Analysis of the evolution of selected enzyme activities revealed adaptations in both the growing and resting cell states. In fact, the energetic state of cells and the management of the acetyl-CoA/CoA pool are the most likely control points. To

the best of our knowledge, ATP is necessary in the metabolism of L(-)-carnitine for transport and activation of trimethylammonium compounds. Moreover, ProU and ProP, two transport systems devoted to the accumulation of osmoprotectants under stress conditions are ATP-dependent. On the other hand, although CaiT has been described as an exchanger (antiporter) of betaines (Jung et al., 2002; Vinothkumar et al., 2006), de-energized *E. coli* cells have been shown to display decreased L(-)-carnitine uptake rates (Cánovas et al., 2003a; Cánovas et al., 2003b). It seems possible that ATP levels indirectly affect the transport process, very probable at the level of conversion into their CoA derivatives by the ATP dependent ligase (Chapter 6), which is required for the biotransformation to proceed (Elssner et al., 2000).

In addition to the energy dependent CoA ligase activity of CaiC, the CoA transferase CaiB cycles the CoA moiety between substrates and products, allowing the biotransformation to proceed in an ATP-independent way (Elssner et al., 2001). Thus, the joint role exerted by CaiB and CaiC in substrate activation, together with the metabolism of acetyl-CoA/CoA, managing fluxes through ATP-producing (acetate metabolism) and anaplerotic (Krebs cycle and glyoxylate shunt) pathways, are proposed as control points (Chapter 7). Furthermore, an *in vitro* CoA-transferase activity was detected, although experiments showed that the most important *in vivo* role was its ligase activity. The analysis of *caiB* and *caiC* mutants and overexpression strains allowed us to further understand the role of these proteins in the biotransformation (Chapter 6), since, although CaiB was necessary for L(-)-carnitine production, its overexpression did not allow further increase of the biotransformation yield compared with the overproducing *E. coli* O44K74 strain (Chapter 7).

Finally, the information gained in the previous metabolic studies allowed us to genetically engineer *E. coli* to improve L(-)-carnitine production (Chapter 7). The relation between the expression pathways indicates that the availability of free coenzyme A for substrate activation, limiting the biotransformation, needed to be explored as possible bottleneck of the process. Since the relevance of cellular cofactors for the biotransformation had been established (Chapters 4 and 5), modification of their levels, also termed as Cofactor Engineering, was undertaken. For that purpose, single-gene overexpressions and deletions were conducted. The activation of trimethylammonium compounds and the acetyl-CoA/CoA ratio were engineered by overexpression of CaiB and CaiC activities and by the addition of a precursor of CoA, pantothenate, to the growth medium. Overexpression of CaiC allowed increased the biotransformation yield. Further, the racemization of D(+)-

carnitine was not engineered in anaerobic batch cultures, but was demonstrated in microaerobic resting cells experiments, underlining once again the importance of oxygen for this process (Chapters 1 and 6). Gene deletion studies allowed us to determine the limiting role that the expression of certain activities of the central metabolism of *E. coli* plays in the biotransformation. In fact, the phosphotransacetylase/acetate-kinase (PTA-ACK) route was seen to be essential because of its role in ATP production and in order to avoid inhibitory levels of cellular acetyl-CoA. On the other hand, decreased flux through the glyoxylate shunt enhanced productivity, especially in exponentially growing cells (Chapter 7). The PEP-glyoxylate cycle, which has recently been described (Fischer and Sauer, 2003) has been implicated in the limitation of the accumulation of reduced redox cofactors, avoiding the excessive production of NADH/NADPH, which is especially important during slow or limited growth, as is the case of batch resting cell or high cell density continuous cultures.

Though the actual role of the metabolism of L(-)-carnitine in *E. coli* remains unknown, a possible function as an anaerobic respiration pathway has previously been proposed (Kleber, 1997; Elssner et al., 1999). In addition, the intracellular accumulation of betaines and related osmoprotectants has been demonstrated to be a general strategy for bacterial survival under stress conditions, not only in high osmotic media, but also under other stress conditions. In addition, in certain species, such as *Listeria monocitogenes*, a connection with the infection ability has also been stated (Sleator et al., 2003). In *E. coli*, the involvement of two transport systems (ProU and ProP) in the uptake of these osmoprotectants has been proposed (Csonka 1991; Verheul et al., 1998; Mcmillan et al., 1999), while the antiporter CaiT is only devoted to supporting the biotransformation (Verheul et al., 1998; Jung et al., 2002). In fact, the expression of the metabolism of carnitine is repressed by high salt concentrations (Cánovas et al., 2003b), supporting that its expression does not imply an advantage for osmoprotection. Results presented in this PhD thesis further support the possible roles proposed for L(-)-carnitine metabolism in *E. coli*. In fact, both aerobic and anaerobic respiration (with oxygen or fumarate as final electron acceptors) enhanced L(-)-carnitine production by inhibiting the "respiration of crotonobetaine" at the level of the crotonobetaine reductase reaction (CRR, catalyzed by CaiA/CaiB). Further, although L(-)-carnitine metabolism was inhibited by high salt concentrations, an increase in production was ascertained under determined experimental conditions, and the metabolic alterations that followed salt stress favoured the biotransformation.

Both metabolism and cell physiology are closely related with the mode of reactor operation and jointly contribute to determining the performance of the bioprocess. In addition, the reactor configuration must be chosen on the basis of optimal performance of the bioprocess under selected conditions by ensuring optimal expression and minimal limitations due to physical or chemical gradients. In this work, the importance of the correct choice of reactor, because of its effect on cell physiology, has been underlined, and cell physiology should be considered as an important criterion, since maximum specific L(-)-carnitine production, it has been stated, corresponds to exponentially growing cells. In resting cell systems, the cell state is of crucial importance in the biotransformation. In fact, for ensuring high yields, re-energization and biotransformation cycles should be alternated in an integrated process. Under bioprocess conditions, the cell physiology largely correlates with the intracellular state, and so altering the reactor configuration, operation mode or even the occurrence of stress factors, can affect expression and performance of the bioprocess.

The most feasible scenario for the interaction between central and secondary metabolisms during L(-)-carnitine production with *Escherichia coli* is determined by the level of available cofactors. The evolution of the ATP level and the $NADH/NAD^+$ and acetyl-CoA/CoA ratios is closely linked to production (Chapter 4) and is also affected by salt stress (Cánovas et al., 2003b and Chapter 5). The activation and recycling of trimethylammonium compounds in *E. coli* and the metabolic fate of acetyl-CoA are bottlenecks for the biotransformation. Furthermore, the energetic state of the cell affects not only the biotransformation process, but also the physiological state of the bacteria. Summarizing, in this work, the need for overall strategies of bioprocess optimization, taking into account both the reactor (macroscopic) and the microorganism (microscopic) levels, is underlined. Furthermore, for any improvement in the biotransformation, primary and secondary metabolisms should be considered, and an analysis of the metabolic adaptations that occur under standard and stress conditions is a prerequisite in the selection of optimization targets. An analysis of any physiological adaptations undergone should be conducted in order to ensure maximal expression and performance.

Though further work remains to be accomplished, from the results presented here, it can be concluded that the biochemical-microbiological-technical interface should be the *leit motif* in the optimization of a bioprocess, since the selection of proper bioreactor configuration and operation modes finally affects the metabolic state of the cells. Optimization of bioprocesses lies at the interface of chemistry,

biology, engineering and computer science, and a multidisciplinary approach is the most reasonable way. Further, application of analytical techniques to assess the evolution of cell populations as well as the metabolic adaptations suffered by these as a whole (mainly enzymes, cofactors and metabolites), will allow the rational design of strain development strategies. In addition, application of state of the art high-throughput techniques for the analysis of the evolution of cellular transcriptome, proteome and metabolome in different conditions would provide a deeper insight into the complexity of metabolic networks.

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CONCLUSIONS

1. The biotransformation of trimethylammonium compounds for L(-)-carnitine production has been studied:

   - With wild type strains the performance is satisfactory in the batch and continuous systems assayed, while the segregational instability of the plasmid-bearing strain is a mayor drawback for continuous L(-)-carnitine production.

   - Crotonobetaine is a better substrate than D(+)-carnitine because the level of expression of carnitine racemase enzyme activity is lower than the expression of L(-)-carnitine dehydratase.

   - Bacterial respiration increases L(-)-carnitine production in *E. coli* O44K74 and *E. coli* K38 pT7-5KE32 strains by means of (a) enhancement of cellular energetic state and (b) inhibition of crotonobetainyl-CoA reductase (CaiA) activity both in batch and continuous systems.

2. *Escherichia coli* O44K74 undergo important physiological and metabolic switches as a result of the adaptation to the absence and/or limitation of nutrients.

   - In both batch resting cell and continuous cell-recycle systems, different bacterial populations can be ascertained by multistaining flow cytometry, the end of the biotransformation of trimethylammonium compounds being linked to the build-up of damaged cells in the reactor.

   - The evolution of the level of intracellular macromolecules reflects the division rate and the metabolic state of the cells, especially in the case of DNA·cell^{-1}, which increases under decreased division rate conditions and steeply decays in resting cell systems as a result of the lack of nutrients.

3. The genetic instability of *E. coli* K38 pT7-5KE32 in continuous culture systems with suspended cells is due to the combination of the decrease of plasmid copy number and the high cell division rate. The lower division rate in continuous immobilized systems means a lower metabolic burden, allowing adequate plasmid segregation into the daughter cells and contributing to the genetic stabilization of the strain. In the immobilized state, cells experience increased protein levels (40% approx.), whereas the RNA concentration is tightly controlled by the cellular machinery, remaining almost constant during continuous processes.

4. The secondary metabolism of trimethylammonium compounds in *Escherichia coli* O44K74 is connected with its central (carbon) metabolism at the cofactor level, especially the need for ATP for substrate transport and activation and for free coenzyme A. In fact:

   - The energetic state of the cell is correlated with the up-regulation of the glyoxylate shunt (isocitrate lyase) under substrate limitation/absence conditions (resting cells and steady state continuous reactors), and that of the Krebs cycle (isocitrate dehydrogenase) during fast cellular growth (exponentially growing batch cells and continuous reactor start-up), marking the metabolic limitations to L(-)-carnitine production.
   - The expression of the acetate metabolism (phosphotransacetylase and acetyl-CoA synthetase), controlling the acetyl-CoA/CoA ratio is higher in continuous anaerobic systems.

5. Although the metabolism of carnitine in *Escherichia coli* O44K74 is not related to osmoprotection and its expression is repressed by high salt concentrations,

   - Salt stress enhances L(-)-carnitine production through a combination of cell membrane permeabilization and the activation of metabolic pathways for the generation of energy and precursors (glyoxylate shunt, Krebs cycle and acetate metabolism) both in batch resting and continuous growing systems.
   - Carrying out pulses in bioreactors is a very suitable strategy for the dynamic study of metabolism and cell physiology.

6. The fourth ORF of the *cai* operon, CaiC, is an ATP-dependent coenzyme A ligase, which is only active on D,L-carnitine, crotonobetaine and γ-butyrobetaine. Moreover, it was observed that:

   - Though *in vitro* betaine:coenzyme A transferase activity is also exhibited in cell free extracts, *in vivo* data support that this is not its main function.
   - Both carnitine:CoA transferase (CaiB) and carnitine:CoA ligase (CaiC) activities are necessary for L(-)-carnitine production by *Escherichia coli*.
   - The overexpression of CaiC was followed by an enhancement in L(-)-carnitine production by both growing and resting cells from crotonobetaine and D(+)-carnitine, meaning that the activation of betaines is an important bottleneck for the biotransformation.

7. Finally, the availability of free coenzyme A affects the performance of the biotransformation. This work showed that:

   - The overexpression of the substrate activating enzymes of the metabolism of carnitine in *Escherichia coli* (CaiB and CaiC) allowed us to engineer the cellular pool of betainyl-CoAs, meaning a two to ten-fold increase in the biotransformation yield.
   - The expression of the glyoxylate shunt and acetate pathways are control parameters for the metabolism of L(-)-carnitine because of their role in the metabolic fate of acetyl-CoA and the maintenance of the acetyl-CoA/CoA ratio.
   - Single gene knock-out affecting the activity of the glyoxylate shunt increased L(-)-carnitine production by 20-25%, while the suppression of phosphotransacetylase almost completely abolished the biotransformation.