UNIVERSIDAD DE MURCIA

FACULTAD DE QUIMICA

Nuevos aspectos de la regulación del metabolismo de acetato en *Escherichia coli*

New insights into the regulation of acetate metabolism in *Escherichia coli*

Dª Sara Castaño Cerezo

2014

UNIVERSIDAD DE MURCIA

Facultad de Química

Departamento de Bioquímica y Biología Molecular B e Inmunología

Nuevos aspectos de la regulación del metabolismo de acetato en Escherichia coli

TESIS DOCTORAL

Sara Castaño Cerezo

DIRECTORES

Dr. Manuel Cánovas Díaz Dr. Vicente Bernal Sánchez

Murcia, 2014

During her PhD, Sara Castaño Cerezo has been recipient of a Research training Fellowship (FPI Fundación Séneca) and two fellowships for her short stays in Utrecht (EMBO Short Stay Fellowship and Fundación Séneca Short Stay Fellowship).

This Thesis has been funded by the projects MCYT BIO2008-04500-C02-01, MICINN BIO2011-29233-C02-01, Fundación Séneca-CARM 08660/PI/08 and PrimeXS project-0000012

Part of the results obtained in this PhD thesis have been presented as:

Publications (Peer-reviewed journals):

- An insight into the role of phosphotransacetylase (*pta*) and the acetate/acetyl-CoA node in *Escherichia coli*. (2009) S. Castaño-Cerezo, J.M. Pastor, S. Renilla, V. Bernal, J.L. Iborra, M. Cánovas. *Microbial Cell Factories* 8(1):54.
- cAMP-CRP coordinates the expression of the protein acetylation pathway with central metabolism in *Escherichia coli*. (2011). S. Castaño-Cerezo, V. Bernal, J.L. Iborra, M. Cánovas. *Molecular Microbiology* 82(5): 1110-1128.
- Acetate scavenging activities in *Escherichia coli*: interplay of acetyl-CoA synthetase and the glyoxylate shunt in chemostat cultures. (2012) Renilla S, Bernal V, Fuhrer T, Castaño-Cerezo S, Pastor JM, Iborra JL, Sauer U, Cánovas M. *Applied Microbiology and Biotechnology*. 93 (5): 2109-2124
- Regulation of bacterial physiology by lysine acetylation of proteins. V. Bernal, S. Castaño-Cerezo, J. Gallego-Jara, A. Écija-Conesa, T. de Diego, J.L. Iborra, M. Cánovas, (*Under review*).
- Characterizing protein acetylation and acetate overflow in *Escherichia coli* BL21 and K12. S. Castaño-Cerezo, V. Bernal, T. Röhrig, M. Cánovas. (*Under preparation*)
- Protein deacetylation regulates acetate metabolism, motility and acid stress response in *Escherichia coli* S. Castaño-Cerezo, V. Bernal, H. Post, T. Fuhrer, S. Cappadona, N.C. Sánchez-Díaz, U. Sauer, A.J.R. Heck, A.F.M. Altelaar, M.Cánovas (*under preparation*).

Contributions To Congresses: Oral Communications

- Effect if glyoxylate shunt associated gene deletion on the central metabolism energy balance of *Escherichia coli*. J.M. Pastor, S.C. Cerezo, S. Renilla, V. Bernal, M. Cánovas, J.L. Iborra. *4th Meeting of the Spanish Systems Biology Network.* 1st-2nd December 2008. Valencia. Spain.
- Metabolic engineering of acetate metabolism pathways in *Escherichia coli:* an insight from the analysis of knockout strains. S. Castaño-Cerezo, S. Renilla, J.M. Pastor, V. Bernal, J.L. Iborra, M. Cánovas. *8th European Symposium on Biochemical Engineering Science*. 5th-8th September 2010. Bologna, Italy.
- Central metabolism and the cAMP-CRP regulon in *Escherichia coli*. S. Castaño-Cerezo, V. Bernal, J.L. Iborra, M. Cánovas. *European Congress of Applied Biotechnology (ECAB2011)*. 25th-29th September 2011. Berlin (Germany).
- Transcriptional regulation of the acetylation/deacetylation system PatZ/CobB in *Escherichia coli*. S. Castaño-Cerezo, V. Bernal, M. Cánovas. *1st PrimeXS Meeting*. 15th-16th April 2012. Palma de Mallorca (Spain)
- Post-translational regulation of metabolism in bacteria: protein acetylation in *Escherichia coli*. *BioSpain, 6th International Meeting on Biotechnology*. 19th-21th September 2012. Bilbao (Spain).

- Role of cAMP on the regulation of bacterial metabolism by protein acetylation. S. Castaño-Cerezo, V. Bernal, T. Röhrig, J. Blanco-Catalá, J.L. Iborra, M. Cánovas. *15th European Congress on Biotechnology (ESBES-ISPPP).* 24th-26th September 2012. Istambul (Turkey).

- Synthetic Biology and Metabolic Engineering of post-translational regulation mechanisms of central metabolism in *Escherichia coli*. Castaño-Cerezo S., Bernal V., Gallego J., Ecija A.,Sanchez-Diaz NC., . Iborra J.L., Cánovas M. *2nd Applied Synthetic Biology in Europe.* 25th-27th November 2013, Malaga (Spain).

Publications (Conference Proceedings):

- A novel role for cAMP in the metabolism of *Escherichia coli*: regulation of the protein acetylation pathway (2012). Castaño-Cerezo S, Bernal V. Blanco-Catalá J, Iborra J.L., Cánovas M. *FEBS J.* 279(S1):281

- Unravelling the metabolic differences between *Escherichia coli* K12 and BL21 (2012). Castaño-Cerezo S., Bernal V., Röhrig T., Iborra J.L., Cánovas M. *FEBS J.* 279(S1):344.

- System analysis of the role of Acetyl-CoA Synthetase acetylation in *E. coli* transient diauxic system (2012). S. Robaina, S. Castaño-Cerezo, M. Cánovas and N. Torres. *FEBS J.* 279 (S1):523

Contributions to Congresses: Posters

- Role of the PEP-glyoxylate-acetate metabolism in *E. coli* growing under anaerobiosis/aerobiosis. S. Renilla, J.M. Pastor, E. González, S. Castaño, V. Bernal, M. Cánovas, J.L. Iborra. *Meeting of the Spanish Society of Systems Biology*. November 2007. Murcia, Spain.

- Regulación transcripcional del ciclo del glioxilato en *Escherichia coli*. S. Renilla, J.M. Pastor, S.C. Cerezo, V. Bernal, M. Cánovas, J.L. Iborra. *Biotec-BioSpain*. Granada, 2008, Spain.

- El papel fundamental de acetato en el metabolismo central de *Escherichia coli*. S.C. Cerezo, S. Renilla, J.M. Pastor, V. Bernal, M. Cánovas, J.L. Iborra. *Biotec-BioSpain*. Granada, 2008. Spain.

- Efecto de la deleción de genes asociados al ciclo del glioxilato en *Escherichia coli* sobre la bioproducción de metabolitos. J.M. Pastor, S.C. Cerezo, S. Renilla, V. Bernal, M. Cánovas, J.L. Iborra. *Biotec-BioSpain*. Granada, 2008. Spain.

- Acetate overflow role in central metabolism in *Escherichia coli*. S.C. Cerezo, J.M. Pastor, S. Renilla, T. Fuhrer, V. Bernal, U. Sauer, J.L. Iborra, M. Cánovas. *4th Meeting of the Spanish Systems Biology Network.* 1st-2nd December 2008. Valencia. Spain.

- Effect of glyoxylate shunt associated genes deletion on the central metabolism energy balance of *Escherichia coli*. J. M. Pastor, S. C. Cerezo, S. Renilla, V. Bernal, M. Cánovas, J. L. Iborra. *4th Meeting of the Spanish Systems Biology Network.* 1st-2nd December 2008. Valencia. Spain.

- Transcriptional regulation of the glyoxylate shunt in *Escherichia coli*. S. Renilla, T. Fuhrer, J.M. Pastor, S.C. Cerezo, V. Bernal, J.L. Iborra, U. Sauer, M. Cánovas. *4th Meeting of the Spanish Systems Biology Network*. 1st-2nd December 2008. Valencia. Spain.

- A Systems Biology study of the CRP-cAMP regulon. S. Castaño-Cerezo, V. Bernal, J.L. Iborra, M. Cánovas. *BioSpain* 2010. Pamplona (Spain).
- The protein acetylation pathway and central metabolism of *Escherichia coli*: role of cAMP on regulation. S. Castaño-Cerezo, V. Bernal, T. Röhrig, J.L. Iborra, M. Cánovas. Metabo*lic Engineering IX Conference.* 3rd-7th June 2012. Biarritz (France).

Acknowledgements

"Science and everyday life cannot and should not be separated"
Rosalind Franklin

Desde que tengo uso de razón he sabido que quería dedicar mi vida la ciencia. Esta tesis doctoral es la culminación de esa ilusión y el inicio de mi carrera como científica. Este trabajo no habría sido posible sin el apoyo y ayuda de muchísimas personas a las que me gustaría mostrar mi agradecimiento.

En primer lugar me gustaría dar mi mas sincero agradecimiento a mis directores Manuel Cánovas y Vicente Bernal.

Manolo gracias por tu incondicional apoyo y por tus sabios consejos. Gracias por haber sido tan persistente ese ultimo año de carrera, espero no haberte defraudado. Gracias por haber confiado en mí y brindarme la gran oportunidad de trabajar en tu equipo.

Vicente, me faltan palabras para darte las gracias como director, pero también como amigo. Serás siempre mi modelo a seguir, no solo por tu dedicación y amor a la ciencia sino también por ser el mejor jefe que un becario puede tener. Por todas esas canciones al final de los correos, por todos esos videos y sacarme una sonrisa siempre que lo he necesitado. Cada vez que pienso como hacer algo me pregunto "¿Cómo lo haría Vicente?". Muchas Gracias por estas siempre ahí.

A mis grandes compañeros del laboratorio José María, Sergio y Emilia. Fui la última en llegar pero me acogisteis con los brazos abiertos. Sergio, gracias por enseñarme todo lo que sabías, sabes que tu fuiste mi primer "mentor". Siempre que oiga la canción de MacGiver me acordaré de ti. Emilia, gracias por sacarme todos los días una sonrisa, echo de menos trabajar cerca de ti. José María, solo quedamos nosotros. No se como decirte lo importante que has sido. Primero como compañero de fatigas nocturnas y después como amigo. Gracias por todos esos abrazos apretados en momentos de estrés. Gracias por escucharme y ayudarme incondicionalmente.

A las chicas del 113, y David, muchas gracias. Bere, tu mera presencia relaja mi mente. Gracias por ser como eres y creer tanto en mi. Juana Mari, somos tan parecidas que hemos chocado en multitud de ocasiones, pero aun así el sólo hecho de hablar contigo me ha ayudado a seguir para delante, muchas gracias por comprenderme. David, gracias por ayudarme en todo lo que te he pedido, con pequeños gestos has influido en toda este trabajo.

A todas aquellas chicas que he han pasado por mis manos estos años de tesis. A mis chicas alemanas, danke!. Teresa, you put a smile on my face everyday, it was a pleasure to hear your laugh in the lab. Svenja, gracias por ser tan dulce. A las "chicas de Sari" muchas gracias. Theng, thanks for giving so much happiness to the lab last year. Nerea, eres la alumna que todo profesor querría, gracias por tu dedicación. Ana y Julia gracias a las dos por hacer ese año 2013

tan difícil en algo que recordar con cariño y alegría. Julia eres de lo mejor que he conocido en un laboratorio, gracias haberme dejado disfrutar de tu presencia. Ana gracias a tí he descubierto lo bonito que es iniciar una vocación. Gracias por dejarme enseñarte mi pasión por la ciencia y enseñarme a ser mas optimista. Gracias a todas por todo.

Gracias a todos aquellos que han pasado por el departamento. Narci, gracias por tu fuerte carácter y por tu fuerte risa. Sigo echando de menos oírte reír desde lo lejos. Gracias Mª José por tus marujeos. Manuel gracias por tu entusiasmo por la ciencia. Sergio, gracias por tus consejos informáticos. Paula, gracias por ser la primera en enseñarme biología molecular. Jorge parte de esta tesis también es tuya, gracias. A todos los demás Antonio, Javi, Francisca, Medhi, Loredana…todos habéis compartido tiempo de esta tesis y por lo tanto influido en ella. Muchísimas gracias.

Al resto de profesores del departamento gracias por todos esos pequeños gestos que han hecho el proceso de esta tesis un poco mas sencilla.

Me gustaría agradecer a Pedro Lozano por haberme dado la oportunidad impartir las practicas de Biología Molecular con las que tanto he disfrutado.

A mi vecino Pepe muchas gracias. Desde que supiste mi vocación y planes me inculcaste la importancia de las notas para poder cumplirla y me resolviste muchas dudas durante mis estudios. Gracias por siempre estar ahí.

Al servicio de Biología Molecular de la Universidad de Murcia, en especial a César, María José, Vanesa y Alejandro muchas gracias vuestra ayuda y consejos. También me gustaría agradecer a las chicas del servicio de cromatografía María José y Lola, por su inestimable ayuda. David Cerezo, gracias por tu ayuda este último año en la realización de mis primeros western blots. Muchas gracias a todos.

I would like to specially thank Albert Heck, Maarten Altelaar and Uwe Sauer for giving me the great opportunity to work in their labs.

Harm many thanks for being such a nice lab mate, thanks for singing everyday. Ana and Teck thanks for sharing chocolate and also really nice moments in the office. Chiara thanks for being my friend; you were the best support I could ever imagine during those "proteomics" months. Salvo I could never know how to thank you all the support you gave me last summer, many thanks for being there. Alba, gracias por toda tu ayuda y sobretodo apoyo que me diste durante esos meses en Utrecht. Toby gracias por acogerme en Zurich y enseñarme tantas cosas nuevas. Thank you!

A mi amigos de siempre, Kiki, Ñoño, Jorge, Gloria, Miguel, gracias por comprenderme. Espero que sepáis que aunque me vaya lejos nunca me olvidaré de que habéis sido y siempre seréis mi gran apoyo.

A mis amigos de la universidad. Desde que nos conocimos hace 12 años nuestros caminos han ido divergiendo pero aun así, hemos permanecido juntos, manteniendo la amistad y brindando siempre apoyo los unos a los otros. Gracias Fuen, Vicky, Marta G, Marta A, Ángel, Mar. Gracias a todos, simplemente por ser como sois y comprenderme mejor que nadie.

A mis hermanos María, Antonio y Concha. Gracias por apoyarme. Me habéis hecho sentir arropada durante estos años y sentir vuestro orgullo por vuestra hermana pequeña me hace sentir muy feliz.

A mis sobrinos gracias. Probablemente no te acuerdes Julia, pero hubo una vez que dijiste que querías ser científica como tu tita. Ese día me sentí muy feliz. Gracias.

Gracias Diego. Mi compañero, mi familia. Esta tesis es tan tuya como mía. Durante estos años has sido mi mayor apoyo, diariamente me has levantado cuando estaba triste y has celebrado mis éxitos. Esta tesis tiene también tu sudor y esfuerzo. Tu apoyo, sacrificio, amor y sobretodo compresión ha sido esencial. Gracias por entenderme y empezar esta nueva vida conmigo. Te quiero.

Mamá gracias. Eres la mujer mas comprensiva y cariñosa que he conocido. Durante estos años siempre me has apoyado y mostrado tu mas sincero interés aun no entendiendo muy bien que hace tu hija trabajando con bacterias. Gracias mamá por el mejor regalo que se puede hacer, tu amor incondicional.

Papá gracias. Gracias por como me has educado. Por enseñarme a ser perseverante, a trabajar duro. Tal vez durante mis años de adolescencia no creí en estos valores, pero gracias a ellos me han llevado ser lo que quería, investigadora. Supongo que después de todo nos parecemos mas de lo que pensaba. Misma nariz y mismo cabeza. Cada vez que trabajo por las noches, recuerdo todos los sacrificios que has hecho en tu vida por nosotros. Gracias por el regalo que llevare siempre conmigo, ser como tu. Gracias papá.

A mi familia

Resumen

Introducción

Escherichia coli es una bacteria Gram-negativa muy utilizada en biotecnología para la producción de proteínas y metabolitos de interés industrial (1). Su fácil cultivo y manipulación genética han hecho de ella el caballo de batalla de muchos laboratorios. Aun así, su uso biotecnológico posee algunas desventajas, como es la producción de acetato en cultivos con glucosa como única fuente de carbono. Una alta concentración de este metabolito en el medio provoca una inhibición del crecimiento, afectando al rendimiento máximo en biomasa, al igual que al del producto deseado (2, 3). De hecho, la cepa más utilizada para la producción heteróloga de proteínas es *E. coli* BL21 debido a su menor producción de este metabolito (4).

Desde la aparición de las disciplinas de la Ingeniería Metabólica y, más tarde, de la Biología de Sistemas, la optimización racional de bioprocesos se ha visto incrementada en las ultimas décadas (5–7). Para la optimización de un bioproceso en un determinado organismo, en nuestro caso *E. coli*, es necesario incrementar el conocimiento sobre la fisiología del éste y los mecanismos reguladores que la controlan.

La regulación transcripcional en *E. coli* ha sido ampliamente estudiada, mientras que otros procesos reguladores (8, 9) como la regulación post-traduccional del metabolismo en procariotas, apenas están empezando a ser estudiados en los últimos años (10). De hecho hasta la ultima década, la regulación del metabolismo por modificaciones post-traduccionales había estado restringida a la enzima isocitrato deshidrogenasa, que se inactiva por fosforilación en *E. coli* (11). A pesar de esto, estudios publicados años más tarde han mostrado evidencias de que el metabolismo del acetato, en especial la enzima acetil-CoA sintetasa, está regulado por acetilación de proteínas en *Salmonella entérica* (12) y otros microorganismos (13–15).

Objetivos

El objetivo principal de esta Tesis doctoral ha sido el estudio de la relación del metabolismo del acetato con el metabolismo central y el efecto de la acetilación de

proteínas sobre la regulación de la fisiología de *E. coli*. Para ello, se plantearon los siguientes subobjetivos: (i) caracterizar los mutantes delecionados en las rutas de consumo/producción de acetato y determinar las consecuencias de su deleción a distintos niveles celulares, (ii) estudiar la regulación *in vivo* del metabolismo del acetato por acetilación de lisinas en *E. coli*, (iii) describir el contexto genómico de los genes de las enzimas responsables de la acetilación de lisinas en *E. coli* (*cobB* y *patZ*) y estudiar su regulación transcripcional, (iv) discernir el papel de la acetilación de lisinas en las diferencias metabólicas observadas entre las cepas de *E. coli* K12 y BL21, (v) identificar los patrones de acetilación de proteínas en distintas condiciones y fondos genéticos en *E. coli* y (vi) caracterizar la funcionalidad de la acetilación de proteínas en el metabolismo del acetato y la regulación transcripcional.

Resultados y discusión

En el primer capítulo de esta Tesis se caracterizó fisiológicamente a los mutantes que carecen de las vías de producción/consumo de acetato (Δ*pta* y Δ*acs*). El objetivo principal de este estudio fue conocer la importancia de la relación entre el metabolismo del acetato y el metabolismo central y, por tanto, las consecuencias fisiológicas de la deleción de estas vías en el fenotipo de *E. coli*. La deleción de la vía principal de producción de acetato (Δ*pta*) alteró el metabolismo central a distintos niveles, desde la transcripción de numerosos genes hasta la homeostasis energética de *E. coli*. En cultivos con glucosa, la deleción de la mayor vía de producción de acetato incrementó los niveles de acetil-CoA inhibiendo la actividad de la enzima piruvato deshidrogenasa alostéricamente. Esta inhibición provocó un incremento de la concentración intracelular de piruvato, produciendo diversas modificaciones transcripcionales y metabólicas. Este hecho mostró la relación directa entre el metabolismo central y del acetato y, por tanto, la importancia de que exista un buen control de este metabolismo en *E. coli*. Algunas de las actividades enzimáticas determinadas en este capítulo, como las de acetil-CoA sintetasa y piruvato deshidrogenasa, mostraron patrones de expresión y actividad que no se correlacionaban entre ellos. Esto sugirió la hipótesis de la posible existencia de algún tipo de regulación post-traduccional no conocida hasta el momento en *E. coli*. Esta regulación podría estar

desequilibrada en el mutante Δ*pta*, pudiendo estar causada por una alteración de los niveles de acetil-fosfato o algún otro metabolito relacionado, como el acetil-CoA.

En el segundo capítulo se investigó el papel que tiene la acetilación de proteínas en *E. coli*. En particular, se analizó el papel de las proteínas involucradas en esta regulación post-traduccional, la sirtuina CobB y la proteín-acetiltransferasa PatZ (anteriormente *yfiQ*), en la regulación *in vivo* de la principal vía de consumo de acetato (la enzima acetil-CoA sintetasa). Se caracterizó fisiológicamente a los mutantes simples Δ*cobB*, Δ*patZ* y el doble Δ*cobB*Δ*patZ* en distintas fuentes de carbono (glucosa y acetato). En cultivos con glucosa, se observó una mayor producción de acetato en el mutante Δ*cobB*, demostrando que una mayor acetilación de proteínas en *E. coli* podría haber desequilibrado el metabolismo del acetato en este mutante. Además, en cultivos con acetato, el mutante delecionado en el gen *cobB* manifestó un fenotipo muy alterado. Éste no pudo consumir todo el acetato presente en el medio y apenas creció a bajas concentraciones de acetato, demostrando con este fenotipo la inactivación *in vivo* de la enzima acetil-CoA sintetasa. No obstante, este fenotipo se revertió tras la deleción del gen *patZ* en el mutante Δ*cobB*. Este último hecho permite concluir que la acetil-CoA sintetasa está inactivada por la proteín-acetiltransferasa PatZ.

Se estudió el contexto genómico de la única desacetilasa anotada en el genoma de *E. coli*, *cobB*, y de la proteín-acetiltransferasa *patZ*. El gen *cobB* se expresó constitutivamente, al igual que la del gen localizado aguas arriba, *nagK*. El gen *patZ* puede expresarse desde dos promotores distintos. El primero, de expresión constitutiva, está localizado aguas arriba del gen *yfiP*, de función desconocida. El segundo, aguas arriba del propio gen *patZ*, está regulado positivamente por el factor de transcripción CRP, teniendo este factor de transcripción dos sitios de unión localizados a -41.5 y -81.5 pares de bases del inicio de la transcripción.

En el tercer capitulo, se estudiaron las diferencias metabólicas entre las cepas de *E. coli* K12 y BL21, poniendo especial énfasis en el metabolismo de acetato. El objetivo principal fue discernir el papel de la acetilación de lisinas en dichas diferencias metabólicas. La principal diferencia entre estas dos cepas consiste en una mayor producción de acetato en la cepa K12 respecto a BL21. Se demostró que la acetilación

de proteínas es distinta en ambas cepas, siendo mas abundante en la cepa K12 que en la BL21. La ausencia de la sirtuina CobB y la proteín-acetiltransferasa PatZ en la cepa BL21 alteró de forma más severa el metabolismo de acetato que en la cepa K12. En los mutantes delecionados, Δ*cobB* y Δ*patZ*, de *E. coli* BL21 la producción/consumo de acetato estaba más alterada que en la cepa y mutantes de K12. El mutante Δ*cobB* mostró una producción mayor y más rápida de acetato que el mismo mutante en K12. Además, no se observó producción de acetato en cultivos del mutante Δ*patZ*, mostrando la importancia de ambas proteínas en el *acetate overflow* en la cepa BL21. Este hecho mostró que la regulación metabólica diferencial de las enzimas CobB y PatZ puede ser la clave del diferente metabolismo del acetato mostrado entre ambas cepas.

En el último capitulo de esta Tesis doctoral se estudió la acetilación diferencial de proteínas en distintas condiciones fisiológicas y mutantes para determinar la función de este mecanismo de regulación post-traduccional en *E. coli* y de esta forma conocer los actores moleculares participantes en ella.

Se realizó un estudio proteómico para cuantificar los niveles de acetilación de proteínas en *E. coli* en cuatro condiciones diferentes (en fase exponencial y estacionaria de cultivos discontinuos con glucosa, fase exponencial de cultivos discontinuos de acetato y quimiostatos limitados en glucosa a baja velocidad de dilución) y en tres fondos genéticos distintos (*E. coli* cepa silvestre y sus mutantes delecionados en *cobB* y *patZ*). Se identificaron y cuantificaron más de 2000 sitios únicos de acetilación pertenecientes a 809 proteínas, de las cuales casi el 65% eran proteínas relacionadas con el metabolismo. Además de observar proteínas con una función metabólica, se observó un alto porcentaje de factores de transcripción acetilados en nuestro estudio. Con todas las secuencias acetiladas obtenidas de este estudio se realizó un análisis de la secuencia consenso de acetilación. Esta mostró que rodeando la lisina acetilada hay una probabilidad alta de encontrar aminoácidos de cadena lateral ácida, como son el acido glutámico y el acido aspártico. Esto explicaría la alta reactividad de las lisinas para acetilarse químicamente, ya que en un entorno ácido, éstas podrían actuar como un grupo nucleófilo pudiendo atacar al grupo carbonilo de agentes acilantes como acetil-CoA o acetil-fosfato sin la mediación de una proteín-acetiltransferasa.

En las cuatro condiciones estudiadas, el mutante Δ*cobB* fue el que mostró una mayor alteración en su fenotipo. Esto se reflejó en una mayor acetilación de proteínas en todas las condiciones. De hecho, cuanto más afectado estaba el mutante, más acetilado estaba su proteoma. Estos resultados mostraron la funcionalidad de esta modificación post-traduccional y la función global de esta sirtuina en *E. coli*. Además, se determinaron casi 1000 lisinas que podrían ser sustratos naturales de la sirtuina CobB. Al contrario de lo observado en la deleción de *cobB*, la ausencia de la proteín-acetiltransferasa *patZ* no mostró cambios tan reseñables en la acetilación de proteínas en la fase exponencial de cultivos con glucosa y en quimiostatos limitados por glucosa a baja velocidad de dilución. Los cambios en la acetilación de proteínas sólo fueron apreciables en cultivos con acetato y, en menor medida, en la fase estacionaria de cultivos discontinuos de glucosa. En acetato, el 75% de los péptidos acetilados identificados estaban más acetilados en el mutante Δ*patZ* que en la cepa silvestre. El incremento en la acetilación en este mutante pudo deberse al aumento de la concentración de algún agente acetilante, como acetil-fosfato o acetil-CoA. De hecho, el aumento de las actividades de las enzimas acetil-CoA sintetasa y fosfotransacetilasa puede indicar que la función de PatZ es la regulación de la acetilación química.

Aparte de determinar los niveles de acetilación entre distintas condiciones y mutantes, se estudiaron las implicaciones fisiológicas del aumento o disminución de la acetilación en las proteínas, y por tanto, en procesos fisiológicos. Se verificó que la enzima acetil-CoA sintetasa se inactiva por acetilación mediante ensayos de desacetilación *in vitro*, y se determinó que la lisina 609 es la responsable de esta inactivación. También se observó que la menor actividad de la enzima isocitrato liasa, observada en el mutante Δ*cobB,* se tradujo en una disminución fisiológica del ciclo del glioxilato. Mediante estudios fisiológicos y proteómicos se verificó que dicha disminución en el ciclo del glioxilato no se debió a la mayor acetilación del regulador post-traduccional AceK, al contrario de lo observado en *Salmonella enterica*. A pesar de las evidencias mostradas de la regulación por acetilación del ciclo del glioxilato, la desacetilación de la isocitrato liasa no se pudo demostrar *in vitro*.

En el estudio proteómico realizado en este capítulo también se observaron que otras proteínas relacionadas con la maquinaria transcripcional están acetiladas. Por esta razón, se realizó un estudio transcriptómico en la cepa silvestre de *E. coli* y sus mutantes Δ*cobB* y Δ*patZ* para determinar si las variaciones en acetilación de algún factor de transcripción modificaba la expresión génica. Los datos presentados mostraron grandes cambios en el mutante Δ*cobB*. La expresión de esos genes desregulados en Δ*cobB* también se observaron en Δ*patZ*, aunque en menor grado. Los genes más expresados en ambos mutantes en comparación con la cepa silvestre, estaban relacionados con la biosíntesis y el funcionamiento de los flagelos y los menos expresados estaban relacionados con el estrés ácido. Los cambios en expresión se verificaron fisiológicamente. Se observó que el mutante Δ*cobB* poseía mayor número de flagelos, mayor motilidad y menor supervivencia frente a estrés por acido. Todos estos datos sugirieron que estas variaciones en la expresión génica y, por tanto, en la fisiología, se debían a la mayor acetilación de la lisina 154 del factor de transcripción RcsB. En el mutante Δ*cobB* esto se produjo por ser uno de sus sustratos y en el mutante Δ*patZ* de forma indirecta, probablemente por acetilación química provocada por la alteración de los niveles de algún agente acetilante. Para verificar que estas observaciones fisiológicas se debían a la acetilación de esta lisina, se mutó la lisina 154 de RcsB por arginina y glutamina para mimetizar el efecto de la desacetilación/acetilación permanente. Los mutantes se caracterizaron fisiológicamente para comprobar que la acetilación de dicho residuo afecta a la unión de este factor de transcripción al DNA. Se determinó que la acetilación de la lisina 154 de RcsB inhibe la actividad de este factor de transcripción, lo que provoca un incremento en la expresión de los genes de flagelos y motilidad y disminuye la de los de resistencia a estrés acido.

Los resultados fisiológicos y proteómicos mostrados en esta Tesis doctoral ofrecen nuevos conocimientos sobre el papel del metabolismo del acetato y de la acetilación de lisinas en *E. coli*. El metabolismo de acetato está directamente unido al metabolismo central, regulando los niveles de metabolitos clave, como el acetil-CoA y el acetil fosfato, que además están involucrados en la acetilación de proteínas. Esta regulación post-traduccional es importante para el control del metabolismo, pero

también se ha mostrado que es importante en otros procesos fisiológicos como la motilidad y la supervivencia a estrés. La proteín-acetiltransferasa PatZ no es una acetiltransferasa general en *E. coli*, pero puede tener un papel esencial en la regulación de la acetilación química, controlando las actividades de acetil-CoA sintetasa o la vía fosfotransacetilasa-acetato kinasa.

A pesar de todo el conocimiento aportado en esta Tesis Doctoral, se deberían estudiar que otras implicaciones fisiológicas tiene la acetilación de proteínas en *E. coli*. Como afecta la acetilación *per se* en la función proteica no es suficiente para determinar que implicaciones tiene la acetilación en la fisiología celular. Las técnicas actuales de cuantificación de modificaciones post-traduccionales de proteínas no pueden determinar la abundancia relativa de ninguna modificación post-traduccional en la población total de una proteína en la célula. Determinar esto es fundamental para intentar entender cómo una regulación post-traduccional impacta en la fisiología de un organismo. Además, junto con el conocimiento de cómo la regulación de la síntesis y degradación de los agentes acetilantes en *Escherichia coli*, ayudaría a tener una imagen más real del funcionamiento y regulación de esta modificación post-traduccional y su impacto en los seres vivos.

Conclusiones

- El metabolismo del acetato regula la homeostasis celular a través de su implicación en la regulación de metabolitos clave, debido a la participación de estos últimos en la regulación post-traduccional,
- La proteín-acetiltransferasa inhibe la actividad a la enzima acetil-CoA sintetasa por acetilación y esta inactivación se revierte por la desacetilasa sirtuina CobB,
- El gen *cobB* se transcribe desde la región aguas arriba del gen *nagK*, siendo la expresión de ambos constitutiva. La expresión de *patZ* se realiza desde la región aguas arriba del propio gen y está regulada transcripcionalmente por el factor de transcripción CRP,

- La sirtuina CobB es la principal desacetilasa en *E. coli*, teniendo mas de 1000 sustratos. La proteín-acetiltransferasa PatZ no es la principal vía de acetilación de lisinas en la célula. Probablemente su función sea el control de la acetilación química en *E. coli*, y
- La acetilación de lisinas en *E. coli* controla funciones fisiológicas como el metabolismo del acetato, la movilidad celular y la supervivencia a estrés.

Bibliografía

1. Martinko, J. M., Madigan, M. T., and Parker, J. (2003) *Brock: Biology of Microorganisms* (Pearson Education). 10th Ed.
2. Sørensen, H. P., and Mortensen, K. K. (2005) Advanced genetic strategies for recombinant protein expression in *Escherichia coli*. *J. Biotechnol.* 115, 113–28
3. Frazzetto, G., Louis, S., Poirier, Y., and Biotechnology, P. (2003) White biotechnology. *EMBO* 4, 835–837
4. Shiloach, J., Reshamwala, S., Noronha, S. B., and Negrete, A. (2010) Analyzing metabolic variations in different bacterial strains, historical perspectives and current trends - example *E. coli*. *Curr. Opin. Biotechnol.* 21, 21–26
5. McCloskey, D., Palsson, B. Ø., and Feist, A. M. (2013) Basic and applied uses of genome-scale metabolic network reconstructions of *Escherichia coli*. *Mol. Syst. Biol.* 9, 661
6. Heinemann, M., and Sauer, U. (2010) Systems biology of microbial metabolism. *Curr. Opin. Microbiol.* 13, 337–43
7. Patil, K. R., Akesson, M., and Nielsen, J. (2004) Use of genome-scale microbial models for metabolic engineering. *Curr. Opin. Biotechnol.* 15, 64–9
8. Zaslaver, A., Bren, A., Ronen, M., Itzkovitz, S., Kikoin, I., Shavit, S., Liebermeister, W., Surette, M. G., and Alon, U. (2006) A comprehensive library of fluorescent transcriptional reporters for *Escherichia coli*. *Nat. Methods* 3, 623–628
9. Gerosa, L., Kochanowski, K., Heinemann, M., and Sauer, U. (2013) Dissecting specific and global transcriptional regulation of bacterial gene expression. *Mol. Syst. Biol.* 9, 658
10. Norvell, A., and McMahon, S. (2010) Rise of the Rival. *Science (80-.).* 327, 964–965
11. LaPorte, D. C., and Koshland, D. E. (1983) Phosphorylation of isocitrate dehydrogenase as a demonstration of enhanced sensitivity in covalent regulation. *Nature* 305, 286–290
12. Starai, V. J., Celic, I., Cole, R. N., Boeke, J. D., and Escalante-Semerena, J. C. (2002) Sir2-dependent activation of acetyl-CoA synthetase by deacetylation of active lysine. *Science (80-.).* 298, 2390–2392
13. Gardner, J. G., Grundy, F. J., Henkin, T. M., and Escalante-Semerena, J. C. (2006) Control of acetyl-coenzyme A synthetase (AcsA) activity by acetylation/deacetylation without NAD+ involvement in *Bacillus subtilis*. *J. Bacteriol.* 188, 5460–5468
14. Crosby, H. A., Heiniger, E. K., Harwood, C. S., and Escalante-Semerena, J. C. (2010) Reversible N-ε-lysine acetylation regulates the activity of acyl-CoA synthetases involved in anaerobic benzoate catabolism in *Rhodopseudomonas palustris*. *Mol Microbiol* 76, 874–888
15. Hua, X., Hegde, S. S., Blanchard, J. S., and Xu, H. (2012) The Reversible Acetylation and Inactivation of *Mycobacterium tuberculosis* Acetyl-CoA Synthetase is Dependent on cAMP. *Biochemistry* 50, 5883–5892

Summary

One of the biggest disadvantages of using *E. coli* for biotechnological purposes is the acetate production that inhibits growth and decreases production yields. Apart from the biotechnological implications, the link of acetate metabolism to central carbon metabolism is not clearly known. Increasing the knowledge of the regulatory mechanisms that control *E. coli* metabolism is an important task, in order to perform rational bioprocess optimization. Several regulatory mechanisms exist in bacteria. Until some years ago, little was known about post-translational modifications in prokaryotes. Evidences in *Salmonella enterica* showed that acetate metabolism is regulated by reversible acetylation of the ε-amino group of lysine residue of proteins.

For these reasons, the main objective of this thesis was to describe the link between acetate and central carbon metabolism, focusing in the role of lysine acetylation in the regulation of this metabolism and in other physiological events.

The deletion of the main acetate-producing pathway altered central metabolism at different levels, from the transcripts to the energetic levels, showing the link between this metabolism and the central carbon metabolism, and also the importance of proper-balance of the acetate metabolism.

The sirtuin CobB and the acetyltransferase PatZ altered the activity of acetyl-CoA synthetase *in vivo*. *cobB* was expressed constitutively while the *patZ* gene expression is activated transcriptionally by the transcription factor CRP, similarly to what has been described for the acetyl-CoA synthetase gene. Two putative binding sites were found in the upstream region of *patZ* gene, at −41.5 and −81.5 bp from the transcription start site.

The absence of the CobB and PatZ proteins in the BL21 strain altered the acetate metabolism more severely than in the K12 strain. This indicates that probably the differential metabolic regulation of these enzymes could be the key for this different acetate production.

The deacetylase activity of CobB is global, contributes to the deacetylation of a big number of substrates and has major impact on bacterial physiology. Acetylation of

isocitrate lyase contributes to the fine-tuning regulation of the glyoxylate shunt and acetylation of lysine 154 of RcsB prevents DNA binding. This last effect activates flagella biosynthesis and motility proteins, and increases susceptibility to acid stress.

Deletion of the acetyltransferase *patZ* increased protein acetylation, especially in acetate cultures. This suggested that the role of PatZ could be the regulation of the acetylating agents levels in the cell. In fact this last finding would explain the simultaneous transcriptional activation of the protein acetyltransferase (*patZ*) and acetyl CoA synthetase (*acs*).

The results presented in this thesis offered new insights into the roles of acetate metabolism and lysine acetylation in *E. coli*. The acetate metabolism is linked to the central metabolism by regulating the levels of key metabolites, such as acetyl-CoA and acetyl-phosphate, which are involved in chemical protein lysine acetylation. This mechanism of post-translational modification of proteins is important in the control of the metabolism, but also in other important physiological roles like motility and stress survival. Also, the protein acetyltransferase, PatZ that does not act as the main acetylating agent in *E. coli*, it might play a role in the regulation of chemical acetylation in *E. coli*.

Index

Chapter 1

Introduction

Part of the information presented in this chapter is based in the manuscript under review:

Bernal V, Castaño-Cerezo S, Gallego-Jara J., Ecija-Conesa A., De Diego T., Iborra JL, Cánovas M.
"*Regulation of bacterial physiology by lysine acetylation of proteins*"

Escherichia coli: the Gram-negative model. Its relevance in Biochemistry and Biotechnology

E. coli is a Gram negative bacterium that normally lives in the gut of warm-blooded animals. Most of the *E. coli* strains are harmless but some can cause infections and intoxications. Harmless *E. coli* in the gut is part of a microbial community that provides the host with vitamins and is a defence barrier against pathogen infections (*1*).

In addition to its ecological function, its easy genetic manipulation and cultivation have made of *E. coli* a useful tool in modern research and biotechnology (*2*). It is used in the industry for metabolite and protein production. The use of bacteria is essential for bioproducts that cannot be produced chemically or if the production should be more efficient and/or green/environmentally friendly (*3*). Industrially relevant metabolites can be directly related to the central carbon metabolism, as in the case of succinate and amino acids (*4*, *5*) or to the secondary metabolism, like L-carnitine or xylitol (*6*, *7*). Furthermore, *E. coli* is the host of choice for the production of heterologous proteins which do not need special post-translational modifications (*8*, *9*).

The study of the central carbon metabolism in *E. coli* is essential in order to improve production yields or to find new ways to obtain these bioproducts. The knowledge of metabolism regulation will increase the development of new rational strategies for the use of *E. coli* in bioprocess.

Central carbon metabolism (CCM) could be defined as the complex series of enzymatic steps that convert carbon sources into metabolic precursors like acetyl-CoA (*10*). The pathways considered to be part of the CCM are glycolysis, gluconeogenesis, pentoses phosphate pathway, tricarboxylic acids cycle, glyoxylate shunt and those related to the consumption of other carbon sources (*10*, *11*). Although the metabolism of *E. coli* is considered the better-studied bacterial model, the mechanisms of regulation of CCM, especially those related with post-translational regulation, are still in their infancy.

In 1970, Francis Crick established the central dogma of Molecular Biology (*12*). This dogma illustrates the unidirectionality of information transference. DNA is

transcribed into RNA, and this RNA is translated into proteins that finally yield a given phenotype of an organism. Nowadays, this dogma has been completed with the discovery of reverse transcriptase (*13*). All these steps (replication, transcription, translation etc.…) are regulated, and these regulation mechanisms provide organisms with an instrument for the adaptation to new environments and survival to stress conditions.

In this PhD Thesis the mechanisms of metabolism regulation, especially those related to acetate metabolism, will be further explored. In the following sections, detailed explanation of the state of the art of some regulatory mechanisms, such as transcriptional and post-translational regulation will be provided. Also, the main metabolic pathways related to acetate metabolism will be thoroughly described.

Central Carbon Metabolism in *Escherichia coli*

Catabolite repression

E. coli is a heterotrophic organism. It can consume several carbon sources such as glycerol, acetate, glucose, fructose or galactose. Free-living bacteria have global systems of regulation in order to consume preferably a compound besides other carbon sources. This phenomenon is called catabolite repression (*14*) .

The system that *E. coli* uses to distinguish between different carbon sources is the PTS system (Phosphoenolpyruvate Transport System) (*15*). The carbon sources that *E. coli* can use can be classified into two different classes: PTS substrates (*i.e.* glucose, fructose) and Non-PTS substrates (*i.e.* glycerol and acetate). This classification is based on how the substrate is transported into the cell (dependent or independently of the PTS system). The PTS system consists of a multi-protein complex that couples a protein phosphorylation cascade that ends in the phosphorylation of the carbon source keeping it inside the cell. Two different parts compose the complex. The common part for all the PTS substrates composed by two proteins: the H-Pr and EI. The specific part for each PTS substrate that is composed of three different proteins: EIIA, EIIB and EIIC.

In the case of the glucose PTS system, the $EIIB^{Glc}$ and $EIIC^{Glc}$ components belong to the same protein that is linked to the plasma membrane, while the $EIIA^{Glc}$

component is located in the cytoplasm (**Fig. 1**). The presence of a high concentration of glucose activates a phosphorylation cascade that follows this scheme: PEP→H-Pr→EIIA^{Glc}→EIIB^{Glc}→Glucose. When glucose concentration is almost null in the medium, the EIIA^{Glc} protein accumulates in its phosphorylated form, activating the adenylate cyclase enzyme (encoded by the *cyaA* gene). This activation causes an increase in the concentration of cyclic AMP (cAMP) that is the signal for the end of catabolite repression. cAMP binds to the transcription factor CRP (Catabolite Repression Protein), inducing its dimerization and activation, being able to activate the transcription of those genes related to the consumption of non-PTS carbon sources (*e.g.*, acetate) and inhibiting those genes related to the glucose consumption (*14*).

Acetate metabolism of *Escherichia coli*

Acetate production has a big importance in biotechnology because it affects bioprocess performance. This metabolite has a negative effect in physiology. It inhibits cell growth in high density cultures because of its toxic effects (*16*), limiting recombinant protein production and being crucial for bioprocess design, affecting the metabolism of glycerol, succinate, ethanol, 3-hydroxypropionate, polyhidroxyalcanoates (PHAs) and also affecting some biotransformations such as that of L-carnitine (*17*).

E. coli can use acetate as the sole carbon source. In anaerobiosis, *E. coli* produces and excretes fermentation products in order to maintain cellular redox homeostasis, being acetate the main product (*18*). In aerobic conditions, acetate is the main by-product of glucose metabolism, decreasing the maximum biomass yield of glucose oxidation. This phenomenon is commonly known as "acetate overflow". Big efforts have been made in order to unravel the physiological function of the acetate overflow in *E. coli*, why acetate is produced is still to be deciphered (*19–21*).

The principal metabolic pathways that are involved in the production and consumption of acetate are those catalysed by the acetyl-CoA synthetase (Acs), phosphotransacetylase (Pta) and acetate kinase (AckA), enzymes of the glyoxylate shunt and, to a lower extent, the pyruvate oxidase. A description of some of them is detailed below.

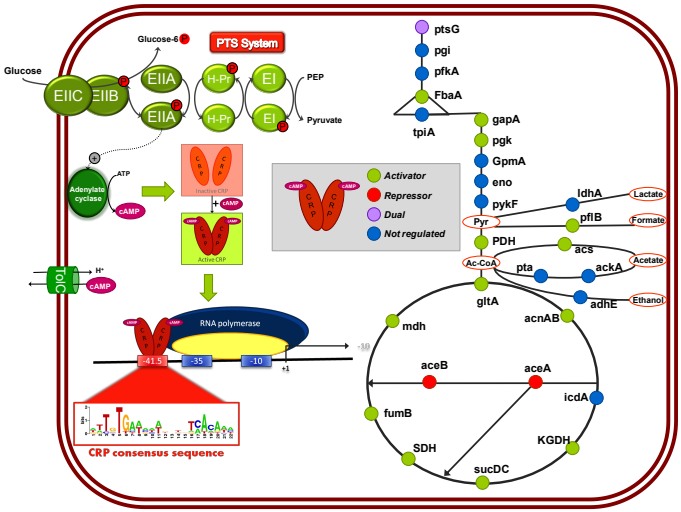


Fig. 1. PTS system in *E. coli* scheme and its link to transcriptional regulation by the transcription factor CRP. This protein regulates the transcription of most of the enzymes of the CCM in *E. coli*.

Acetyl-CoA synthetase pathway (Acs). This enzyme catalyses the irreversible transformation of acetate to acetyl-CoA though an acetyl-AMP intermediate (*22*). This is a high affinity pathway due to the low K_m of this enzyme for acetate (200 μM). Its main function is scavenging acetate from the medium when it is present at low concentration (*23*). The gene that encodes for this enzyme is located in an operon with other two genes (*24*): *yjcH* of unknown function, and *actP*, that encodes for an acetate permease (*25*). The organization of this operon is represented in **Fig.2.**

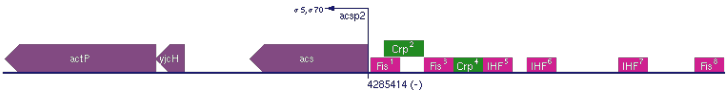


Fig. 2. Transcriptional unit of the *acs-yjcH-actP* operon in *E. coli*. (*26*). Boxes in pink represent transcriptional repressors and green boxes represent transcriptional activators *Source: Ecocyc (www.ecocyc.org)*

The transcription of this operon can start from two different promoter regions, namely *acsp1* and *acsp2* (*27*, *28*), being the latter the most important. Transcription from *acsp2* is σ70-dependent and it has also been described that σ38 can

inhibit the transcription from this promoter indirectly (*29*, *30*). The transcriptional regulators acting directly on this operon are CRP, IHF and Fis, while other regulators, such as Fnr and IclR, act indirectly (*29*). For the expression of this operon it is at least need the binding of CRP at position -69.5 from the transcription start site. Maximal expression of this operon is observed when another CRP molecule binds at position -122.5pb (*27*, *28*). It has also been described an internal promoter that regulates the σ54 dependent expression of *actP* (*31*).

Phosphotransacetylase-acetate kinase pathway (Pta-AckA). These two enzymes catalyse the reversible production and consumption of acetate. This pathway is mainly used for acetate production in a mechanism known as "acetate overflow" (*32*). This is a low affinity (K_m for acetate is 10 mM) and high capacity pathway (*33*), for this reason it is also used for the consumption of acetate when it is present in the medium at high concentration.

The genes that encode for these two proteins belong to the same operon and their expression is considered to be constitutive, although its transcription is slightly stimulated by the transcription factor Fnr (*34*). It has been also recently observed that this operon can produce two different transcripts, one containing both enzymes and another one that only contains the *pta* gene (*35*).

The unstable intermediate of this pathway, acetyl-phosphate, is recognized for its importance in signalling pathways. It has been demonstrated that this metabolite can phosphorylate proteins related to signal transduction, especially two component systems (*36*). *In vivo* it has been demonstrated that it acts in the CheY-CheA system, related to chemotaxis (*37*), and in the PhoB-PhoR system, involved in phosphate metabolism (*38*). Recently, acetyl phosphate has also been demonstrated to be an acetyl donor, further information on this function is detailed below (*39*).

Glyoxylate shunt. This pathway has two enzymes, isocitrate lyase (AceA) and malate synthase (AceB). Isocitrate is the substrate of AceA. It splits isocitrate into glyoxylate and succinate. Glyoxylate is condensed with an acetyl-CoA molecule to form malate, a reaction catalysed by the malate synthase enzyme (AceB). This pathway

shortcuts the tricarboxylic acids cycle (TCA) to prevent the loss of the two carbons of acetyl-CoA when bacteria are growing under gluconeogenic conditions. In fact, the *aceA* gene is essential for growth on acetate minimal medium (*40*). Even more, it has been described that this pathway also forms part of another catabolic pathway named the PEP-GOX cycle. This pathway is mainly active when cells are limited by the carbon source and its main function is to reduce the overproduction of NADPH and favour the production of NADH, and then energy, under these conditions (*41*, *42*).

The genes that encode these enzymes, isocitrate lyase and malate synthase are located in the same operon, named *aceBAK*. The third product of the operon is the enzyme isocitrate dehydrogenase kinase/phosphatase (AceK) (*43*). This is a regulatory enzyme that modulates the activity of the isocitrate dehydrogenase (Icd) by phosphorylation/dephosphorylation. The phophorylation of Icd inhibits its activity, increasing the concentration of isocitrate. The increase of this metabolite is important due to the different affinity of both enzymes of the isocitrate node for isocitrate. AceA has a higher K_m for this metabolite (*i.e.* lower affinity) being only possible the flux through this shunt by an inactivation of Icd and an increase in the isocitrate concentration (*44*, *45*).

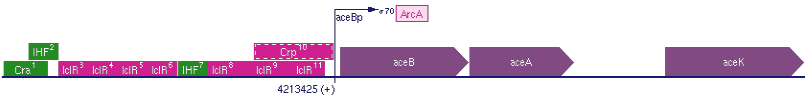


Fig. 3. Representation of the *aceBAK* transcriptional unit. Boxes in pink represent transcriptional repressors and green boxes represent transcriptional activators (*26*). *Source: Ecocyc (www.ecocyc.org)*

The expression of this operon can occur from two different promoters, one which transcribes the three genes (*aceBp)* and an internal promoter that only transcribes the *aceK* gene (*aceKp)* (*46*). The main promoter is *aceBp*, that is σ70 dependent (**Fig. 3**). The activity of this promoter is regulated by several transcription factors. IHF, has two binding sites in the operator region and activates transcription when acetate is present in the medium (*47*). The transcription factor Cra (also known as FruR) also activates the transcription of the operon (*48*, *49*). Furthermore, three different transcription inhibitors have been found, two general transcription factors (CRP and ArcA) and another which is specific for this operon, IclR (*50*).

The overflow metabolism. In the last years several studies have been published that tried to shed more light on the acetate overflow phenomenon in *E. coli* (*41*, *51*). This metabolism is observed when *E. coli* grows in glucose batch cultures and at high dilution rates in glucose-limited chemostat. Although it was previously thought that acetate production was related to a high growth rate, some authors have described that it is due to a high glucose consumption rate (*41*, *52*).

Recently it has been demonstrated that *E. coli* produces acetate continuously, independently of the growth and the glucose consumption rates (*41*, *51*). These authors demonstrated that acetate accumulation was due to an unbalance of the acetate producing (Pta-AckA and PoxB) and consuming (through Acs) pathways. Even more, they identified that catabolite repression (mediated by cAMP-CRP) of the *acs* expression in batch cultures and high dilution rate glucose limited cultures is the main unbalance factor for the acetate accumulation. The acetate metabolism in *E. coli* produces a futile cycle because of simultaneous production and consumption of acetate. The reasons explaining the existence of this futile cycle are unclear; it spills energy, thereby decreasing the cellular yield. It has been hypothesized that the maintenance of a acetyl-phosphate concentration in the cell could be the clue for this process (*41*, *51*).

Regulatory mechanisms in *Escherichia coli*

Transcription regulation

Sigma factors and Transcriptional regulators

Most of the genes in prokaryotes are organized in transcriptional units called operons. Operons are formed by functionally related genes and are coordinately regulated. Gene expression depends on the promoter activity and this depends on the sequence up-stream the operon that can also be regulated by specific transcription factors. This strategy allows cells transcribing, at the same time, genes that form part of the same metabolic or signalling pathway, which constitute a regulon. Recently, it has been demonstrated that in many operons have internal promoters. This allows bacteria to fine tune gene expression of some genes independently of the rest the transcriptional unit (*46*).

Binding of the RNA polymerase to the promoter region is needed for the transcription of an operon. Even more, the binding of other proteins to this region, called transcription factors, modulate the activity of the RNA polymerase. Besides this, transcription factors can act as activators or inhibitors of the transcription.

The RNA polymerase in *E. coli* is formed by five different subunits: two catalytic subunits (β and β´), two α subunits that regulate transcription initiation frequency, and the σ subunit, which recognizes and interacts with the transcription start site (TSS). There are seven different σ subunits that recognize different types of promoters. The general σ subunit is σ70, that is considered constitutive (*53*). Another important is σ32, that binds to those promoters that should be transcribed under stressing conditions(*54*).

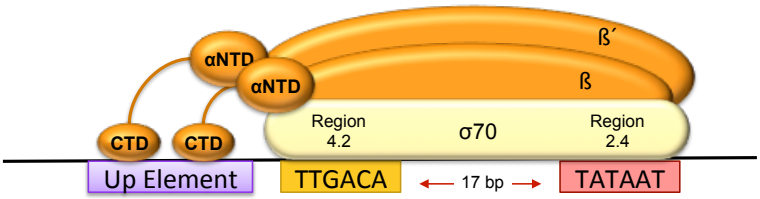


Fig. 4 Structure of the RNA polymerase in prokaryotes and its interaction with DNA sequences (*53*).

One of the best-known transcription factors in *E. coli* is CRP. This protein is 24 kDa and it is synthesized as inactive monomers. The presence of cAMP changes the conformation of CRP, forming dimers and getting the active conformation (*55*).

Consensus sequence

The term "consensus sequence" is used with the aim of describing the strength of the binding of proteins to their target DNA sequences. A consensus sequence is the DNA sequence that binds to RNA polymerase or a transcription factor with the highest affinity. In the case of σ70 subunit of the RNA polymerase, the consensus sequence has two different parts (**Fig. 4**): the -10 box (TATAAT) and the -35 box (TTGACA), both spaced by 17 pair of bases. Nowadays, it is known that there are other characteristics that increase the affinity of the RNA polymerase for its promoter. This is the case of the

extended -10 box, which makes the -35 box dispensable (*56*). For the transcription factor CRP (**Fig. 1**), the consensus sequence is 22 base pair long and it is the sequence palindrome TGTGA------TCACA (*57*).

In the last years, bioinformatic techniques have evolved providing better tools for the analysis of genomic sequences. Since then, it is easier to obtain consensus sequences from a big number of sequences building position weight matrixes. These are constructed aligning all the binding sequences known of a particular transcription factor. After alignment, it is possible to calculate the relative frequency of each base in each position. These values allow the calculation of a score for each sequence from the position weight matrix (*58*, *59*). These matrices can also be used to search in the genome for new putative binding sites for a given transcription factor or sigma factor.

Post-translational regulation

Post-translational modification (PTM) of proteins is an evolutionarily conserved strategy used by organisms for the efficient control of their biological activities, allowing them to exert rapid adaptive responses to environmental changes. Several types of PTMs exist in Nature. Among the more prominent are serine, threonine, and tyrosine phosphorylation; lysine ubiquitination, sumoylation, and neddylation; lysine acylation; lysine and arginine methylation; and proline isomerization (*60*).

Until some years ago, the better-known PTM in *E. coli* was protein phosphorylation. This modification was observed mainly in the two-component regulatory systems where aspartic acid and histidine residues are phosphorylated. These systems generally consist in two proteins, one that acts as signal receptor and another that acts as transducer of the signal. For example, in the ArcA/ArcB (Anoxic Redox Control) two-component regulatory system ArcB senses the signal, and ArcA translates this signal into transcriptional regulation (**Fig. 5**). In conditions where the oxygen is limiting this system activates. The protein ArcB autophophorylates and triggers a phosphorylation cascade through ArcA. The phosphorylated form of ArcA inhibits genes related to the aerobic metabolism and activates the expression of fermentative

genes (*61*). Some fermentation products like D-lactate, pyruvate or acetate, also activate ArcB autophosphorylation (*62*). In aerobic conditions, the autokinase activity of ArcB it is inhibited by the presence of quinones producing the dephophorylation cascade similar to the described before for phosphorylation (*63*, *64*).

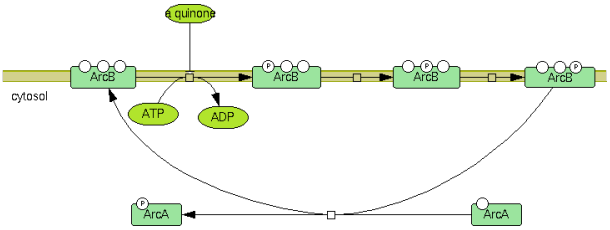


Fig. 5. ArcA/ArcB two component system (*26*). *Source: Ecocyc (www.ecocyc.org)*

Furthermore, until some years ago isocitrate dehydrogenase was the only enzyme known to be affected by a PTM in *E. coli*. This enzyme belonging to the Tricarboxylic cycle (TCA) is reversibly phosphorylated by a specific kinase/phosphatase belonging to the glyoxylate shunt operon (AceK) (*65*, *66*).

Protein lysine acetylation

In the last years, protein acetylation is receiving more attention due to its involvement in several mitochondrial, nuclear and cytosolic processes (*67–70*). Protein acetylation can be defined as the transference of an acetyl group from a convenient biochemical donor molecule to an amino group on a protein. Activated acyl groups such as acyl-CoAs and acyl-phosphates can acetylate many proteins. Thus, proteins can be acetylated, propionylated, butyrylated or succinylated (*71*, *72*). Although some of these processes are catalysed by specific transferases, they can also occur non-enzymatically. It is not clear yet whether these events are spontaneous or auto-catalytical. Among all these acylation modifications, protein acetylation is by far the best known.

Acetylation of lysine residues of proteins was first identified in histones more than 40 years ago. Recent reports have shown that a high number of metabolic enzymes are acetylated, which has led to suggesting an evolutionarily conserved mean

of regulation of intermediary metabolism (*73*, *74*). Protein acetylation and deacetylation is catalysed by protein acetyltransferases and deacetylases, respectively, of which several families exist. In addition, several proteins acetylate in the presence of reactive acetyl derivatives, such as acetyl-phosphate, acetyl-CoA or acetyl-AMP (*39*, *75*, *76*).

Based on the chemical nature of the acetylated amino group, two types of protein acetylation can be considered, each one exhibiting specific characteristic features. The acetylation of the α-amino group of the N-terminal amino acid of proteins is very rare in bacteria (*77*) but frequent in eukaryotes and archaea (30-80 and 14-29% of proteins, respectively) (*78*). On the other hand, the acetylation of proteins at the ε-amino group of internal lysine residues is a widely distributed PTM, frequent in all domains of life. This is a reversible post-translational process which has now been found on over 1500 eukaryotic proteins with diverse functions and locations (*69*, *79*, *80*). Lysine acetylation exerts various effects on many of their critical functional properties, including interaction with other proteins and DNA, stability and sub-cellular distribution (*67*, *69*).

Diversity of bacterial protein acetyltransferases and deacetylases

N-acetyltransferases catalyse the transfer of an acetyl group from acetyl-CoA to a primary amine on a substrate. The acetylation of the ε-amino group of lysine residues of proteins is catalysed by lysine acetyltransferases (often abbreviated to KATs or LATs), formerly known as histone acetyltransferases (HATs). Several KATs are known in eukaryotes, which are structurally diverse. All families contain a homologous acetyl-CoA binding core segment, which raises the possibility that they have evolved from a common ancestral protein (*81*) .

Enzymes belonging to the Gcn5-related N-acetyltransferase (GNAT) superfamily (pfam00583) are conserved in all domains of life and are best described as enzymes that utilize acyl-coenzyme A (CoA) as a donor for the acylation of the ε-amino group of lysine residues of proteins and small molecules. Many of the bacterial KATs described belong to this group. This is the case of *Salmonella enterica*: in 2004, the gene encoding the KAT *Se*Pat was identified in this organism. This protein is responsible for

the acylation of acetyl-CoA and propionyl-CoA synthetases (*82*, *83*). There is controversy on the acetylation of other metabolic proteins by *Se*Pat in *S. enterica*. Wang and col. described that the metabolic enzymes GapA, AceA and AceK are acetylated by *Se*Pat (*74*), but it has been claimed that these results cannot be reproduced (*84*).

*Se*Pat possesses two domains: a C-terminal domain homologous to GNAT acetyltransferases and a N-terminal domain with high homology to NDP-forming acetyl-CoA synthetase. However, it lacks acetyl-CoA forming activity due to the replacement of a histidine residue which is critical for catalysis (*83*, *85*). *Se*Pat shows sigmoidal kinetics and positive cooperativity for acetyl-CoA and a biphasic interaction with acetyl-CoA, which are unusual in GNATs. The structural basis for this behaviour is the tetramerization of the protein in the presence of acetyl-CoA (*85*). This biphasic acetyl-CoA binding relies on the acetyl-CoA synthetase-like domain, which allows it to respond to small variations in the acetyl-CoA concentration. This characteristic contributes to maintaining acetyl-CoA homeostasis during acetate uptake (*85*, *86*).

Other microorganisms contain KATs from the GNAT superfamily that lack the putative acetyl-CoA synthetase domain found in *Se*Pat (such as AcuA from *B. subtilis*) or have a regulatory domain absent in the *Se*Pat (such as *Mt*Pat from *Mycobacterium tuberculosis* which is responsive to cyclic nucleotides). In addition, many organisms that contain homologs to protein lysine deacetylases lack Pat homologs, an observation that suggests the existence of functional orthologous with distinct evolutionary lineage (*83*).

Lysine deacetylases (KDACs) catalyse the cleavage of N-acetyl amide moieties. Eukaryotic KDACs are subdivided into four classes on the basis of phylogenetic criteria (*87*). According to their reaction mechanism, two types of deacetylases can be discerned: simple hydrolases, which release the acetyl group as free acetate, and sirtuins, which are nicotinamide adenine dinucleotide (NAD^+)-dependent protein deacetylases. Sirtuins use NAD^+ as a co-substrate, which is cleaved in the catalytic cycle, generating nicotinamide and 2'-O-acetyl-ADP-ribose (OAADPr) as co-products. KDACs are conserved proteins with an ancient origin, and they are expected to participate in basic processes well conserved across organisms (*87*).

Sirtuins typically consist of a conserved catalytic core domain (~250 aa) and two optional and highly variable N- and C-terminal domains (50-300 aa) (*87*, *88*). It is now evident that sirtuin protein sequences are broadly conserved within the three domains of bacteria, archaea and eukaryotes. Of special relevance is the involvement of sirtuins in the response to nutrients availability (calorie restriction response) and, therefore, in metabolic regulation. In fact, it is that what is thought to be the major functions of sirtuins (*67*).

Both types of KDACs are found in bacteria. The CobB protein in *S. enterica* is the best characterized sirtuin in bacteria (*89*), although it has also been found in *E. coli* (*90*), *B. subtilis* (*91*), *Rhodopseudomonas palustris* (*92*), *Streptomyces coelicolor* (*93*) and other genera. Phylogenetic analysis reveals that bacterial sirtuins are related to the mitochondrial Sirt4 and Sirt5 from humans, while archaeal sirtuins are closer to eukaryotic Sirt6 and Sirt7, and Sirt1, Sirt2 and Sirt3 cluster separately, together with yeast sirtuins (*88*). No recognizable hydrolytic (non-sirtuin) deacetylases have been found in many individual eubacterial species (*e.g. E. coli*) (*87*), although they have been described in *B. subtilis* (*94*), *R. palustris* [28] and *M. tuberculosis* (*95*).

Protein acetylation in bacteria: targeted pathways

Modulation of the activity of bacterial proteins by site-specific acetylation has long been known. One classical example is CheY, which regulates chemotaxis through the acetylation of two specific lysine residues (*96*, *97*). Nevertheless, it was not until 2002, that the regulation of a bacterial enzyme by reversible acetylation/deacetylation was described for the first time (*89*). Major advances in the identification of acetylated proteins have been done in *E. coli*, *S. enterica*, *B. subtilis*, *R. palustris* and *Mycobacterium* species. In this Section, pathways targeted by protein acetylation in these model microorganisms and their physiological roles will be summarized.

AMP-forming acyl-CoA synthetases

The members of the AMP-forming acyl-CoA synthetases family are the best-characterized substrates of the protein acetylation system (**Table 1**). This large and diverse group of enzymes can activate several carboxylic acids into coenzyme A

thioesters, including short-, medium- and long-chain fatty acids, dicarboxylic acids, aromatic acids and other carboxylated metabolites (*92*, *98–100*).

Acetyl-CoA synthetase (Acs) is a wide spread enzyme which activates acetate, allowing it to be scavenged and metabolized (*41*, *98*). In 2002, Acs from *S. enterica* was the first bacterial protein which activity was described to be regulated by reversible acetylation (*89*). Currently, Acs orthologues in other bacterial species are also regulated in the same way.

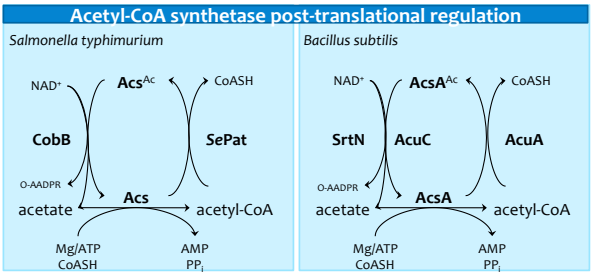


Fig. 6. Acetyl-CoA synthetase post-translational modification by lysine acetylation in *Salmonella enterica* and *Bacillus subtilis*.

a) Acetylation of Acs in Enterobacteria: *S. enterica*.

Acs from *S. enterica* is acetylated at a specific lysine residue in the vicinity of its active site, inactivating the enzyme. A careful analysis of the modified enzyme showed that formation of the reaction intermediary acetyl-AMP is blocked, while its transformation to acetyl-CoA proceeds normally (*89*). Inactivation of Acs is reversed by deacetylation (**Table 1**). The KAT and the KDAC responsible for this reversible modification of Acs were identified (*83*, *89*). The acetylation of Acs at high acetyl-CoA concentrations prevents its accumulation, while avoiding the depletion of free coenzyme A and metabolic energy (*85*, *86*).

b) Acetylation of AcsA in the Firmicute *Bacillus subtilis*.

In *B. subtilis*, the metabolism of acetate and acetoin are highly related. The acetate activating enzyme (encoded by *acsA*) is specifically acetylated by the KAT from the GNAT family AcuA (*94*, *101*) and is deacetylated by the KDACs AcuC and SrtN

(*91*) (**Fig. 6**). AcuA and AcuC are products of the *acuABC* operon (*ac*etoin *u*tilization), while an independent gene encodes SrtN. The *acuABC* operon and the *acsA* gene are adjacent in *B. subtilis* genome and these two transcriptional units are divergently co-transcribed (*91*).

The existence of two independent KDACs in *B. subtilis* and other bacteria such as *R. palustris* and *Mycobacterium sp* suggests that these microorganisms may have evolved divergent strategies to deal with protein acetylation *in vivo*. It has been argued that the activity of NAD^+-dependent and independent KDACs is differently affected by small molecules such as nicotinamide nucleotides. Therefore these two strategies might be active under different growth conditions (*91*). The existence of NAD^+-independent deacetylases has not been so far demonstrated in other microorganisms such as *E. coli*.

c) Acetylation of acyl-CoA synthetases in *R. palustris*

R. palustris is a gram-negative purple non-sulfur α-proteobacterium that grows photoheterotrophically on aromatic compounds available in aquatic environments rich in lignin derived residues. When growing photosynthetically in the absence of oxygen, *R. palustris* converts aromatic compounds to acetyl-CoA, which is then used as carbon and energy source. At least 40 genes are annotated in *R. palustris* genome to encode putative AMP-forming acyl-CoA synthetases and many of them have the conserved lysine which is acetylated in Acs (*69*, *98*). Two KATs (*Rp*Pat and *Rp*KatA) and two KDACs (*Rp*LdaA and *Rp*SrtN) have been identified in *R. palustris*. Crosby and col. identified 24 putative acetylated proteins, 14 of which were over-acetylated in a mutant devoid of both deacetylases. Nine of these proteins were AMP-forming acyl-CoA synthetases (*84*). The activity of the aryl-CoA synthetases BadA (which activates benzoate) and HbaA (which activates 4-hydroxybenzoate) and the alicyclic acyl-CoA synthetase AliA (which activates cyclohexanecarboxylate) are regulated by N-ε-lysine acetylation (*84*, *92*). The acetylation of these acyl-CoA synthetases avoids uncontrolled consumption of large amounts of ATP and free CoA, which might lead to growth arrest. In addition, these enzymes are reactivated by SrtN, which suggests that under high NAD^+/NADH ratio conditions, NAD^+ might signal a need to catabolize benzoate (and related compounds) to generate carbon and reducing power. In addition, it has

been suggested that the transcriptional activator of the benzoate catabolism operon (BadR) may sense benzoyl-CoA rather than benzoate and, therefore, this regulation of benzoyl-CoA synthesis would co-ordinate post-translational and transcriptional regulation of aromatic compounds degradation (*92*).

d) Acetylation of AcsA in Mycobacteria.

Mycobacteria are of high biomedical interest because they are the causative agents of diseases such as tuberculosis and leprosy. Acetylation of proteins has been described recently in *M. tuberculosis*, which causes tuberculosis, and in the non-pathogenic *M. smegmatis*. Of special interest are the KATs from *M. tuberculosis* (*Mt*Pat) and *M. smegmatis* (*Ms*Pat), which possess a C-terminal GNAT domain fused to an N-terminal cyclic nucleotide-binding domain. This is the only bacterial KAT allosterically regulated by cAMP binding (*102*). Cyclic nucleotides are universal second messengers, which elicit diverse responses in bacteria. It has been reported that cAMP reprograms mycobacterial physiology, and its production by *M. tuberculosis* is critical for host-pathogen interaction and disease (*102*). In fact, mycobacteria contain over a dozen genes identified as adenylate cyclases (ACs) (*103*), which activity increases under various physiological conditions. In particular, in *M. tuberculosis*, several of the ACs are specifically responsive to nitrogen and carbon limitation, pH, and bicarbonate (*103*). The phagolysosome is an acidic, nutrient poor, and oxidatively hostile environment, and bacteria slow down their metabolism in order to survive (*103*). When *M. tuberculosis* is phagocyted by macrophages, cAMP increases dramatically, and this cAMP is secreted to the phagolysosome, exerting responses in the host which are involved in pathogenesis (*104*).

Other pathways targeted by protein acetylation

Other metabolic enzymes

Important acetylation targets have been identified lately thanks to high throughput proteomics. This is the case of glyceraldehyde phosphate dehydrogenase (GapA), isocitrate lyase (AceA) and the isocitrate dehydrogenase (Icdh)

kinase/phosphatase (AceK) in *S. enterica* (*74*). However, it has been claimed that these results cannot be reproduced (*84*) and should be critically revised.

Non-metabolic targets: gene transcription, cell motility and chemotaxis

In addition to metabolic proteins, other interesting targets have been the subjects of classical molecular level studies in bacteria. In this section, we will focus on the regulation by acetylation of cell motility and chemotaxis, gene transcription and RNA stability.

Modulation of gene transcription: acetylation of RNA polymerase. In *E. coli*, the RNA polymerase (RNAP) is acetylated on almost 30 residues on different subunits (α, β and β'), and this acetylation pattern depends on the growth conditions (*105*). Of special interest is the acetylation of the α-carboxy-terminal domain (α-CTD), which is involved in promoter recognition by interacting with a DNA region upstream the transcription initiation site known as the UP element and/or by interacting with certain TFs. In fact, this specific domain is acetylated in response to glucose and other carbon sources. Moreover, PatZ (YfiQ) and CobB mutations affected acetylations, although there are no direct evidences that these proteins are responsible for these events. Interestingly, *cpxP* transcription responds to an acetyl-CoA to CoA imbalance that the cell perceives as a stress and may contribute to the regulation of protein acetylation and, probably, also to NAD^+ availability which would lead to competition of the deacetylase CobB with other enzymes for the free oxidized form of the nucleotide (*105*).

Modulation of cellular motility and chemotaxis: RcsB and CheY. The multicomponent RcsF/RcsC/RcsD/RcsA-RcsB phosphorelay system is involved in *E. coli* in the regulation of several genes related to the synthesis of the colanic acid capsule, cell division and motility, among others (*106*). The DNA binding protein RcsB is the response regulator of this system. Acetylation or substitution by site-directed mutagenesis of a specific lysine residue of the protein (which lies within a helix-turn-helix motif and is essential for DNA binding) impedes *in vitro* binding to the RcsB box of a regulated promoter (*107*). The active form of RcsB is phosphorylated (*108*) and, therefore, regulation involves two concurrent types of PTMs.

CheY protein, which regulates chemotaxis, is also subjected to a similarly complex regulation by acetylation and phosphorylation. CheY is phosphorylated in response to environmental conditions and acetylated in response to altered metabolic state of cells. CheY is deacetylated by CobB (*109*). These modifications shift the isoelectric point of the protein, avoiding the interaction with its partner proteins and regulating chemotaxis (*109*).

The concurrence of two independent PTMs regulating the same protein is intriguing. Both PTMs are linked and co-regulated. CheZ, dephosphorylates CheY and also increases its acetylation. These authors proposed that the physiological role of these mutual effects is at two levels: linking chemotaxis to the metabolic state of the cell, and serving as a tuning mechanism that compensates for cell-to-cell variations in the concentrations of regulatory proteins such as CheA and CheZ (*110*, *111*). Therefore it seems possible that these two modes of regulation may have complementary roles.

The complementary regulation of protein activity by two PTMs (as described in CheY and RcsB) demonstrates the existence of a cross-talk between regulatory networks in *E. coli* (*60*), which has also been recently evidenced in the genome reduced bacterium *Mycoplasma pneumoniae* (*112*) and indicates that we have a very limited picture of the actual complexity of cellular regulatory networks.

Modulation of protein stability by acetylation: RNase R. RNase R is an exoribonuclease important for the degradation of structured RNAs in *E. coli*. This normally unstable protein increases its half-life in the stationary phase and certain stress conditions. Acetylation by PatZ (YfiQ) in the exponential phase (but not in the stationary phase) results in tighter binding of tmRNA-SmpB (the *trans*-translation machinery and its associated protein) and subsequent proteolytic degradation. Acetylation of RNase R only affects its stability and does not affect its catalytic activity (*113*).

Table 1. Lysine acetylated bacterial proteins

Species	Protein	Position[a]	Effect of acetylation	References
Escherichia coli	Acetyl-CoA synthetase (AMP forming)	K609	Deletion of deacetylase impairs growth on acetate as sole carbon source.	(*90*)
	CheY	K91 K109	Acetylation represses the binding of CheY to FliM, a component of the Flagellar Motor Switch Complex. Acetylation of CheY results in clockwise rotation of the flagella. CobB regulates chemotaxis by deacetylating CheY.	(*109*, *111*)
	Regulator of capsule synthesis (RcsB)	K180 K154	RcsB is a global regulatory protein that controls cell division and capsule and flagellum biosynthesis. Acetylation of RcsB in both lysines blocks DNA binding. Substitution of K180 by A, R or Q results in an increased motility and substitution of K154 by Q decrease transcription of the small RNA *rprA*.	(*107*, *114*)
	RNase R	K544	Acetylation by YfiQ destabilizes the protein by increasing the strength of the binding of tmRNA-SmpB to the C-terminal region of RNase R in the exponential phase, leading to proteolytic degradation. Deacetylation in the stationary phase leads to the stabilization of the protein.	(*115*)
	N-hydroxyarylamine O-acetyltransferase (NhoA)	K214 K281	The mutation of both lysines decreased N-hydroxyarylamine O-acetyltransferases and aromatic amine N-acetyltransferases activity. These lysines are deacetylated by CobB	(*116*)
	α-subunit of RNAP	K298	Acetylation of the α-subunit of RNAP enhances the expression at the *cpx*P promoter in response to acetyl-CoA to CoA imbalance.	(*117*)
Salmonella enterica	Acetyl-CoA synthetase (AMP forming) (Acs)	K609	Acetylation blocks the adenylating activity of the enzyme. Deletion of deacetylase impairs growth on acetate as sole carbon source.	(*89*)
	Propionyl-CoA synthetase (AMP forming) (Pcs)	K592	Acetylation blocks the adenylating activity of the enzyme.	(*83*)
	Glyceraldehyde-3-phosphate dehydrogenase (GapA)	K321	Acetylation increases GapA enzymatic activity	(*74*)
	Isocitrate lyase (AceA)	K34	AceA is inactivated by acetylation.	(*74*)
	Isocitrate dehydrogenase phosphatase/kinase (AceK)	K205	Acetylation may activate the phosphatase activity toward ICDH. Deacetylation of AceK by CobB increases AceK's ability to inactivate ICDH.	(*74*)
Bacillus subtilis	Acetyl-CoA synthetase (AMP forming) (Acs)	K549	The acetylation of AcsA by AcuA is reversed by the deacetylases AcuC (NAD-independent) and SrtN (sirtuin).	(*91*, *94*)

[a] Position of acetylated lysine in protein sequence.

Table 1 Lysine acetylated bacterial proteins**.**

R. palustris	Acetyl-CoA synthetase (Acs) Benzoyl-CoA synthetase (BadA) 4-hydroxybenzoyl-CoA synthetase (HbaA) Cyclohexanecarboxyl-CoA synthetase (AliA)	K512 (BadA)	RpPat acetylates all 4 acyl-CoA synthetases. Two protein deacetylases are reported: SrtN (sirtuin-like, NAD^+-dependent) and LdaA (NAD^+ independent). SrtN and LdaA activate BadA. LdaA activates HbaA and AliA.	(*92*)
	9 AMP-forming acyl-CoA synthetases; glyceraldehyde-3-phosphate dehydrogenase; 4-oxalocrotonate tautomerase		RpPat acetylates 9 acyl-CoA synthetases and RpLdaA deacetylates 8 of them. Acetylation decreases activity of the enzymes by >70%.	(*84*)
Streptomyces coelicolor	Acetyl-CoA synthetase (Acs)		Acs is reactivated by CobB1-mediated deacetylation.	(*93*)
Streptomyces lividans	Acetoacetyl-CoA synthetase	K617	This enzyme is inactivated by the acetylation of residue 617 by *Sl*Pat.	(*118*)
Mycobacterium tuberculosis	Acetyl-CoA synthetase (Acs)	K617	Acs is inactivated by acetylation.	(*103*)
	Fatty acyl-CoA synthetase (FadD13)	K487	MtPat acetylates this enzyme in a cAMP dependent manner; the substitution of this residue to a glutamine decreases this enzyme acetylation and its activity.	)
Mycobacterium smegmatis	Universal Stress Protein (USP)	K104	Unknown role.	(*102*)
	Acetyl-CoA synthetase (Ms-Acs)	K589	This enzyme is inactivated by the acetylation in a cAMP dependent manner by MsPat and reverted this effect by the deacetylase SrtN	(*95*)
	Propionyl-CoA synthetase (Ms5404)	K586	The absence of any deacetylase in this organism alters growth in propionate minimal medium.	(*95*)

[a] Position of acetylated lysine in protein sequence.

High throughput analysis of cellular acetylomes

The number of proteins known to be post-translationally acetylated is constantly increasing due to the improvement in mass spectrometry (MS)-based proteomics techniques (*39*, *69*, *72*, *120*). These methods can be targeted for the identification of acetylated proteins by using antibodies to specifically immunoprecipitate acetylated peptides or increase the sensitivity of the technique by applying fractionation techniques to reduce the complexity of peptide mixtures subjected to mass spectrometric analysis (*39*, *121*). In general, MS-based strategies allow the non-biased identification of a high number of targeted proteins/pathways. Other techniques, such as protein microarrays, have also been used for the identification of substrates of acetyltransferases, although *in vitro* specificities may not mirror what happens *in vivo* (*107*).

Several systems-wide studies of protein acetylation have been published to date, both in eukaryotic and prokaryotic model organisms (**Table 2**). Overall, these works have demonstrated that acetylation in bacteria is more common and widespread than previously thought (maximum percentage of organisms ORFs, open reading frames, found acetylated: 20% in rat, 24% in *E. coli*, 34% in *M. pneumoniae*) (*39*, *112*, *122*). The percentage of acetylated ORFs is highly variable, from one organism to another, or even between different studies performed on the same organism. In fact, the output of these studies, *i.e.* the number or acetylation targets identified, is highly dependent on the techniques used previous to chromatographic analysis (*e.g.* immunoprecipitation with anti-acetyl-lysine antibodies, sample fractionation strategies…), the power and sensitivity of the MS/MS detection system used and the data analysis software package used to inspect LC-MS/MS data.

A thorough validation of the targets identified by these high throughput analysis techniques is necessary in order to determine the impact of the acetylation on the functionality of the protein. In some of the works published, limited validation was done to confirm that the targets were actually acetylated or that these acetylation events had an effect on the protein targeted (**Table 2**).

Table 2. Overview of lysine acetylation peptides/proteins identified in LC-MS/MS-based studies in different organisms. Studies in model eukaryotic systems (*A. thaliana*, *D. melanogaster*, *H. sapiens*, *R. norvegicus*, *S. cerevisiae* and *T. gondii*) are shown for comparison.

Organism	Number acetylated peptides/proteins	% Acetylated ORF	Validation Targets	Ref
Arabidopsis thaliana	91/71 [a]	0.3	Y	(*123*)
	64/57 [a]	0.2	Y	(*124*)
Bacillus subtilis	332/185 [a]	4.4	N	(*125*)
Drosophila melanogaster	1981/1013 [a,b,c]	7.3	Y	(*126*)
Erwinia amylovora	141/96 [a]	2.6	N	(*127*)
Escherichia coli	8284/1000 [a,c,*]	24.1	N	(*39*)
	1070/ 349 [a]	8.4	N	(*128*)
	138/91 [a]	2.2	N	(*120*)
	125/85 [a]	2.1	N	(*129*)
Geobacillus kaustophilus	253/114 [a]	3.2	N	(*130*)
Homo sapiens	3600/1750 [a,b,*]	8.7	N	(*131*)
Mycoplasma pneumoniae	719/221 [a,*]	34.1	N	(*112*)
Rattus novergicus	15474/4541 [a,c,*]	19.6	Y	(*122*)
Plasmodium falciparum	421/230	4.3	Y	(*132*)
Rhodopseudomonas palustris	244/292	6.1	Y	(*133*)
Saccharomyces cerevisiae	2878/ 1059[a,b,*]	17.9	N	(*134*)
Salmonella enterica	235/191 [a,*]	4.2	Y	(*74*)
Thermus thermophilus	197/128[a]	5.8	N	(*135*)
Toxoplasma gondii	411/274 [a]	3.4	N	(*136*)
	571/386 [a]	4.8	N	(*137*)

Data have been obtained using different techniques previous to LC-MS/MS:

[a]Immunoprecipitation (for enrichment in acetylated peptides)
[b]Isoelectric focusing fractionation (reduction of sample complexity).
[c]Micro-strong cation exchange fractionation (reduction of sample complexity).
*Studies that quantified the acetylated peptides.

It is quite remarkable that all these studies coincide in underling that approximately 50% of the targeted proteins were related to metabolism. In principle this is due to the fact that these proteins usually are highly abundant in the cells and, therefore, detection of acetylated peptides is more straightforward. However, it also supports that protein acetylation has a role in the regulation of metabolism in bacteria.

Despite these efforts, the biological substrates and/or functions for a majority of bacterial KATs and KDACs and the particular effects of acetylation on the activity of most of the target enzymes remain largely unknown.

Chemical acetylation of proteins

The unexpectedly high abundance of protein acetylation has underlined the importance of chemical acetylation of proteins in eukaryotes and prokaryotes. First *in vitro* evidences of non-enzymatic acetylation of histones by high energy metabolites acetyl phosphate and acetyl-AMP were reported in the 70s (*75*). Later on physiological roles of chemical acetylation of proteins in bacteria were reported. In *E. coli*, the chemotactic response regulator protein CheY was the first protein demonstrated to be chemically acetylated or autoacetylated (*97*, *110*, *138*, *139*). Eisenbach and col. showed that CheY could be acetylated at different lysine residues with acetyl-CoA as acetyl donor. Even more, it was observed that the acetylation of CheY decreased in the absence of acetyl-CoA synthetase (Acs), indicating its involvement, probably by supplying the acetylating agent. In yeast, low levels of histones acetylation were observed after deletion of the *acs1* and *acs2* genes, altering gene transcription (*140*). More recently, the slow kinetics of non-enzymatic acetylation of human histone H3 by acetyl-CoA have been shown (*76*). Although the kinetics of chemical lysine acetylation have not been extensively studied, it seems reasonable that the rate of chemical acetylation may be protein and residue dependent. Moreover, chemical acylation of proteins is highly dependent on pH, probably due to higher reactivity of lysil groups at high pH values. The acetylation of proteins in the mitochondrial matrix by acetyl-CoA is stimulated by a pH increase (*71*).

Other acyl-CoAs can also act as sources of activated acyl groups. In fact, proteins can also be modified at lysines by succinylation, malonylation, propionylation or butyrylation. It has been observed that lysine succinylation can be enzyme independent. Succinylation overlaps lysine acetylation and an increase of succinyl-CoA levels in the cells can increase this post-translational modification in proteins (*71*, *121*).

In bacteria, acetylation of various proteins by acetyl-CoA has been described. More recently, the involvement of acetyl-phosphate in chemical acetylation of proteins in *E. coli* has been demonstrated. The authors of this study altered the pool of acetyl-phosphate deleting the enzymes involved in its production: acetate kinase (AckA) and

phosphotransacetylase (Pta) (*39*). Therefore, unbalanced synthesis and degradation of acetyl-phosphate may also alter the acetylation state of proteins in *E. coli*.

In *B. subtilis*, analysis of single and multiple mutants in the deacetylase genes showed that both genes are required for efficient growth on acetate (*91*). In addition, these authors failed to construct a *pta acuC srtN* triple mutant, which suggests that this is a synthetic lethal mutant, probably due to an over-acetylation of proteins due to accumulated acetyl-phosphate. In fact, although an *acuA acuC srtN* mutant showed improved growth when compared to the *acuC srtN* strain, wild type growth was not recovered, suggesting either other KATs may be active in those conditions or that chemical acetylation occurred to a certain extent.

Altogether, these observations indicate that chemical acylation of proteins may be directly regulated by the levels of acyl-CoAs and acetyl phosphate and, therefore, depends on the metabolic state of the cells.

Bibliography

1. Martinko, J. M., Madigan, M. T., and Parker, J. (2003) *Brock: Biology of Microorganisms* (Pearson Education). 10th Ed.
2. Lee, S. Y. (1996) High cell-density culture of *Escherichia coli*. *Trends Biotechnol.* 14, 98–105
3. Frazzetto, G., Louis, S., and Poirier, Y. (2003) White biotechnology. *EMBO* 4, 835–837
4. Lin, H., Bennett, G. N., and San, K.-Y. (2005) Genetic reconstruction of the aerobic central metabolism in *Escherichia coli* for the absolute aerobic production of succinate. *Biotechnol. Bioeng.* 89, 148–156
5. Lee, K. H., Park, J. H., Kim, T. Y., Kim, H. U., and Lee, S. Y. (2007) Systems metabolic engineering of *Escherichia coli* for L-threonine production. *Mol. Syst. Biol.* 3, 149
6. Bernal, V., Masdemont, B., Arense, P., Canovas, M., and Iborra, J. L. (2007) Redirecting metabolic fluxes through cofactor engineering: Role of CoA-esters pool during L(-)-carnitine production by *Escherichia coli*. *J Biotechnol* 132, 110–117
7. Khankal, R., Chin, J. W., Ghosh, D., and Cirino, P. C. (2009) Transcriptional effects of CRP* expression in *Escherichia coli*. *J. Biol. Eng.* 3, 13
8. Shiloach, J., Kaufman, J., Guillard, A. S., and Fass, R. (1996) Effect of glucose supply strategy on acetate accumulation, growth, and recombinant protein production by *Escherichia coli* BL21 (lambda DE3) and *Escherichia coli* JM109. *Biotechnol. Bioeng.* 49, 421–428
9. Waegeman, H., De Lausnay, S., Beauprez, J., Maertens, J., De Mey, M., and Soetaert, W. (2013) Increasing recombinant protein production in *Escherichia coli* K12 through metabolic engineering. *N. Biotechnol.* 30, 255–61
10. Noor, E., Eden, E., Milo, R., and Alon, U. (2010) Central Carbon Metabolism as a Minimal Biochemical Walk between Precursors for Biomass and Energy. *Mol. Cell* 39, 809–820
11. Papagianni, M. (2012) Recent advances in engineering the central carbon metabolism of industrially important bacteria. *Microb. Cell Fact.* 11, 50
12. Crick, F. (1970) Central Dogma of Molecular Biology. *Nature* 227, 561–563
13. Cox, M. M., and Nelson, D. L. (2005) *Lehninger: Principles of Biochemistry* (Omega). 4th Ed.
14. Deutscher, J. (2008) The mechanisms of carbon catabolite repression in bacteria. *Curr. Opin. Microbiol.* 11, 87–93
15. Rojo, F. (2010) Carbon catabolite repression in *Pseudomonas*: optimizing metabolic versatility and interactions with the environment. *FEMS Microbiol Rev* 34, 658–684
16. Chong, H., Yeow, J., Wang, I., Song, H., and Jiang, R. (2013) Improving acetate tolerance of *Escherichia coli* by rewiring its global regulator cAMP receptor protein (CRP). *PLoS One* 8, e77422
17. Cánovas, M., Bernal, V., Torroglosa, T., Ramirez, J. L., and Iborra, J. L. (2003) Link between primary and secondary metabolism in the biotransformation of trimethylammonium compounds by *Escherichia coli*. *Biotechnol. Bioeng.* 84, 686–99
18. Neidhardt, F. C., Curtiss, I. I. R., Ingraham, J. L., Lin, E. C., Low Jr, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M., and Umbarger, H. E. (1996) Escherichia coli *and* Salmonella, *Cellular and Molecular Biology* (American Society for Microbiology, Washington, D.C). 2nd Ed.
19. Doelte, H. W., Ewings, K. N., Hollywood, N. W., and Doelle, H. (1982) Regulation of glucose metabolism in bacterial systems. *Adv Biochem Eng Biotechnol, Microb. React.*, 1–35
20. Takeda, S., Matsushika, A., and Mizuno, T. (1999) Repression of the Gene Encoding Succinate Dehydrogenase in Response to Glucose Is Mediated by the EIICB. *J. Biochem.* 360, 354–360

21. Chohnan, S., Izawa, H., Nishihara, H., and Takamura, Y. (1998) Changes in size of intracellular pools of coenzyme A and its thioesters in *Escherichia coli* K-12 cells to various carbon sources and stresses. *Biosci Biotechnol Biochem* 62, 1122–1128
22. Berg, P. (1956) Acyl adenylates enzymatic mechanism of acetate activation. *J Biol Chem* 222, 991–1013
23. Kumari, S., Tishel, R., Eisenbach, M., and Wolfe, A. J. (1995) Cloning, characterization, and functional expression of acs, the gene which encodes acetyl coenzyme A synthetase in *Escherichia coli*. *J Bacteriol* 177, 2878–2886
24. Serres, M. H., Gopal, S., Nahum, L. A., Liang, P., Gaasterland, T., and Riley, M. (2001) A functional update of the *Escherichia coli* K-12 genome. *Genome Biol* 2, 31–35
25. Gimenez, R., Badia, J., Aguilar, J., Baldoma, L., and Nunez, M. F. (2003) The gene yjcG, cotranscribed with the gene acs, encodes an acetate permease in *Escherichia coli*. *J Bacteriol* 185, 6448–6455
26. Keseler, I. M., Mackie, A., Peralta-Gil, M., Santos-Zavaleta, A., Gama-Castro, S., Bonavides-Martínez, C., Fulcher, C., Huerta, A. M., Kothari, A., Krummenacker, M., Latendresse, M., Muñiz-Rascado, L., Ong, Q., Paley, S., Schröder, I., Shearer, A. G., Subhraveti, P., Travers, M., Weerasinghe, D., Weiss, V., Collado-Vides, J., Gunsalus, R. P., Paulsen, I., and Karp, P. D. (2013) EcoCyc: fusing model organism databases with systems biology. *Nucleic Acids Res.* 41, D605–12
27. Beatty, C. M., Browning, D. F., Busby, S. J. W., and Wolfe, A. J. (2003) Cyclic AMP Receptor Protein-Dependent Activation of the *Escherichia coli acsP2* Promoter by a Synergistic Class III Mechanism. *J. Bacteriol.* 185, 5148–5157
28. Browning, D. F., Beatty, C. M., Sanstad, E. A., Gunn, K. E., Busby, S. J. W., and Wolfe, A. J. (2004) Modulation of CRP-dependent transcription at the *Escherichia coli acsP2* promoter by nucleoprotein complexes: anti-activation by the nucleoid proteins FIS and IHF. *Mol Microbiol* 51, 241–254
29. Kumari, S., Simel, E. J., and Wolfe, A. J. (2000) σ70 is the principal sigma factor responsible for transcription of acs, which encodes acetyl coenzyme A synthetase in *Escherichia coli*. *J Bacteriol* 182, 551–554
30. Kumari, S., Beatty, C. M., Browning, D. F., Busby, S. J. W., Simel, E. J., Hovel-Miner, G., and Wolfe, A. J. (2000) Regulation of acetyl coenzyme A synthetase in *Escherichia coli*. *J Bacteriol* 182, 4173–4179
31. Zhao, K., Liu, M., and Burgess, R. R. (2009) Promoter and regulon analysis of nitrogen assimilation factor, sigma 54, reveal alternative strategy for *Escherichia coli* MG1655 flagellar biosynthesis. *Nucleic Acids Res* 38, 1273–1283
32. Wolfe, A. J. (2005) The acetate switch. *Microbiol Mol Biol Rev* 69, 12–50
33. Brown, T. D. K., Jonesmortimer, M. C., and Kornberg, H. L. (1977) Enzymic interconversion of acetate and acetyl-Coenzyme-A in *Escherichia coli*. *J Gen Microbiol* 102, 327–336
34. Shalel-Levanon, S., San, K. Y., and Bennett, G. N. (2005) Effect of ArcA and FNR on the expression of genes related to the oxygen regulation and glycolysis pathway in *Escherichia coli* under growth conditions. *Biotechnol Bioeng* 92, 147–159
35. Kakuda, H., Hosono, K., Shiroishi, K., and Ichihara, S. (1994) Identification and characterization of *ackA* (acetate kinase A)-*pta* (phosphotransacetylase) Operon and complementation analysis of acetate utilization by and *ackA-pta* deletion mutant of *Escherichia coli*. *J Biochem* 116, 916–922
36. McCleary, W. R., Stock, J. B., and Ninfa, A. J. (1993) Is acetyl phosphate a global signal in *Escherichia coli*? *J Bacteriol* 175, 2793–2798
37. Dailey, F. E., and Berg, H. C. (1993) Change in direction of flagellar rotation in *Escherichia coli* mediate by acetate kinase. *J Bacteriol* 175, 3236–3239
38. McCleary, W. R., and Stock, J. B. (1994) Acetyl phosphate and the activation of 2-component response regulators. *J Biol Chem* 269, 31567–31572

39. Weinert, B. T. T., Iesmantavicius, V., Wagner, S. A. A., Schölz, C., Gummesson, B., Beli, P., Nyström, T., Choudhary, C., Scho, C., and Nystro, T. (2013) Acetyl-Phosphate Is a Critical Determinant of Lysine Acetylation in *E . coli*. *Mol. Cell* 51, 1–8
40. Maharjan, R. P., Yu, P.-L. L., Seeto, S., Ferenci, T., and Prasad Maharjan, R. (2005) The role of isocitrate lyase and the glyoxylate cycle in *Escherichia coli* growing under glucose limitation. *Res Microbiol* 156, 178–183
41. Renilla, S., Bernal, V., Fuhrer, T., Castaño-Cerezo, S., Pastor, J. M., Iborra, J. L., Sauer, U., and Cánovas, M. (2012) Acetate scavenging activity in *Escherichia coli*: interplay of acetyl-CoA synthetase and the PEP-glyoxylate cycle in chemostat cultures. *Appl Microbiol Biotechnol* 95, 2109–2124
42. Fischer, E., and Sauer, U. (2003) A novel metabolic cycle catalyzes glucose oxidation and anaplerosis in hungry *Escherichia coli*. *J Biol Chem* 278, 46446–46451
43. Cortay, J. C., Bleicher, F., Duclos, B., Cenatiempo, Y., Gautier, C., Prato, J. L., and Cozzone, A. J. (1989) Utilization of acetate in *Escherichia coli*: structural organization and differential expression of the ace operon. *Biochimie* 71, 1043–1049
44. LaPorte, D. C., and Koshland, D. E. (1982) A protein with kinase and phosphatase activities involved in regulation of tricarboxylic acid cycle. *Nature* 300, 458–460
45. Chung, T., Resnik, E., Stueland, C., and LaPorte, D. C. (1993) Relative expression of the products of glyoxylate bypass operon: contributions of transcription and translation. *J. Bacteriol.* 175, 4572–4575
46. Zaslaver, A., Bren, A., Ronen, M., Itzkovitz, S., Kikoin, I., Shavit, S., Liebermeister, W., Surette, M. G., and Alon, U. (2006) A comprehensive library of fluorescent transcriptional reporters for *Escherichia coli*. *Nat. Methods* 3, 623–628
47. Resnik, E., Pan, B., Ramani, N., Freundlich, M., and LaPorte, D. C. (1996) Integration host factor amplifies the induction of the *aceBAK* operon of *Escherichia coli* by relieving IclR repression. *J. Bacteriol.* 178, 2715–2717
48. Zhang, Z., Gosset, G., Barabote, R., Gonzalez, C. S., Cuevas, W. A., and Saier Jr., M. H. (2005) Functional Interactions between the Carbon and Iron Utilization Regulators, Crp and Fur, in *Escherichia coli*. *J. Bacteriol.* 187, 980–990
49. Cortay, J. C., Nègre, D., Scarabel, M., Ramseier, T. M., Vartak, N. B., Reizer, J., Saier, M. H., and Cozzone, A. J. (1994) In vitro asymmetric binding of the pleiotropic regulatory protein, FruR, to the ace operator controlling glyoxylate shunt enzyme synthesis. *J Biol Chem* 269, 14885–14891
50. Lorca, G. L., Ezersky, A., Lunin, V. V, Walker, J. R., Altamentova, S., Evdokimova, E., Vedadi, M., Bochkarev, A., and Savchenko, A. (2007) Glyoxylate and pyruvate are antagonistic effectors of the *Escherichia coli* IclR transcriptional regulator. *J Biol Chem* 282, 16476–16491
51. Valgepea, K., Adamberg, K., Nahku, R., Lahtvee, P.-J., Arike, L., and Vilu, R. (2010) Systems biology approach reveals that overflow metabolism of acetate in *Escherichia coli* is triggered by carbon catabolite repression of acetyl-CoA synthetase. *BMC Syst Biol* 4, 166
52. Vemuri, G. N., Altman, E., Sangurdekar, D. P., Khodursky, A. B., and Eiteman, M. A. (2006) Overflow metabolism in *Escherichia coli* during steady-state growth: transcriptional regulation and effect of the redox ratio. *Appl. Environ. Microbiol.* 72, 3653–3661
53. Huerta, A. M., Francino, M. P., Morett, E., and Collado-Vides, J. (2006) Selection for Unequal Densities of sigma 70 Promoter-Like Signals in Different Regions of Large Bacterial Genomes. *PLoS Genet* 2, e185
54. Lange, R., and Hengge-Aronis, R. (1994) The cellular concentration of the sigma S subunit of RNA polymerase in *Escherichia coli* is controlled at the levels of transcription, translation, and protein stability. *Genes Dev* 8, 1600–1612
55. Deutscher, J., Francke, C., and Postma, P. W. (2006) How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. *Microbiol. Mol. Biol. Rev.* 70, 939–1031

56. Huerta, A. M., and Collado-Vides, J. (2003) Sigma70 Promoters in *Escherichia coli*: Specific Transcription in Dense Regions of Overlapping Promoter-like Signals. *J. Mol. Biol.* 333, 261–278
57. Gunasekera, A., Ebright, Y. W., and Ebright, R. H. (1992) DNA sequence determinants for binding of the *Escherichia coli* catabolite gene activator protein. *J Biol Chem* 267, 14713–14720
58. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673–80
59. Hertz, G. Z., and Stormo, G. D. (1999) Identifying DNA and protein patterns with statistically significant alignments of multiple sequences. *Bioinformatics* 15, 563–577
60. Yang, X.-J., and Seto, E. (2008) Lysine acetylation: codified crosstalk with other posttranslational modifications. *Mol. Cell* 31, 449–61
61. Iuchi, S., and Lin, E. C. (1992) Purification and phosphorylation of the Arc regulatory components of *Escherichia coli*. *J. Bacteriol.* 174, 5617–5623
62. Georgellis, D., Kwon, O., and Lin, E. C. C. (1999) Amplification of Signaling Activity of the Arc Two-component System of *Escherichia coli* by Anaerobic Metabolites. *J Biol Chem* 274, 35950–35954
63. Georgellis, D., Kwon, O., and Lin, E. C. C. (2001) Quinones as the Redox Signal for the Arc Two-Component System of Bacteria. *Science (80-.).* 292, 2314–2316
64. Malpica, R., Franco, B., Rodriguez, C., Kwon, O., and Georgellis, D. (2004) Identification of a quinone-sensitive redox switch in the ArcB sensor kinase. *Proc Natl Acad Sci U S A* 101, 13318–13323
65. LaPorte, D. C., and Chung, T. (1985) A single gene codes for the kinase and phosphatase which regulate isocitrate dehydrogenase. *J Biol Chem* 260, 15291–15297
66. El-Mansi, M., Cozzone, A. J., Shiloach, J., and Eikmanns, B. J. (2006) Control of carbon flux through enzymes of central and intermediary metabolism during growth of *Escherichia coli* on acetate. *Curr Opin Microbiol* 9, 173–179
67. Finkel, T., Deng, C.-X., and Mostoslavsky, R. (2009) Recent progress in the biology and physiology of sirtuins. *Nature* 460, 587–591
68. Glozak, M. A., Sengupta, N., Zhang, X., and Seto, E. (2005) Acetylation and deacetylation of non-histone proteins. *Gene* 363, 15–23
69. Kim, G.-W., and Yang, X.-J. (2011) Comprehensive lysine acetylomes emerging from bacteria to humans. *Trends Biochem. Sci.* 36, 211–20
70. Norris, K. L. K., Lee, J.-Y. J., and Yao, T.-P. T. (2009) Acetylation Goes Global: The Emergence of Acetylation Biology. *Sci. Signal.* 2, 1–7
71. Wagner, G. R., and Payne, R. M. (2013) Metabolism : Widespread and enzyme-independent N ε-acetylation and N ε-succinylation in the chemical conditions of the mitochondrial matrix. *J. Biol. Chem.* 288, 29036–45
72. Mischerikow, N., and Heck, A. J. R. (2011) Targeted large-scale analysis of protein acetylation. *Proteomics* 11, 571–589
73. Zhao, S., Xu, W., Jiang, W., Yu, W., Lin, Y., Zhang, T., Yao, J., Zhou, L., Zeng, Y., Li, H., Li, Y., Shi, J., An, W., Hancock, S. M., He, F., Qin, L., Chin, J., Yang, P., Chen, X., Lei, Q., Xiong, Y., and Guan, K.-L. (2010) Regulation of cellular metabolism by protein lysine acetylation. *Science* 327, 1000–4
74. Wang, Q., Zhang, Y., Yang, C., Xiong, H., Lin, Y., Yao, J., Li, H., Xie, L., Zhao, W., Yao, Y., Ning, Z.-B., Zeng, R., Xiong, Y., Guan, K.-L., Zhao, S., and Zhao, G.-P. (2010) Acetylation of metabolic enzymes coordinates carbon source utilization and metabolic flux. *Science* 327, 1004–7
75. Ramponi, G., Manao, G., and Camici, G. (1975) Nonenzymatic Acetylation of Histones with Acetyl Phosphate and Acetyl Adenylate. *Biochemistry* 14, 2681–2685

76. Kuo, Y.-M., and Andrews, A. J. (2013) Quantitating the specificity and selectivity of Gcn5-mediated acetylation of histone H3. *PLoS One* 8, e54896
77. Polevoda, B., and Sherman, F. (2003) N-terminal Acetyltransferases and Sequence Requirements for N-terminal Acetylation of Eukaryotic Proteins. *J. Mol. Biol.* 325, 595–622
78. Soppa, J. (2010) Protein acetylation in archaea, bacteria, and eukaryotes. *Archaea* 2010, 820681
79. Hu, L. I., Lima, B. P., and Wolfe, A. J. (2010) Bacterial protein acetylation: the dawning of a new age. *Mol. Microbiol.* 77, 15–21
80. Thao, S., and Escalante-Semerena, J. C. (2011) Control of protein function by reversible Nε-lysine acetylation in bacteria. *Curr Opin Microbiol* 14, 200–204
81. Friedmann, D. R., Marmorstein, R., Expression, G., Children, C., Type, A., and Article, R. (2013) Structure and mechanism of non-histone protein acetyltransferase enzymes. *FEBS J.* 280, 5570–81
82. Garrity, J., Gardner, J. G., Hawse, W., Wolberger, C., and Escalante-Semerena, J. C. (2007) Nε-lysine propionylation controls the activity of propionyl-CoA synthetase. *J Biol Chem* 282, 30239–30245
83. Starai, V. J., and Escalante-Semerena, J. C. (2004) Identification of the protein acetyltransferase (Pat) enzyme that acetylates acetyl-CoA synthetase in *Salmonella enterica*. *J Mol Biol* 340, 1005–1012
84. Crosby, H. a, Pelletier, D. a, Hurst, G. B., and Escalante-Semerena, J. C. (2012) System-wide studies of N-lysine acetylation in *Rhodopseudomonas palustris* reveal substrate specificity of protein acetyltransferases. *J. Biol. Chem.* 287, 15590–601
85. Thao, S., and Escalante-semerena, J. C. (2011) Biochemical and Thermodynamic Analyses of *Salmonella enterica* Pat , a Multidomain , Multimeric N -Lysine Acetyltransferase Involved in Carbon and Energy Metabolism. *MBio* 2, 1–8
86. Chan, C. H., Garrity, J., Crosby, H. A., and Escalante-Semerena, J. C. (2011) In *Salmonella enterica*, the sirtuin-dependent protein acylation/deacylation system (SDPADS) maintains energy homeostasis during growth on low concentrations of acetate. *Mol Microbiol* 80, 168–183
87. Gregoretti, I. V, Lee, Y.-M., and Goodson, H. V (2004) Molecular evolution of the histone deacetylase family: functional implications of phylogenetic analysis. *J. Mol. Biol.* 338, 17–31
88. Frye, R. a (2000) Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. *Biochem. Biophys. Res. Commun.* 273, 793–798
89. Starai, V. J., Celic, I., Cole, R. N., Boeke, J. D., and Escalante-Semerena, J. C. (2002) Sir2-dependent activation of acetyl-CoA synthetase by deacetylation of active lysine. *Science (80-.).* 298, 2390–2392
90. Zhao, K., Chai, X., and Marmorstein, R. (2004) Structure and substrate binding properties of CobB, a Sir2 homolog protein deacetylase from *Escherichia coli*. *J Mol Biol* 337, 731–741
91. Gardner, J. G., and Escalante-Semerena, J. C. (2009) In *Bacillus subtilis*, the sirtuin protein deacetylase, encoded by the *srtN* gene (formerly yhdZ), and functions encoded by the *acuABC* genes control the activity of acetyl coenzyme A synthetase. *J. Bacteriol.* 191, 1749–1755
92. Crosby, H. A., Heiniger, E. K., Harwood, C. S., and Escalante-Semerena, J. C. (2010) Reversible N-ε-lysine acetylation regulates the activity of acyl-CoA synthetases involved in anaerobic benzoate catabolism in *Rhodopseudomonas palustris*. *Mol Microbiol* 76, 874–888
93. Mikulik, K., Felsberg, J., Kudrnáčová, E., Bezoušková, S., Šetinová, D., Stodůlková, E., Zídková, J., Zídek, V., and Setinová, D. (2012) CobB1 deacetylase activity in *Streptomyces coelicolor*. *Biochem. Cell Biol.* 90, 179–87

94. Gardner, J. G., Grundy, F. J., Henkin, T. M., and Escalante-Semerena, J. C. (2006) Control of acetyl-coenzyme A synthetase (AcsA) activity by acetylation/deacetylation without NAD+ involvement in *Bacillus subtilis*. *J. Bacteriol.* 188, 5460–5468
95. Hayden, J. D., Brown, L. R., Gunawardena, H. P., Perkowski, E. F., Chen, X., and Braunstein, M. (2013) Reversible acetylation regulates acetate and propionate metabolism in *Mycobacterium smegmatis*. *Microbiology* 159, 1986–99
96. Barak, R., and Eisenbach, M. (2001) Acetylation of the response regulator, CheY, is involved in bacterial chemotaxis. *Mol. Microbiol.* 40, 731–743
97. Barak, R., Welch, M., Yanovsky, A., Oosawa, K., and Eisenbach, M. (1992) Acetyladenylate or its derivative acetylates the chemotaxis protein CheY *in vitro* and increases its activity at the flagellar switch. *Biochemistry* 31, 10099–10107
98. Starai, V. J., and Escalante-Semerena, J. C. (2004) Acetyl-coenzyme A synthetase (AMP forming). *Cell Mol Life Sci* 61, 2020–2030
99. Soupene, E., and Kuypers, F. A. (2008) Mammalian long-chain acyl-CoA synthetases. *Exp. Biol. Med. (Maywood).* 233, 507–21
100. Carmona, M., and Zamarro, M. (2009) Anaerobic catabolism of aromatic compounds: a genetic and genomic view. *Microbiol Mol Biol Rev.* 73, 71–133
101. Gardner, J. G., and Escalante-Semerena, J. C. (2008) Biochemical and mutational analyses of AcuA, the acetyltransferase enzyme that controls the activity of the acetyl coenzyme a synthetase (AcsA) in *Bacillus subtilis*. *J. Bacteriol.* 190, 5132–5136
102. Nambi, S., Basu, N., and Visweswariah, S. (2010) cAMP-regulated Protein Lysine Acetylases in Mycobacteria. *J Biol Chem* 285, 24313–24323
103. Hua, X., Hegde, S. S., Blanchard, J. S., and Xu, H. (2012) The Reversible Acetylation and Inactivation of *Mycobacterium tuberculosis* Acetyl-CoA Synthetase is Dependent on cAMP. *Biochemistry* 50, 5883–5892
104. Bai, G., Knapp, G. S., and McDonough, K. A. (2011) Cyclic AMP signalling in mycobacteria: redirecting the conversation with a common currency. *Cell. Microbiol.* 13, 349–58
105. Lima, B. P., Antelmann, H., Gronau, K., Chi, B. K., Becher, D., Brinsmade, S. R., and Wolfe, A. J. (2011) Involvement of protein acetylation in glucose-induced transcription of a stress-responsive promoter. *Mol. Microbiol.* 81, 1190–204
106. Huang, Y.-H., Ferrières, L., and Clarke, D. J. (2006) The role of the Rcs phosphorelay in Enterobacteriaceae. *Res. Microbiol.* 157, 206–212
107. Thao, S., Chen, C.-S., Zhu, H., and Escalante-Semerena, J. C. (2010) Nε-lysine acetylation of a bacterial transcription factor inhibits Its DNA-binding activity. *PLoS One* 5, e15123
108. Majdalani, N., and Gottesman, S. (2005) The Rcs phosphorelay: a complex signal transduction system. *Annu. Rev. Microbiol.* 59, 379–405
109. Li, R., Gu, J., Chen, Y.-Y., Xiao, C.-L., Wang, L.-W., Zhang, Z.-P., Bi, L.-J., Wei, H.-P., Wang, X.-D., Deng, J.-Y., and Zhang, X.-E. (2010) CobB regulates Escherichia coli chemotaxis by deacetylating the response regulator CheY. *Mol. Microbiol.* 76, 1162–1174
110. Barak, R., Prasad, K., Shainskaya, A., Wolfe, A. J., and Eisenbach, M. (2004) Acetylation of the chemotaxis response regulator CheY by acetyl-CoA synthetase purified from *Escherichia coli*. *J Mol Biol* 342, 383–401
111. Acetylation represses the binding of CheY to its target proteins. *Mol. Microbiol.* 76,
112. Van Noort, V., Seebacher, J., Bader, S., Mohammed, S., Vonkova, I., Betts, M. J., Kühner, S., Kumar, R., Maier, T., O'Flaherty, M., Rybin, V., Schmeisky, A., Yus, E., Stülke, J., Serrano, L., Russell, R. B., Heck, A. J. R., Bork, P., and Gavin, A.-C. (2012) Cross-talk between phosphorylation and lysine acetylation in a genome-reduced bacterium. *Mol. Syst. Biol.* 8, 571

113. Liang, W., Malhotra, A., and Deutscher, M. P. (2011) Acetylation regulates the stability of a bacterial protein: growth stage-dependent modification of RNase R. *Mol. Cell* 44, 160–6
114. Hu, L. I., Chi, B. K., Kuhn, M. L., Filippova, E. V, Walker-Peddakotla, A. J., Bäsell, K., Becher, D., Anderson, W. F., Antelmann, H., and Wolfe, A. J. (2013) Acetylation of the Response Regulator RcsB Controls Transcription from a Small RNA Promoter. *J. Bacteriol.*,
115. Liang, W., and Deutscher, M. P. (2012) Transfer-messenger RNA-SmpB protein regulates ribonuclease R turnover by promoting binding of HslUV and Lon proteases. *J. Biol. Chem.* 287, 33472–9
116. Zhang, Q., Gu, J., Gong, P., Wang, X., Tu, S., Bi, L., Yu, Z., Zhang, Z., Cui, Z., Wei, H., Tao, S., and Zhang, X. (2013) Reversibly acetylated lysine residues play important roles in the enzymatic activity of *Escherichia coli* N-hydroxyarylamine O-acetyltransferase. *FEBS J.* 280, 1966–79
117. Lima, B. P., Thanh Huyen, T. T., Bäsell, K., Becher, D., Antelmann, H., and Wolfe, A. J. (2012) Inhibition of acetyl phosphate-dependent transcription by an acetylatable lysine on RNA polymerase. *J. Biol. Chem.* 287, 32147–60
118. Tucker, A. C., and Escalante-semerena, J. C. (2013) Acetoacetyl-CoA synthetase activity is controlled by a protein acetyltransferase with unique domain organization in *Streptomyces lividans*. *Mol. Microbiol.* 87, 152–167
119. Nambi, S., Gupta, K., Bhattacharyya, M., Ramakrishnan, P., Ravikumar, V., Siddiqui, N., Thomas, A. T., and Visweswariah, S. S. (2013) Cyclic AMP-dependent protein lysine acylation in Mycobacteria regulates fatty acid and propionate metabolism. *J. Biol. Chem.* 288, 14114–24
120. Zhang, J., Sprung, R., Pei, J., Tan, X., Kim, S., Zhu, H., Liu, C.-F., Grishin, N. V, and Zhao, Y. (2009) Lysine acetylation is a highly abundant and evolutionarily conserved modification in *Escherichia coli*. *Mol Cell Prot* 8, 215–225
121. Weinert, B. T., Schölz, C., Wagner, S. a, Iesmantavicius, V., Su, D., Daniel, J. a, Choudhary, C., and Scho, C. (2013) Lysine succinylation is a frequently occurring modification in prokaryotes and eukaryotes and extensively overlaps with acetylation. *Cell Rep.* 4, 842–51
122. Lundby, A., Lage, K., Weinert, B. T., Bekker-Jensen, D. B., Secher, A., Skovgaard, T., Kelstrup, C. D., Dmytriyev, A., Choudhary, C., Lundby, C., and Olsen, J. V (2012) Proteomic analysis of lysine acetylation sites in rat tissues reveals organ specificity and subcellular patterns. *Cell Rep.* 2, 419–31
123. Friedman, N., Vardi, S., Ronen, M., Alon, U., and Stavans, J. (2005) Precise temporal modulation in the response of the SOS DNA repair network in individual bacteria. *PLoS Biol.* 3, e238
124. Wu, X., Oh, M.-H., Schwarz, E. M., Larue, C. T., Sivaguru, M., Imai, B. S., Yau, P. M., Ort, D. R., and Huber, S. C. (2011) Lysine acetylation is a widespread protein modification for diverse proteins in *Arabidopsis*. *Plant Physiol.* 155, 1769–78
125. Kim, D., Yu, B. J., Kim, J. A., Lee, Y.-J., Choi, S.-G., Kang, S., and Pan, J.-G. (2013) The Acetylproteome of Gram-positive Model Bacterium *Bacillus subtilis*. *Proteomics J.* 13, 1–28
126. Weinert, B. T., Wagner, S. a, Horn, H., Henriksen, P., Wenshe, R., Olsen, J. V, Jensen, L. J., July, C. C., Liu, W. R., and Choudhary, C. (2011) Proteome-wide mapping of the *Drosophila* acetylome demonstrates a high degree of conservation of lysine acetylation. *Sci. Signal.* 4, ra48
127. Wu, X., Vellaichamy, A., Wang, D., Zamdborg, L., Kelleher, N. L., Huber, S. C., and Zhao, Y. (2012) Differential lysine acetylation profiles of *Erwinia amylovora* strains revealed by proteomics. *J. Proteomics* 79, 60–71

128. Zhang, K., Zheng, S., Yang, J. S., Chen, Y., and Cheng, Z. (2012) Comprehensive Profiling of Protein Lysine Acetylation in *Escherichia coli*. *J. Proteome Res.* 12, 844–51
129. Jo, B., Kim, J. A., Moon, J. H., Ryu, S. E., Pan, J. G., Yu, B. J., and Yu, Byung Jo, Jung Ae Kim, Jeong Hee Moon, Seong Eon Ryu, A. J.-G. P. (2008) The Diversity of Lysine-Acetylated Proteins in *Escherichia coli*. *J. Microbiol. Biotechnol.* 18, 1529–1536
130. Lee, D.-W., Kim, D., Lee, Y.-J., Kim, J., Choi, J. Y., Kang, S., and Pan, J.-G. (2013) Proteomic analysis of acetylation in thermophilic *Geobacillus kaustophilus*. *Proteomics* 13, 2278–2282
131. Choudhary, C., Kumar, C., Gnad, F., Nielsen, M. L., Rehman, M., Walther, T. C., Olsen, J. V, and Mann, M. (2009) Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science* 325, 834–40
132. Miao, J., Lawrence, M., Jeffers, V., Zhao, F., Parker, D., Ge, Y., Sullivan, W. J., and Cui, L. (2013) Extensive lysine acetylation occurs in evolutionarily conserved metabolic pathways and parasite-specific functions during *Plasmodium falciparum* intraerythrocytic development. *Mol. Microbiol.* 89, 660–75
133. Crosby, H. a, Rank, K. C., Rayment, I., and Escalante-Semerena, J. C. (2012) Structural insights into the substrate specificity of the *Rhodopseudomonas palustris* protein acetyltransferase RpPat: identification of a loop critical for recognition by RpPat. *J. Biol. Chem.* 287, 41392–404
134. Henriksen, P., Wagner, S. a, Weinert, B. T., Sharma, S., Bacinskaja, G., Rehman, M., Juffer, A. H. A. H., Walther, T. C., Lisby, M., and Choudhary, C. (2012) Proteome-wide analysis of lysine acetylation suggests its broad regulatory scope in *Saccharomyces cerevisiae*. *Mol. Cell. Proteomics* 11, 1510–22
135. Okanishi, H., Kim, K., Masui, R., and Kuramitsu, S. (2013) Acetylome with Structural Mapping Reveals the Significance of Lysine Acetylation in *Thermus thermophilus*. *J. Proteome Res.*,
136. Jeffers, V., and Sullivan, W. J. (2012) Lysine Acetylation Is Widespread on Proteins of Diverse Function and Localization in the Protozoan Parasite *Toxoplasma gondii*. *Eukaryot. Cell* 11, 735–42
137. Xue, B., Jeffers, V., Sullivan, W. J., and Uversky, V. N. (2013) Protein intrinsic disorder in the acetylome of intracellular and extracellular *Toxoplasma gondii*. *Mol. Biosyst.* 9, 645–57
138. Barak, R., Yan, J., Shainskaya, A., and Eisenbach, M. (2006) The chemotaxis response regulator CheY can catalyze its own acetylation. *J. Mol. Biol.* 359, 251–65
139. Yan, J., Barak, R., Liarzi, O., Shainskaya, A., and Eisenbach, M. (2008) In vivo acetylation of CheY, a response regulator in chemotaxis of *Escherichia coli*. *J. Mol. Biol.* 376, 1260–71
140. Kozak, B. U., van Rossum, H. M., Benjamin, K. R., Wu, L., Daran, J.-M. G., Pronk, J. T., and van Maris, A. J. a. (2013) Replacement of the *Saccharomyces cerevisiae* acetyl-CoA synthetases by alternative pathways for cytosolic acetyl-CoA synthesis. *Metab. Eng.* 21, 46–59

Chapter 2

Objectives

"Nothing is impossible in our world, we must only discover the means to achieve it."
Hermann Oberth

Objectives

Escherichia coli has a high importance in industry and basic research laboratories. It is used for many biotechnological processes, such as protein and metabolite production, being these last derivatives from the primary or secondary metabolism. Since the emergence of the field of Metabolic Engineering, the design of rational strategies to improve the production of metabolic products has impelled the field of Biotechnology. In order to improve bioprocesses in any organism, a better understanding of metabolism and its regulation is needed. One of the major drawbacks of using *E. coli* as a microbial cell factory is acetate production. Acetate production decreases *E. coli* efficiency in terms of biomass yield and metabolite production. In fact, low acetate producer strains, such as *E. coli* BL21, are preferred for protein production. In addition, a better knowledge of regulatory mechanisms involved in central carbon metabolism, especially on acetate metabolism, would help for bioprocess optimization. In the last years, evidences in *Salmonella enterica, Bacillus subtilis* and other microorganisms have shown that lysine acetylation play a role in the regulation of metabolism. The regulation of metabolism in *E. coli* by this post-translational modification of proteins has not been demonstrated thus far. For all these reasons, the general objectives of this PhD thesis are:

- To characterize the effect of the deletion of the main acetate pathways of *E. coli* in different growth conditions. The consequences of the deletion of these enzymes at different levels (enzyme activities, gene expression and energy levels) will provide new insights of *E. coli* acetate and central metabolism (**Chapter 3**).

- To study the regulation of the acetate metabolism by lysine acetylation in *E. coli*. **(Chapter 4)**

- To describe the genomic context of the genes involved in lysine acetylation, *cobB* and *yfiQ*, in *E. coli* and to identify the genomic regions and proteins that control their expression. **(Chapter 4)**

- To investigate the role of lysine acetylation on the different acetate overflow shown in the *E. coli* strain K12 and the protein producer strain BL21. (**Chapter 5**)
- To identify new targets of lysine acetylation in *E. coli* K12 under different conditions. **(Chapter 6)**
- To characterize the functional effect of lysine acetylation in proteins related to metabolism and transcriptional regulation in *E. coli.* **(Chapter 6)**

Chapter 3

An insight into the role of phosphotransacetylase (*pta*) and the acetate/acetyl-CoA node in *Escherichia coli*

The results presented in this chapter are based in the publication:

Castaño-Cerezo S, Pastor JM, Renilla S, Bernal V, Iborra JL, Cánovas M.
"An insight into the role of phosphotransacetylase (pta) and the acetate/acetyl-CoA node in *Escherichia coli*."
Microbial Cell Factories 2009;8:54.

"What we know is a drop, what we don't know is an ocean"
Isaac Newton

Abstract

Acetate metabolism in *Escherichia coli* plays an important role in the control of the central metabolism and in bioprocess performance. The main problems related to the use of *E. coli* as cellular factory are i) the deficient utilization of carbon source due to the excretion of acetate during aerobic growth, ii) the inhibition of cellular growth and protein production by acetate and iii) the need for cofactor recycling (namely redox coenzymes and free Coenzyme A) to sustain balanced growth and cellular homeostasis.

This work analyses the effect of mutations in the acetate excretion/assimilation pathways, acetyl-CoA synthase (*acs*) and phosphotransacetylase (*pta*), in *E. coli* BW25113 grown on glucose or acetate minimal media. Biomass and metabolite production, redox ($NADH/NAD^+$) and energy (ATP) state, enzyme activities and gene expression profiles related to the central metabolism were analysed. The knockout of *pta* led to a more altered phenotype than that of *acs*. Deletion of *pta* reduced the ability to grow on acetate as carbon source and strongly affected the expression of several genes related to central metabolic pathways.

Results showed that *pta* limits biomass yield in aerobic glucose cultures, due to acetate production (overflow metabolism) and its inefficient use during glucose starvation. Deletion of *pta* severely impaired growth on acetate minimal medium and under anaerobiosis due to decreased acetyl CoA synthetase, glyoxylate shunt and gluconeogenic activities, leading to lower growth rate. When acetate is used as carbon source, the joint expression of *pta* and *acs* is crucial for growth and substrate assimilation, while *pta* deletion severely impaired anaerobic growth. Finally, at an adaptive level, *pta* deficiency makes the strain more sensitive to environmental changes and de-regulates the central metabolism.

Introduction

When *E. coli* grows on excess of glucose it excretes acetate, a phenomenon known as the Crabtree Effect or "acetate overflow", and which has several causes. When glucose is in excess, the TCA cycle is limited, acetyl-CoA accumulates and 15-30% is excreted as acetate, allowing the regeneration of Coenzyme A (*1–3*), although it is not clear how the regulation works (*4*). Moreover, *E. coli* is also able to metabolize the acetate produced and even use it as sole carbon source. The enzymes intervening (**Fig. 1**) are acetyl-CoA synthetase (Acs, non-reversible assimilation) and phosphotransacetylase-acetate kinase (Pta-Ack, reversible assimilation). It has previously been demonstrated that Pta-AckA and Acs are the sole pathways responsible for acetate assimilation, since a double *pta/acs* deletion mutant does not grow on acetate medium (*5*).

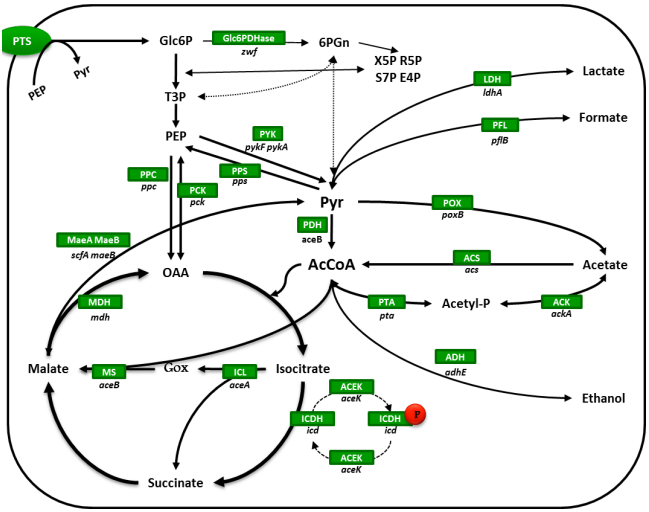


Figure 1. Simplified model for the central metabolic network of *E. coli* metabolism. The enzymes involved (and their codifying genes) are shown in the figure, ACEK (*aceK*) isocitrate dehydrogenase phosphatase/kinase; ACK (*ackA*), acetate kinase; ACS (*acs*), acetyl-CoA synthetase; ICDH (*icd*), isocitrate dehydrogenase; ICL (*aceA*), isocitrate lyase; ICLR (*iclR*), repressor of the glyoxylate shunt; MDH (*maeB*), malate dehydrogenase; ME (*sfcA*), malic enzyme, PFL (*pfl*) pyruvate:formate lyase; PTA (*pta*), phosphotransacetylase (Ecocyc-Metacyc (6)).

Acs irreversibly transforms acetate to acetyl-CoA, consuming ATP (7). This is a high affinity pathway with a low K_m for acetate (200 µM) therefore suitable for metabolizing low concentrations of acetate (*5*). When growing on glucose, it is expressed during the stationary phase, although its regulation is complex, since it involves several transcription factors, two sigma factors and two promoters (8). The gene encoding this enzyme, *acs*, is within an operon, together with another two genes: *yjcH*, an inner membrane conserved protein of unknown function, and *actP*, which codes for an acetate permease (9, 10).

On the other hand, the phosphotransacetylase-acetate kinase (Pta-AckA) pathway is characterized by its reversibility. Under aerobiosis, this pathway excretes acetate and produces ATP, a mechanism known as "acetate overflow" (11). It is characterized by its low affinity (K_m 7-10 mM) (12), thus being able to dissimilate high concentrations of acetate. These two genes belong to the same operon (13) and are constitutively expressed, although *ackA* is slightly activated by Fnr (14). The reaction catalysed by these two enzymes generates an unstable intermediate, acetyl-phosphate (**Fig. 1**), which phosphorylates proteins related to signal transduction pathways (15), such as the double component systems. *In vivo*, acetyl-phosphate acts on CheY-CheA, related to the flagellar function (16) and PhoB-PhoR, which are phosphate concentration regulators (17). Additionally, mutations on *pta* and/or *ackA* have been demonstrated to affect repair-deficient mutants of *E. coli* (18).

Besides acting as substrate or product in a large number of reactions, acetyl-CoA connects the glycolysis and the acetate metabolism pathways (namely, Acs and Pta-Ack) with the TCA cycle and the glyoxylate shunt. Thus, this metabolite is a key factor in determining biomass synthesis, the redox balance and energy yield. Moreover, the acetate/acetyl-CoA node also largely determines the control exerted by the central metabolism on the performance of many microbial-based bioprocesses. Many authors have reported the decreased efficiency of acetate over-producing strains for the high-yield production of recombinant proteins (19) and also acetate affects biotransformations(20). In addition, acetyl-CoA is the precursor of many biosynthetic pathways (21), and the engineering of the acetyl-CoA/CoA ratio (cofactor engineering)

has been demonstrated to be a valuable strategy for metabolic engineering (22, 23). In fact, the main problems related to the use of *E. coli* as cellular factory are i) the loss of carbon in the form of acetate during aerobic growth at high growth rates, ii) the inhibition of cellular growth and protein production by acetate (*3*) and iii) the need for cofactor recycling, (namely redox coenzymes and free CoA) to sustain balanced growth and cellular homeostasis. Moreover, the role of acetate production and CoA regeneration in the central metabolism of *E. coli* remains to be unveiled.

In this work, we provide a further insight into the connection between the acetate/acetyl-CoA node and the central metabolism of *E. coli*. The behaviour of *E. coli* BW25113 strains carrying deletions in the acetate assimilation/excretion pathways (Acs and Pta-Ack) was studied based on the analysis of growth and metabolite production/consumption, the energy and redox state and the expression of genes and enzyme activities related to the central metabolism. The strains were analysed in three different metabolic scenarios.

Materials and Methods

Bacterial strains and cultures

E. coli BW25113 strains (wild-type and *acs* and *pta* deletion mutants) were used throughout this study (**Table 1**). The *E. coli* BW25113 derivatives carry complete gene deletions and belong to the KO- collection (http://www.ecoli.aist-nara.ac.jp/; (*24*)). The standard minimal media (pH 7.4) contained: 2.6 g/L $(NH_4)_2SO_4$, 1.0 g/L NH_4Cl, 0.5 g/L NaCl, 15.0 g/L $Na_2HPO_4 \cdot 12\ H_2O$, 3.0 g/L KH_2PO_4, 50.0 mg/L $FeCl_3 \cdot 6\ H_2O$, 65.0 mg/L EDTA Na_2, 1.8 mg/L $ZnSO_4 \cdot 7\ H_2O$, 1.8 mg/L $CuSO_4 \cdot 5\ H_2O$, 1.2 mg/L $MnSO_4 \cdot H_2O$, 1.8 mg/L $CoCl_2 \cdot 6\ H_2O$, 2.0 mM $MgSO_4$, 0.2 mM $CaCl_2$, and 0.3 µM thiamine·HCl. As carbon source, 20 mM glucose or 60 mM acetate were used. Aerobic 200 mL batch cultures were grown in 1 L flasks at 37°C on a rotary shaker at 150 rpm. Frozen 20% glycerol stock cultures were used to inoculate glucose-supplemented minimal media precultures. Cultures were inoculated to an optical density (OD_{600nm}) of 0.05 with exponentially growing precultures.

Table1. Strains used in this work

Strain	Genotype	Antibiotics	Ref.
E. coli BW25113	*rrnB3 ΔlacZ4787 hsdR514Δ(araBAD)567 Δ(rhaBAD)568 rph-1*	None	(*25*)
E. coli BW25113 Δ*acs*	[BW25113] Δ*acs*	Kanamycin	(*24*)
E. coli BW25113 Δ*pta*	[BW25113] Δ*pta*	Kanamycin	(*24*)

Analytical procedures

To estimate cell concentration, cells were resuspended in 65 mM phosphate buffer pH 7.5 and absorbance was measured at 600 nm (Pharmacia Biotech Novaspec II Spectrophotometer, Uppsala, Sweden). A_{600} values and dry cell weight were correlated for each strain.

Extracellular metabolites (acetate, formate, ethanol, succinate, pyruvate and lactate) were analysed by HPLC (Shimadzu Scientific Instruments, Columbia, MD),

equipped with differential refractive (Shimadzu Scientific Instruments, Columbia, MD) and UV (Waters, Milford, MA) detectors, using a cation-exchange column (HPX-87H, BioRad Labs, Hercules, CA). The mobile phase was 5 mM H_2SO_4 at 0.4 ml·min^{-1} flow rate and 65ºC. Glucose was assayed by a glucose (HK) assay kit (Sigma Aldrich, Saint Louis, MO) according to the manufacturer's recommendations. Measurements were performed in a Microplate Spectrophotometer Synergy HT (Bio-Tek, Winooski, VT).

Preparation of RNA and RT-PCR

Total RNA was isolated from $3 \cdot 10^8$ cells by Qiagen Rneasy® Mini Kit (QIAGEN Ibérica, Madrid, Spain) according to the manufacturer's recommendations. Additionally, Dnase I digestion of the isolated RNA was performed using the Rnase-Free Dnase Set (QIAGEN Ibérica, Madrid, Spain) to avoid DNA interferences during PCR steps. Isolated RNA purity and concentration were assessed in a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA quality was evaluated by microfluidic capillary electrophoresis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) using Agilent RNA 6000 Pico kit. Chips were prepared and loaded according to the manufacturer's instructions. Isolated RNA was stored at -80ºC for no longer than three days.

One microgram of high quality RNA (rRNA ratio [23S/16S] ≈ 1.6, RNA integrity number [RIN] > 9.0, and A^{260}/A^{280} ratio > 2.0) was reverse transcribed with TaqMan® Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol and stored at -20ºC prior to use. Briefly, a 50 µL reaction mixture was incubated in a Peltier Thermal Cycler 200 (MJ Research Inc., Boston, MA) for 10 min at 25ºC, 30 min at 48ºC and 5 min at 95ºC.

The primers used in this work (see **Appendix**) were designed using the Primer Express® Software v3.0 (Applied Biosystems, Foster City, CA) and ordered from Applied Biosystems (Cheshire, UK). The *polA*, *dnaA* and *rrsA* genes (encoding DNA polymerase I, transcriptional dual regulator and 16S ribosomal RNA, respectively) were used as internal control for relative quantification.

Quantitative PCR was performed in a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) using Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Briefly, 50 μL reactions mixtures, with 10 ng template cDNA and 15 pmol of each primer, were incubated for 2 min at 50ºC, 10 min at 95ºC and 40 PCR cycles (15 s at 95ºC and 1 min at 60ºC). An additional dissociation step (15 s at 95ºC, 30 s at 60ºC and 15 s at 95ºC) was added to assess non-specific amplification. PCRs were run in triplicate. Raw data were transformed into threshold cycle (C_t) values. Relative gene expression for each mutant compared to wild type was calculated by the comparative C_t Method ($\Delta\Delta C_t$).

Enzyme assays

The enzyme activity assays were optimized for the conditions and media. All measurements were carried out in a microplate spectrophotometer Synergy HT (Bio-Tek, Winooski, VT). Enzyme activity was defined as μmol of substrate consumed per minute and mg of protein (U/mg). All enzyme activities were measured at 37ºC.

In each case, reactor bulk liquid samples were withdrawn and centrifuged at 16,000 xg at 4ºC. The supernatant was removed and cells were resuspended in 65 mM phosphate buffer (pH 7.5). Cells were sonicated on ice for 3 cycles (20 s each), with a probe of 3 mm diameter of a Vibra Cell VC 375 ultrasonic processor (Sonics Materials, Danbury, CT). The extract was centrifuged for 15 min at 20,000 xg and 4ºC to remove cell debris and the supernatant was used for subsequent activity measurements. Protein content was determined by the method of Lowry modified by Hartree (*26*).

Isocitrate dehydrogenase (Icdh) The method was described by Aoshima et al. (*27*). The measurement buffer was 65 mM potassium phosphate (pH 7.5). The reaction components were 5 mM $MgCl_2$, 2 mM $NADP^+$ and 2.5 mM D,L-isocitrate. The enzyme activity was followed by the increase in NADPH absorbance at 340 nm (ε_{NADPH}=6.220 $M^{-1}cm^{-1}$). One unit of enzyme activity was that required for the generation of 1 μmol of NADPH per min.

Isocitrate lyase (Icl) The assay was that described by Aoshima et al. (*27*), using the same buffer as above. The reaction mixture was composed of 5 mM $MgCl_2$, 20 mM phenylhydrazine and 5 mM D,L-sodium isocitrate. The enzyme activity was followed by the increase in absorbance at 324 nm due to the reaction of the glyoxylate produced with phenylhydracine (ε_{adduct}=16,8 $M^{-1}cm^{-1}$). One unit of enzyme activity was taken as that needed to generate 1 μmol of adduct per min.

Acetyl-CoA synthetase (Acs) The method used was that established by Lin et al. (*28*). The measurement buffer was 100 mM Tris-HCl (pH 7.8). The reaction mixture contained 5 mM D,L-Malate, 1 mM ATP, 2.5 mM $MgCl_2$, 0.1 mM coenzyme A, 3 mM NAD^+, 2.5 U/mL malate dehydrogenase, 1.25 U/mL citrate synthase and 100 mM sodium acetate. The acetyl-CoA synthetase activity was followed as the increase in NADH absorbance at 340 nm (ε_{NADH}=6.220 $M^{-1}cm^{-1}$). Enzyme activity unit was defined as the enzyme generating 1 μmol of NADH per min.

Glucose 6-phosphate dehydrogenase (Zwf) The method was that of Peng et al. (*29*). The measurement buffer was Tris-HCl 100mM (pH 7.5), and the reaction mixture contained 10 mM $MgCl_2$, 1.5 mM $NADP^+$ and 10 mM glucose 6-phosphate. The enzyme activity was followed for 5 min as the increase in NADPH absorbance at 340 nm (ε_{NADH}=6.220 $M^{-1}cm^{-1}$), one unit being the enzyme required to generate 1 μmol of NADPH per min.

Phosphotransacetylase (Pta) The assay was carried out as in Peng et al. (*29*).The measurement buffer was 250 mM Tris-HCl, pH 7.8. The reaction components were 1 mM $MgCl_2$, 10 mM D,L-malic acid, 3 mM NAD^+, 0.5 mM coenzyme A, 2.5 U/ mL malate dehydrogenase, 1.25 U/mL citrate synthase and 10 mM acetyl-phosphate. The enzyme activity was followed as the increase in NADH absorbance at 340 nm (ε_{NADH}=6.220 $M^{-1}cm^{-1}$), one unit being taken as the enzyme required for the generation of 1 μmol of NADH per min.

Pyruvate dehydrogenase complex (Pdh) The method was that of Brown et al. (*30*). The measurement buffer was 50 mM potassium phosphate (pH 8.0), and the reaction components were 1 mM $MgCl_2$, 0.5 mM thyamine pyrophosphate, 0.5 mM L-cysteine,

2.5 mM NAD^+, 0.1 mM coenzyme A and 10 mM sodium pyruvate. The enzyme activity was followed as the increase in NADH absorbance at 340 nm (ε_{NADH}=6.220 $M^{-1}cm^{-1}$). One enzyme activity unit was taken to be the enzyme required to generate 1 μmol of NADH per min.

Malate dehydrogenase (Mdh) The method was that of Park et al., (*31*). The measurement buffer was 65 mM potassium phosphate (pH 7.5), with 0.5 mM NADH, and 0.2 mM oxaloacetic acid as substrates. The enzyme activity was followed as the decrease in NADH absorbance at 340 nm (ε_{NADH}=6.220 $M^{-1}cm^{-1}$), one unit being taken as the enzyme required for the consumption of 1 μmol of NADH per min.

Pyruvate oxydase (PoxB) The method was that of Abdel-Hamid et al. (*32*) with minor modifications. The measurement buffer was 65 mM potassium phosphate buffer (pH 6.0), and the reaction mixture was 5 mM $MgCl_2$, 0.25 mM thyamine pyrophosphate, 2.5 mM potassium ferricyanide and 100 mM sodium pyruvate. The enzyme activity was followed for 10 min as the increase in potassium ferricyanide absorbance at 405 nm ($\varepsilon_{ferricyanide}$=0.093 $M^{-1}cm^{-1}$). One enzyme activity unit was taken as the enzyme generating 1 μmol of ferricyanide per min.

Pyruvate kinase (Pyk) The method was that of Peng et al. (*29*)with minor modifications. The measurement buffer was 50mM (pH 6.5) Bis-Tris buffer, and the reaction mixture was 25 mM $MgCl_2$, 10mM KCl, 0.25 mM Dithiotreitol, 0.5 mM NADH, 2.5 mM ADP, 2.5 U/ml L-lactic dehydrogenase and 5 mM phosphoenolpyruvate. The enzyme activity was followed as the decrease in NADH absorbance at 340 nm (ε_{NADH}=6.220 $M^{-1}cm^{-1}$), one unit being taken as the enzyme required for the consumption of 1 μmol of NADH per min.

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 microplate spectrophotometer Synergy HT (Bio-Tek,

Winooski, VT) was used. DMSO was used for cell lysis. Cell content was determined assuming an intra-cellular volume of 1.63 µL/mg (*33*).

Reducing power, taken as the $NADH/NAD^+$ ratio, was determined as in Snoep et al. (*34*). For the measurements, an enzymatic method based on alcohol dehydrogenase was used. The extraction of the reduced or the oxidized forms was carried out by two different methods, involving alkali or acid extraction.

Results

The effect of *pta* and *acs* deletion was assessed in three different scenarios: (i) aerobic growth on glucose, (ii) aerobic growth on acetate and (iii) anaerobic growth on glucose. The parent strain, *E. coli* BW25113, was used as control (**Table 1**).

Glucose as the carbon source in aerobic batch cultures

Kinetics of cell growth

The growth and metabolism of *E. coli* BW25113 and its knockout strains, Δ*pta* and Δ*acs*, were characterized in aerobic cultures on glucose minimal medium. None of the mutations impaired growth; similar growth rates were observed for all the three strains, while biomass yield was slightly higher for the Δ*pta* mutant (**Table 2**). The production of lactate during the exponential growth phase in this mutant was nearly 10-fold higher than that of the control. Glucose exhaustion caused a lag in growth and lactate began to be consumed, leading to diauxic growth (**Fig. 2**). The Δ*pta* mutant presented lower metabolite production rates than the *E. coli* wild type and Δ*acs* strains, and acetate production was 14-fold lower in this strain. However, residual acetate production indicated that pathways other than the Pta-AckA were active. Acetate was always consumed by all three strains in the stationary phase, but only after glucose/lactate exhaustion (**Fig. 2**). Substantial differences were observed in the Δ*pta* mutant and further analyses were performed.

Table 2. Metabolic and growth parameters during growth on glucose minimal medium and aerobiosis. Culture conditions are expressed in the Materials and Methods section.

Growth parameters	BW25113	Δ*pta*	*Δacs*
Biomass yield ($g \cdot g^{-1}$)	0.27±0.06	0.31±0.02	0.26±0.06
Growth rate (h^{-1})	0.74±0.03	0.76±0.05	0.74±0.03
Glucose uptake rate [$mmol \cdot (g \cdot h)^{-1}$]	10.64±0.96	13.60±0.91	11.31±1.21
Acetate production rate [$mmol \cdot (g \cdot h)^{-1}$]	7.70±0.62	0.52±0.07	8.60±0.49
Ethanol production rate [$mmol \cdot (g \cdot h)^{-1}$]	2.63±0.27	1.23±0.28	2.36±0.01
Formate production rate [$mmol \cdot (g \cdot h)^{-1}$]	7.20±0.58	1.18±0.01	7.78±0.01
Lactate production rate [$mmol \cdot (g \cdot h)^{-1}$]	0.71±0.02	7.31±0.54	0.74±0.16

Energetic and redox state

The energetic and redox states of the wild type and Δ*pta* strains were determined during the early exponential phase of growth and at the onset of the stationary phase. The intracellular ATP concentration of the Δ*pta* strain was higher than that of the wild type strain, especially in the late exponential phase (**Table 3**). On the other hand, the redox state of both strains was nearly the same in the exponential phase and slightly reduced in the Δ*pta* strain at the onset of the stationary phase (**Table 3**).

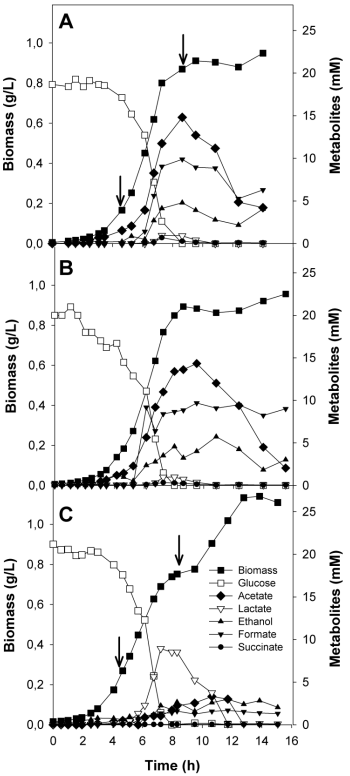


Fig. 2. Growth and metabolite production of A) E. coli BW25113 (wild type.) and its B) Δ*acs* and C) Δ*pta* knockout derivative strains.
Experiments were performed using glucose as the carbon source. (■) Biomass, (□) glucose, (◆) acetate, (▽) lactate, (▲) ethanol, (▼) formate and (●) succinate. Sampling times for enzyme activities and/or gene expression are indicated by arrows. Assays were carried out as indicated in the Materials and Methods section.

Table 3. Energetic and redox state in *E. coli* BW25113 and its *Δpta* knockout strain. ATP values are intracellular concentrations (nM). Culture conditions are expressed in the Materials and Methods section.

	ATP		NADH/NAD$^+$	
	BW25113	**Δ*pta***	**BW25113**	**Δ*pta***
Glucose aerobic *Early exponential phase*	2.27±0.17	2.68±0.11	0.184±0.001	0.173±0.003
Glucose aerobic *Stationary phase*	0.77±0.05	1.22±0.12	0.224±0.003	0.262±0.016
Acetate aerobic *Early exponential phase*	2.22±0.53	2.30±0.58	0.240±0.007	0.260±0.001
Glucose anaerobic *Early exponential phase*	1.04±0.11	1.85±0.04	0.303 ±0.044	0.450 ±0.084

Enzyme activities

Nine enzyme activities related to the central metabolism were studied in the early exponential phase and at the onset of the stationary phase of growth (**Table 4**). Pyruvate kinase (Pyk) and pyruvate dehydrogenase (Pdh) in the stationary phase were higher in the Δ*pta* strain in both stages. As regards the TCA cycle, Icdh was higher in the Δ*pta* mutant while the first enzyme of the glyoxylate shunt (Icl) was lower in the deleted strain in both phases. Taken together, these changes clearly reflect a more active glycolysis and TCA cycle in the mutant strain as a consequence of the mutation and a down-regulation of the glyoxylate shunt, as further supported by the higher Icdh/Icl ratios.

When considering the acetate metabolism, Acs was only detected in the stationary phase of both strains, although expression in the Δ*pta* mutant was three-fold lower, probably as a result of low acetate levels (**Table 4**). Moreover, although the only active acetate-producing enzyme (PoxB) showed high activity in both strains, low acetate production levels were observed in the Δ*pta* strain (**Fig. 2**).

Gene expression by RT-PCR

The relative expression of 29 genes related to the central metabolism of *E. coli* was analysed at the early exponential phase and at the onset of the stationary phase in the wild type and Δ*pta* strains. The results are shown in **Table 5**.

At the early exponential phase, the down regulation of *adhE* and *poxB* explained the lower production of ethanol and acetate in the mutant strain. With

respect to glycolysis, the genes analysed showed similar expression to the parent strain, while the gluconeogenesis enzyme (phosphoenolpyruvate synthase) encoded *pps* was up regulated in the *pta* mutant.

At the onset of the stationary phase, the wild type and the Δ*pta* mutant strains began to adapt to consume the acetate and lactate produced, respectively, after glucose exhaustion. Accordingly, in the Δ*pta* mutant, gluconeogenic and some glycolytic genes (*pykA* and *pykF*) were activated. In this stage, several changes were also observed in genes related to the fermentation pathways. Surprisingly, although *pflA* was up regulated, the mutant produced less formate. The genes related to lactate metabolism also presented a different expression pattern: *ldhA* was up regulated while *lldD* and *dld* were down regulated. The TCA cycle and the glyoxylate shunt were down-regulated in the mutant strain as well as the *acs* gene, which reflects the substantial differences in the pathways involved in the consumption of acetate (wild type strain) and lactate (Δ*pta* mutant) in this phase.

In addition to these alterations, a few transcriptional regulators were also altered in both strains, reflecting the role of general control mechanisms in the alterations observed in the mutant. In fact, the regulation of *rpoS* and *pdhR* reflected these alterations, especially at the onset of the stationary phase.

Table 4. Enzyme activities in the control strain and *pta* mutant. Samples were taken at mid-exponential phase and at the beginning of the stationary phase. All enzyme activities are expressed as mU·mg^{-1} protein. Experimental conditions are expressed in the Materials and Methods section. (*n.a.*- not applicable; *n.d.*- not detected).

	Glucose aerobic batch cultures				**Acetate aerobic batch cultures**				**Glucose anaerobic batch cultures**	
	Exponential phase		***Stationary phase***		***Exponential phase***		***Stationary phase***		***Exponential phase***	
	BW25113	*pta*$^-$	*BW25113*	*pta*-	*BW25113*	*pta*$^-$	*BW25113*	*pta*$^-$	*BW25113*	*pta*$^-$
MDH	132.6±3.1	124.0±25.2	66.7±17.7	74.3±0.4	41.7+10.0	53.9±6.8	57.3±2.2	62.2±5.6	71.6±3.6	60.3±8.7
ICDH	298.3±3.4	432.6±16.7	351.5±51.0	502.2±21.6	376.2±14.4	243.7±33.0	318.5±8.6	158.4±5.5	129.3±4.0	136.6±18.8
ICL	18.4±0.8	15.1±2.3	15.2±3.7	9.6±0.8	276.2±31.9	187.2±4.4	242.7±27.4	211.4±23.7	12.1±0.5	5.9±0.9
ACS	n.a.	n.a.	18.0±1.4	5.6±0.1	28.4±9.8	0.3±0.5	10.5±2.3	4.6±0.2	101.6±1.9	10.0±0.1
PTA	897.7±10.3	n.a.	1716.7±168	n.a.	1140.0±39.1	n.a.	2441.8±121.8	n.a.	1691.8±231.2	n.a.
POX	240.6±1.65	164.6±16.6	231.5±45.9	261.0±14.1	323.6±34.5	431.3±21.7	441.05±24.3	481.4±43.8	251.9±25.1	205.1±8.2
PDH	249.3±3.9	505.9±61.8	293.6±39.4	605.8±106.3	141.3±39.6	96.7±15.4	204.5±5.6	126.3±25.8	211.1±29.0	273.0±16.2
PYK	184.6±51.9	303.4±59.9	273.9±65.3	377.5±17.4	189.6±21.9	176.8±15.9	257.2±8.7	315.0±14.2	461.9±61.9	512.1±17.6
ZWF	124.1±23.1	140.1±7.8	117.4±33.0	173.9±24.3	68.5±14.3	100.2±23.1	82.0±22.5	128.6±4.4	232.2±30.6	129.0±10.7
ICDH/ICL	16.2±0.8	28.7±4.5	23.1±3.6	52.6±4.8	1.4±0.2	1.3±0.2	1.3±0.2	0.7±0.1	10.7±0.5	23.2±4.7
PTA/ACS	n.a.	n.a.	95.4±4.5	n.a.	40.1±13.9	n.a.	231.5±51.2	n.a.	16.6±2.3	n.a.

Table 5. Relative gene expression in *E. coli* Δ*pta* mutant**.** Logarithmic ratios were determined using the ΔΔCt method. *E. coli* BW25113 was used as control under each growth condition. Experimental conditions are expressed in Materials and Methods section. (*n.d.* – not determined). E.E.(early exponential phase) and L.E (Late exponential phase)

			Glucose aerobic batch cultures		**Acetate aerobic batch cultures**	
Gene	***Description***	***Pathway***	***E.E***	***L.E.***	***E.E***	***L.E.***
rpoD	RNA polymerase sigma 70 subunit	Transcriptional regulators	0.05±0.02	-0.15±0.04	-0.46±0.23	-0.36±0.10
rpoS	RNA polymerase sigma 38 subunit		-0.18±0.22	-0.48±0.05	0.36±0.17	-0.18±0.32
ihfA	Integration host factor, α subunit		0.31±0.19	-0.46±0.3	-0.15±0.06	-0.59±0.08
crp	cAMP repression protein		0.04±0.15	0.31±0.11	-0.22±0.25	-0.05±0.04
pdhR	Pyruvate dehydrogenase complex regulator		-0.70±0.15	-1.11±0.15	*n.d.*	*n.d.*
fruR	cAMP independent protein		-0.19±0.01	-0.20±0.16	-0.59±0.06	-0.05±0.04
zwf	Glucose 6-phosphate dehydrogenase	Pentose phosphate	-0.04±0.02	-0.05±0.03	-0.39±0.06	-0.24±0.04
pykF	Pyruvate kinase F	Glycolysis	-0.12±0.08	0.26±0.11	-0.85±0.22	-0.88±0.24
pykA	Pyruvate kinase A		-0.25±0.16	0.67±0.11	-0.83±0.10	-0.13±0.06
aceE	Pyruvate dehydrogenase		-0.07±0.12	0.00±0.05	-1.04±0.18	-0.16±0.07
ldhA	NAD dependent D-Lactate dehydrogenase	Fermentation pathways	-0.02±0.11	0.73±0.18	*n.d.*	*n.d.*
dld	NAD independent Lactate dehydrogenase		0.31±0.26	-0.66±0.05	*n.d.*	*n.d.*
lldD	NAD independent L-lactate dehydrogenase		-0.09±0.12	-0.41±0.03	*n.d.*	*n.d.*
adhE	Alcohol dehydrogenase		-0.76±0.04	0.21±0.04	*n.d.*	*n.d.*
pflA	Pyruvate formate-lyase activating enzyme		-0.09±0.19	1.10±0.10	*n.d.*	*n.d.*
acs	Acetyl-CoA synthethase	Acetate metabolism	0.37±0.10	-0.71±0.01	-1.28±0.39	-1.36±0.77
poxB	Pyruvate oxidase		-0.94±0.30	-0.40±0.05	-0.82±0.10	-1.00±0.11
actP	Acetate Permease		*n.d.*	*n.d.*	-0.28±0.10	-1.13±0.03
ackA	Acetate Kinase		*n.d.*	*n.d.*	-0.10±0.01	-0.44±0.00
icdA	Isocitrate dehydrogenase	TCA cycle	0.27±0.04	-0.30±0.16	-0.82±0.17	0.31±0.47
sucA	2-ketoglutarate dehydrogenase subunit		-0.12±0.19	0.05±0.03	-0.79±0.17	-0.57±0.36
sdhC	succinate dehydrogenase membrane protein		-0.14±0.11	-0.77±0.18	0.43±0.58	-0.13±0.47
mdh	Malate dehydrogenase		0.15±0.03	-0.28±0.08	-0.87±0.08	-0.52±0.17
aceA	Isocitrate lyase	Glyoxylate shunt	0.27±0.06	-1.18±0.09	-0.35±0.25	-0.54±0.23
aceB	Malate synthase		0.01±0.11	-0.85±0.04	-0.83±0.15	-0.31±0.31
maeB	Malate dehydrogenase ($NADP^+$-requiring)	Glucogenogenesis / anaplerosis	0.25±0.09	-0.26±0.01	-0.39±0.27	0.27±0.34
maeA	Malate dehydrogenase (NAD^+-requiring)		0.26±0.04	-0.18±0.03	-0.05±0.23	-0.45±0.28
pck	Phosphoenolpyruvate carboxykinase		0.32±0.07	1.04±0.03	-0.57±0.19	0.36±0.13
ppc	Phosphoenolpyruvate carboxylase		-0.07±0.08	0.68±0.01	0.21±0.25	0.29±0.03
pps	Phosphoenolpyruvate synthase		1.27±0.07	0.64±0.00	-0.39±0.05	-0.07±0.37

Acetate as the carbon source in aerobic batch cultures

The three *E. coli* strains were grown in acetate minimal medium in an attempt to understand the relevance of the Acs and Pta-Ack pathways in the assimilation of acetate and their relation to central metabolism.

Kinetics of cell growth and metabolism

The deletions greatly decreased the efficiency of acetate assimilation in both strains. The growth rate of the Δ*pta* strain in the acetate culture was almost half, compared to the control, while biomass yield was reduced in both mutant strains (**Table 6**). The acetate consumption rate in the Δ*pta* mutant was half that of the wild type strain, while in the Δ*acs* mutant it was slightly higher than in the control. However, the Δ*acs* mutant strain was unable to fully consume the carbon source in the medium, probably as a consequence of the reversibility and low affinity of the Pta-Ack pathway (12). No relevant metabolite production was assessed during culture and only traces of ethanol were detected (**Fig. 3**).

Table 6 - Growth and metabolic parameters of *E. coli* BW25113 (control), Δ*acs* and Δ*pta* mutants in batch aerobic cultures in acetate minimal medium. Culture conditions are expressed in the Materials and Methods section.

Growth parameters	**BW25113**	**Δ*pta***	**Δ*acs***
Biomass yield ($g \cdot g^{-1}$)	0.26±0.01	0.18±0.01	0.13±0.01
Growth rate (h^{-1})	0.28±0.03	0.17±0.02	0.27±0.01
Acetate uptake ($mmol \cdot (g \cdot h)^{-1}$)	24.67±1.59	12.25±0.29	29.10±0.51

Enzyme activities

Key enzyme activities were also studied during the early exponential phase of growth and at the onset of the stationary phase in the Δ*pta* mutant and control strains in acetate cultures (**Table 4**). Enzymes related to energy metabolism (glycolysis and TCA cycle) and the glyoxylate shunt showed altered levels. In fact, Pdh, Icdh and Icl activities were lower in the Δ*pta* strain, while Pyk activity remained steady. A similar Icdh/Icl ratio was observed for both strains in the exponential phase, indicating that the ability to direct acetyl-CoA towards biosynthetic pathways was not altered, while in the late exponential phase, the relative activity of the glyoxylate shunt in the Δ*pta* mutant was

enhanced (**Table 4**). Moreover, the pentose phosphate shunt enzyme, Zwf, presented a higher activity in the mutant.

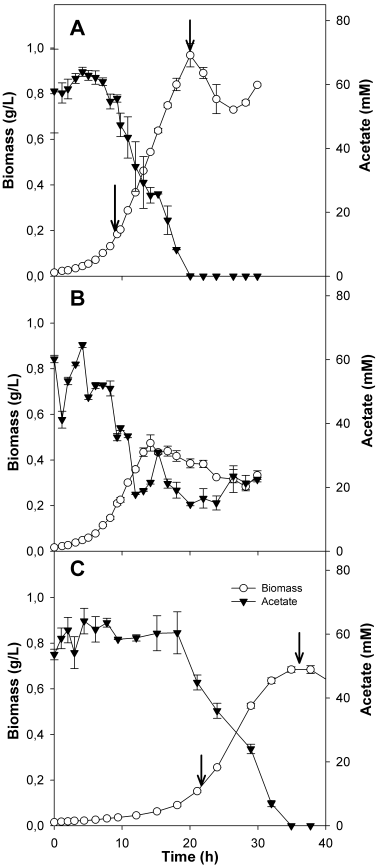


Figure 3 - Growth and metabolite production of A) *E. coli* BW25113 (wild type) and its B) Δ*acs* and C) Δ*pta* knockout mutants. Experiments were performed using acetate as the carbon source. (▲) Biomass and (O) acetate. Sampling times for enzyme activities and/or gene expression are indicated by arrows. Assays were carried out as indicated in the Materials and Methods section.

PoxB and Acs showed a surprising profile since the latter (which is the only feasible pathway for acetate assimilation in the Δ*pta* mutant) decreased in this strain,

especially during the exponential phase, while PoxB (which excretes acetate to the medium) showed much higher activity, especially in the early exponential phase.

Gene expression by RT-PCR

In general, the transcription of the genes analysed was down regulated within the different functional groups. At the early exponential phase some of the genes related to glycolysis, TCA cycle and glyoxylate shunt related were down regulated (**Table 5**). Interestingly, the expression of *acs, actP* and *aceA/aceB* was down-regulated in the Δ*pta* mutant, which supports the low growth rate observed in this strain (**Table 6**). At the onset of the stationary phase, fewer genes were down regulated probably because of the adaptation of the Δ*pta* mutant to the medium; *pykF*, *acs* and *poxB* showed substantially decreased expression.

Among regulatory genes, a noticeable down-regulation of the global regulator *cra* was observed during the exponential phase, which could explain the down regulation of the GS, TCA cycle, and gluconeogenesis related genes. Moreover, a slight increase in *rpoS* expression, related to stress conditions, was observed in the same stage.

Glucose anaerobic batch cultures

Finally, since acetate is the major product during anaerobic growth, *E. coli* BW25113 and the mutant strains were studied under anaerobiosis in glucose minimal medium.

Kinetics of cell growth and metabolites

Results indicate that the Δ*pta* strain grew at a lower rate and with a lower biomass yield than the control (**Table 7**). In addition, a lag phase was observed in the growth of this mutant (**Fig. 4**). Similarly to that observed in the aerobic conditions, acetate, ethanol and formate were produced at lower rates than in the wild type strain, the main by-product being lactate. On the other hand, the Δ*acs* mutant showed a similar growth rate and metabolite production profile to the control.

Table 7 - Growth and metabolic parameters of *E. coli* BW25113 (control) Δ*acs* and Δ*pta* mutants in batch anaerobic cultures in glucose minimal medium. Culture conditions are expressed in the Materials and Methods section.

Growth parameters	**BW25113**	**Δ*pta***	**Δ*acs***
Biomass yield ($g \cdot g^{-1}$)	0.10±0.003	0.05±0.003	0.09±0.003
Growth rate (h^{-1})	0.48±0.03	0.22±0.02	0.43±0.02
Glucose uptake rate [$mmol \cdot (g \cdot h)^{-1}$]	25.40±0.96	29.16±0.15	25.36±1.11
Acetate production rate [$mmol \cdot (g \cdot h)^{-1}$]	19.15±1.65	0.79±0.17	16.15±2.09
Ethanol production rate [$mmol \cdot (g \cdot h)^{-1}$]	17.13±3.14	2.00±0.39	15.72±2.13
Formate production rate [$mmol \cdot (g \cdot h)^{-1}$]	37.34±3.14	4.27±0.64	31.00±3.73
Lactate production rate [$mmol \cdot (g \cdot h)^{-1}$]	0.91±0.05	40.51±5.53	0.30±0.01
Succinate production rate [$mmol \cdot (g \cdot h)^{-1}$]	2.19±0.46	1.31±0.22	1.33±0.15

Energetic and redox state

The intracellular ATP levels and the redox state of the Δ*pta* strain were measured at the exponential phase of growth in anaerobic batch cultures. The Δ*pta* mutant exhibited significantly higher ATP levels than the control strain, while the redox state was almost the same in both strains (**Table 3**).

Enzyme activities

The greatest differences observed in enzyme activities affected energy metabolism in response to the anaerobic conditions: the Icdh, Mdh and Pdh enzymes had lower activity and the glycolysis (Pyk), pentoses phosphate pathway (Zwf) and fermentative pathways (Pta and Acs) had more activity (**Table 4**).

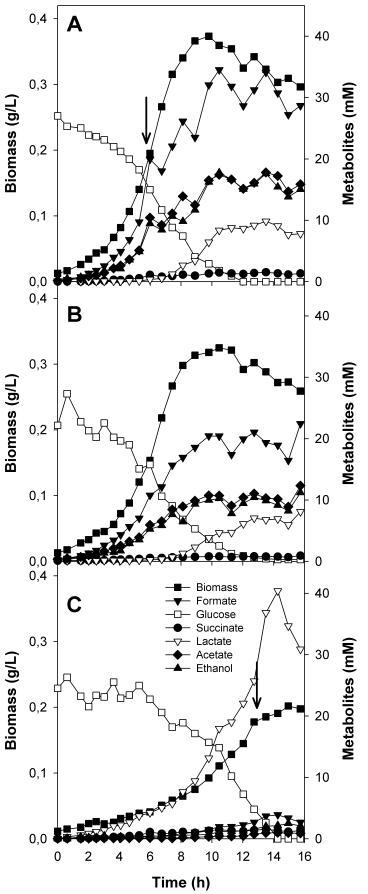


Figure 4 - Growth and metabolite production of A) *E. coli* BW25113 (wild type) and its B) Δ*acs* and C) Δ*pta* knockout mutants. Experiments were performed using glucose as the carbon source and anaerobiosis. (■) Biomass, (□) glucose, (◆) acetate, (▽) lactate, (▲) ethanol, (▼) formate and (●) Succinate. Sampling times for enzyme activities and/or gene expression are indicated by arrows. Assays were carried out as indicated in the Materials and Methods section.

The differences found between the Δ*pta* and the control strains were similar to those found in glucose aerobic batch cultures. The pentose shunt Zwf showed very low activity in the Δ*pta* mutant. As demonstrated in the glucose aerobic cultures, Pdh also had higher activity in the Δ*pta*. The glyoxylate shunt and acetate metabolism were also affected: in Δ*pta* mutant Icl and Pox presented lower activity and almost no Acs activity

was detected. It should be underlined that, under anaerobic conditions, both Pox and Acs activities were high (**Table 4**), meaning that i) Pta-Ack is not the sole acetate-producing pathway in *E. coli* and ii) the co-expression of Pox and Acs pathways could lead to an acetate-consuming cycle with lower energetic efficiency, as previously suggested by Flores et al. (*35*).

Discussion

In this work, further insight into the interrelations between the acetyl-CoA/acetate pathways (Pta-AckA and Acs) and the central metabolism of *E. coli* is presented. The effect of the deletion of *pta* and *acs* was evaluated in three different scenarios: growth on glucose (aerobic and anaerobic conditions) and on acetate (aerobic conditions). While *acs* deletion had only slight effects on bacterial physiology and metabolism, the deletion of *pta* provoked a strong perturbation, indicating its great importance. For that reason, a detailed characterization of this mutant was performed, using a multilevel analysis approach (gene expression, enzyme activities and metabolic rates) to further understand how this mutation affects *E. coli*. Growth on different carbon sources further underlined the role of *pta*, while the comparison of aerobic/anaerobic cultures yielded less valuable information, probably because *pta*, as is known, is constitutively expressed under both conditions (5, 12). Thus, aerobic and anaerobic glucose cultures will be jointly discussed and compared to acetate cultures.

It has been previously described that when *pta* gene is deleted in *E. coli*, pyruvate accumulates in the cell (*36*, *37*), as a result of which the fermentation profile is completely altered. In our work, almost no acetate or ethanol was produced on glucose, under aerobic or anaerobic conditions and was substituted by lactate (**Fig. 2 and 4**). Lactate dehydrogenase (Ldh) in *E. coli* has a high K_m (low affinity) towards pyruvate, and lactate production is only observed when pyruvate accumulates (*38*). Thus, the *pta* strain also suffered an overflow, with lactate being produced instead of acetate and ethanol, in order to restore the NADH/NAD^+ balance and allow for continued glycolysis (**Table 2**). Contrary to what has previously been proposed (*36*), lactate production was not due to the increased expression of lactate dehydrogenase (*ldhA*), but rather to pyruvate accumulation (*37*). In fact, the fermentation pathways genes analysed here, as well as *poxB* (acetate metabolism), were down regulated in the *pta* mutant during the exponential phase, with the exception of *dld*, which codes for a NAD^+-independent lactate dehydrogenase. Interestingly, with entry into the stationary phase, the expression pattern of *ldhA* and *dld* (coding for NAD^+-dependent and NAD^+-independent lactate

dehydrogenases) was inverted, which explains why, in the stationary phase, the *pta* mutant recycled the previously excreted lactate, resulting in diauxic growth and increased biomass yield (**Fig. 2, Table 5**).

The deletion of *pta* caused a further metabolic rearrangement. The analysis of genes and enzyme activities related to the central metabolism threw more light on these alterations, most of which were caused by pyruvate/acetyl-CoA accumulation. In general, data on relative gene expression, enzyme activities and metabolic fluxes fitted well. However, it is well known that multiple levels of regulation exist in the cells, which can lead to non-conclusive results. This is the case of the Pdh complex, which is coded by three structural genes (*aceEF* and *lpdA*) and a regulator (*pdhR*) included within the same operon. This operon is subjected to complex transcriptional regulation, which involves a specific regulator (PdhR) and three different promoters (*39*, *40*). The activity of Pdh is subjected to tight regulation by NADH (*41*) and acetyl-CoA (*42*). The accumulation of acetyl-CoA would inhibit the Pdh complex, resulting in the accumulation of pyruvate. This is known to prevent the binding of PdhR to the promoter of the *pdh* operon (**Fig. 5** (*43*)), finally leading to the expression of *aceEF* and *lpdA*. Consistent with this regulatory scheme, a ten-fold decrease in the *pdhR* transcript level was observed in the *pta* mutant in glucose cultures, while no difference in that of *aceE* was observed (**Table 5**). Furthermore, the Pdh activity in the *pta* mutant was double that of the wild type strain (**Table 4**), which underlines the feasibility of post-transcriptional and post-translational control mechanisms, which have not been described so far in *E. coli*.

In glucose culture, the TCA cycle genes, *icdA* and *mdh* (and the corresponding enzymes Icdh and Mdh), were up regulated (**Tables 4 and 5**). Pyruvate also acts on the phosphatase and kinase activities of AceK (which controls Icdh activity by phosphorylation) and thus, pyruvate accumulation would result in increased Icdh activity (*44*). Moreover, IclR (the repressor of the GS) is also activated by pyruvate (*45*), resulting in *aceBA* down-regulation. In fact, *aceBA* was repressed in the stationary phase on glucose and in both phases on acetate, and lower Isocitrate lyase activity was

detected in all phases. Therefore, pyruvate accumulation would seem to increase TCA cycle fluxes by modulating the two activities, which control the isocitrate node.

Finally, the expression of phosphoenolpyruvate synthase (*pps*), essential during growth in pyruvate and lactate (*46*), was more expressed in the Δ*pta* mutant, controlling the flux through gluconeogenesis and altering the metabolic yields in *E. coli*. In fact, *pps* and *pck* were highly expressed in the glucose cultures. This increase in the gluconeogenic pathways could be a means to compensate the down-regulation of the GS and would explain the excellent growth characteristics of this mutant in aerobic glucose cultures.

Enzyme activities analysed during anaerobic cultures mostly reflected the effect of the absence of oxygen. In general, the rearrangements observed in the *pta* mutant strain in the aerobic and anaerobic conditions were similar.

In the acetate cultures, both Pta and Acs activities were at a high level in the wild type strain, and the Pta/Acs ratio was lower than on glucose, indicating the induction of Acs (**Table 4**). The expression of *acs* is induced by acetate (*47*). In fact, this was verified in the wild type strain, in which the stationary phase on glucose and in both phases on acetate. Acetate production/assimilation pathways were greatly altered in the *pta* mutant strain. Both *acs* and *poxB* gene expression were repressed in the *pta* mutant strain in glucose and acetate cultures. Accordingly, Acs activity was much lower in all the conditions assayed (**Table 4**), a phenomenon already reported (*47*). The down-regulation of *poxB* in the *pta* mutant would allow increasing growth yield in acetate as carbon source. However, i) in glucose cultures, PoxB activity in the *pta* mutant was similar to that of the wild type and, ii) in acetate, it reached values higher than those observed in glucose cultures or by the wild type strain in acetate cultures (**Table 4**). This implies the occurrence of a potential futile cycle *in vivo*, which would lead to decreased energetic efficiency in carbon assimilation. In fact, the biomass yield in acetate cultures decreased by 40% in the *pta* mutant strain compared to that observed in the glucose cultures (**Tables 2** and **6**). Some authors have proposed that the expression of *poxB* under conditions mimicking carbon starvation might be seen as a carbon scavenging response leading to increased biomass yield upon Pdh (aerobic) or Pfl

(anaerobic) repression (*35*). The PoxB/Acs pathway is a bypass of these routes, and all the three involve the formation of a CO_2 molecule. When the PoxB/Acs, the glyoxylate shunt, the TCA cycle and/or the PEP-glyoxylate pathway (*48*) are jointly considered, the stoichiometry of the route changes in terms of energy and redox efficiency. Compared with the TCA cycle, the PEP-glyoxylate pathway allows skipping the formation of one GTP and NADPH per PEP molecule. The metabolization of pyruvate through the PoxB/Acs pathway involves the net consumption of ATP and the formation of ubiquinol (instead of NADH). Interestingly, both PoxB/Acs and the PEP-glyoxylate cycle are related to hunger and/or slow-growth conditions (*32*, *48*). Thus, under slow growth conditions, can lead to (i) lower production of ATP/GTP and (ii) lower production of NADPH and (iii) formation of ubiquinol. This means that in slow growing bacteria the metabolism of cells is switched towards sub-optimal efficiency. This may allow a proper coupling of the production and consumption of energy and reducing power or maybe it is simply the price the bacteria have to pay to be able to adapt to variable stress conditions. This switch to lower efficiency is physiological and crucial during the down-regulation of other pathways, since *poxB* delection leads to inefficient growth, especially in some genetic backgrounds (such as *pts*- strains) (*32*, *35*).

The deletions strongly decreased the efficiency of acetate assimilation and growth, indicating that the Pta-Ack and the Acs pathways must act together. This was especially relevant in the case of the *pta* mutant strain, probably as a result of the specific regulation and characteristics of Acs. The lag observed in the growth of this mutant was probably related to the repression of Acs in the exponential phase (since it is especially related to the stationary phase) (*47*) in the *pta* mutant. On the other hand, the Pta-Ack pathway alone does not allow for an efficient use of acetate as carbon source because of i) its reversibility and ii) lower affinity towards the substrate (K_m 7-10 mM, (*49*)). However, other non-identified factors cannot be ruled out.

Metabolic pathways had to rearrange towards a more efficient use of acetate as carbon source in the Δ*pta* mutant strain. In these cultures, the expression of almost all the genes assayed decreased. A down-regulation of Acs, glycolysis, TCA cycle and GS was observed in the *pta* mutant strain. However, the GS, which is essential for growth

on acetate, showed high activity in acetate cultures in both strains, with levels of the same order of magnitude as those of Icdh (**Table 4**). Further, the Icdh/Icl ratio was similar in both strains during the exponential phase and sharply decreased during the stationary phase in the case of the *pta* strain, revealing the adaptation of this mutant to acetate culture (**Table 4**). Surprisingly, *pta* deletion caused a decrease in Acs expression and activity, which partially explains the low growth and acetate uptake rates and biomass yield observed (**Table 6**).

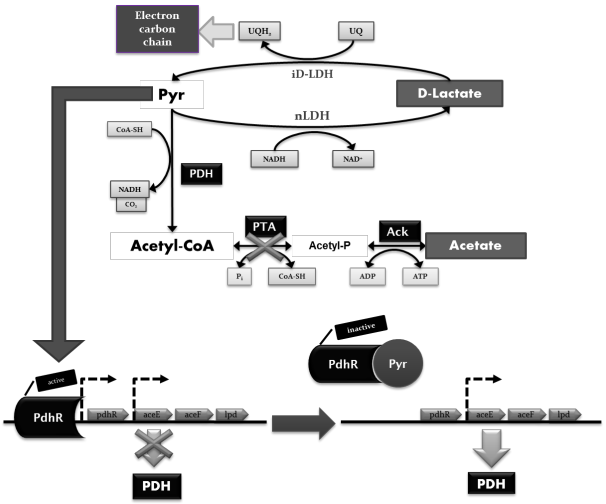


Figure 5 - Simplified model for the alterations in the metabolic network of *E. coli* after *pta* deletion**.** Lactate is produced as a result of the knockout of the *pta* gene and it is used to produce ATP by the combined action of lactate dehydrogenases, nLDH and iDLDH. Moreover, pyruvate accumulation would activate the *pdh* operon. The enzymes involved are shown in the figure [ACK (*ackA*), acetate kinase; PTA (*pta*), phosphotransacetylase, PDH (*aceEF;lpd*) pyruvate dehydrogenase (Ecocyc-Metacyc (*50*)).

These evident effects could stem from changes in the transcriptional regulators of the cellular metabolism. It could be hypothesized that the accumulation of acetyl-phosphate could be responsible for the poor growth characteristics of this strain. However, an *ackA* mutant showed similarly affected growth behaviour even though no acetyl-phosphate accumulation was detected (*51*). Among the regulators analysed, the down-regulation of *cra* (*fruR*) was the most dramatic change observed, revealing that the

mutation in *pta* had a global effect on cellular physiology. In fact, Cra regulates the expression of a number of genes from glycolysis, TCA cycle, GS and gluconeogenesis, and plays an important role in the control of carbon fluxes (*52–54*). Moreover RpoD (σ^{70}) and RpoS (σ^{S}), different sigma subunits of RNA polymerase, were differentially expressed in the exponential and stationary phases of growth. The slight up-regulation of *rpoS* in the exponential phase of growth on acetate, allows us to ascertain why cell growth was so affected, since stress conditions severely compromised cellular physiology. Microarray studies have indicated that the expression of several important genes of the acetate metabolism such as *acs*, *aceAB*, *cysDEK*, *fadR*, etc, are significantly affected by *rpoS* (*55*). During the late exponential phase of the acetate cultures, changes in gene expression were not as noticeable as in the exponential phase. In fact, the levels of expression of the different transcriptional regulators analysed were similar in the wild type strain and Δ*pta* mutant, including *rpoS* and *cra*. In this case, *ihfA* (coding for the integration host factor α subunit), a transcription factor which is relevant in the stationary phase and in the metabolism of acetate, was strongly down-regulated, probably causing the repression of the GS, TCA cycle and acetate metabolism. Moreover, IHF exerts its effect on acetate, while hardly any effect is observed on glucose (*56*).

Oh et al. (*57*) analysed the transcriptional response of *E. coli* to growth on acetate compared to glucose as carbon source, finding that in the presence of acetate most of the altered genes were down-regulated, especially those involved in the cellular machinery. The authors related this to the slow-down in cellular growth and the less active metabolism. These authors also underlined the importance of Acs, GS and gluconeogenesis. Identical responses to acetate were observed in this work for the control strain (data not shown) and even more drastic responses to acetate were determined for the Δ*pta* mutant. In fact, although Acs is the main acetate uptake pathway, it is recognized that Pta-Ack increases acetate influx (*57*).

Contrary to what has previously been described (*58*), we demonstrate herein that *pta* is not essential for anaerobic growth, although its deletion leads to inefficient growth. Moreover, the mutation of *pta* also affected the redox and energy state of *E. coli*

(Table 3). Once the aerobic glucose cultures reached the stationary phase, the redox environment of the *pta* mutant strain was more reduced than that of the wild type strain (while the amount of intracellular ATP was higher), which reflects lactate consumption. Similarly, a more reduced environment was also observed in the exponential phase of the anaerobic glucose culture, suggesting that the Pta-Ack pathway is also important for ensuring a proper redox balance, as further substantiated by the slow growth.

On the other hand, analysis of the metabolism of the Δ*pta* mutant underlines the importance of pyruvate as mediator of the metabolic alterations observed, where it acts not only as an intermediate of the central metabolism but also as an allosteric regulator of several enzymes and activator/repressor of transcription factors. Pyruvate is an allosteric activator of Ldh and the Pfl-activating enzyme, and also activates Pta, AceK (Icdh phosphatase/kinase) and IclR; on the other hand, it inhibits the Pdh complex, AceK and Pfl-deactivase. On top of this, the related PEP inhibits Icl, Ppc, Pck and regulates AceK (*50*). Taken together, the results we present herein further support the close interconnection between the metabolisms of acetate and isocitrate, with pyruvate and PEP as feasible actuators. In fact, the PEP-pyruvate-OAA node is composed of enzymes linking gluconeogenesis with the TCA cycle and GS, allowing anaplerosis (*59*) and were up-regulated in the experiments performed on glucose. Moreover, under these conditions, both Pyk isozymes were down regulated, while Pps, which catalyses the first step of the gluconeogenic pathway, was up regulated. In acetate cultures, only Ppc was slightly up regulated, in the *pta* mutant. On acetate, the gluconeogenic pathway, Pck or the malic enzymes need to be active (*57*) and, in fact, these were repressed (**Table 5**). Additionally, this reveals that the PEP-pyruvate-OAA node acts as a bottleneck for the *pta* mutant when growing on acetate and *E. coli* rearranges flux distributions around the PEP-glyoxylate cycle.

It is likely that the constitutive expression of Pta allows the rapid adaptation and survival of *E. coli* in changing environments. Acetate metabolism has long been regarded as crucial for the use of *E. coli* as cellular factory. Overflow metabolism leads to decreased carbon yield, growth and protein production inhibition (3, 19, 20). The present work provides a further insight into the acetyl-CoA/CoA metabolic node of *E.*

coli. Lactate overflow in Δ*pta* mutant strain shows that metabolic overflow is necessary for adequate metabolic balancing. However, both *pta* and *acs* mutations severely affected the ability of *E. coli* to adapt to changes in their environment (such as the use of acetate as carbon source and/or anaerobiosis). Further work is necessary in order to evaluate the implications of these mutations for the improvement of *E. coli* as a cellular factory.

Summarising, the activity of the central pathways of *E. coli* is affected by deletion of the genes of the acetyl-CoA/acetate metabolism. The mutations alter the co-regulation of the acetate metabolism, glyoxylate shunt and the anaplerotic/gluconeogenic pathways, affecting the efficient assimilation of the carbon sources. The reversibility and low-affinity of the Pta-Ack pathway resulted in the low efficiency of acetate consumption following *acs* deletion, while *pta* deletion severely compromised the adaptation capacity of *E. coli* to anaerobic conditions or to the use of acetate as carbon source. On the other hand, in the Δ*pta* strain, the metabolism had to rearrange in order to buffer the ATP and NADH/NAD^+ pools. The production of lactate and, especially, the altered regulation of the Pdh complex in the Δ*pta* strain reflects that i) metabolic overflow is a crucial mechanism to ensure continued glycolytic activity, ii) the metabolic pools are altered as a consequence of gene deletions and iii) metabolic fluxes can be rearranged in order to ensure redox homeostasis of the cell. Finally, the activation of PoxB in acetate cultures was detected. Moreover, it is demonstrated that Δ*pta* is not essential for growth under anaerobic conditions, although its deletion drastically decreases growth efficiency under certain conditions, underlining that these pathways, and the PEP-pyruvate-OAA node are highly relevant for ensuring the adaptability of this bacterium to environmental changes and for its use in bioprocesses.

Bibliography

1. Doelte, H. W., Ewings, K. N., Hollywood, N. W., and Doelle, H. (1982) Regulation of glucose metabolism in bacterial systems. *Adv Biochem Eng Biotechnol, Microb. React.*, 1–35
2. Chohnan, S., Izawa, H., Nishihara, H., and Takamura, Y. (1998) Changes in size of intracellular pools of coenzyme A and its thioesters in *Escherichia coli* K-12 cells to various carbon sources and stresses. *Biosci Biotechnol Biochem* 62, 1122–1128
3. Luli, G. W., and Strohl, W. R. (1990) Comparison of growth, acetate production, and acetate inhibition of *Escherichia coli* strains in batch and fed-batch fermentations. *Appl. Environ. Microbiol.* 56, 1004–1011
4. Takeda, S., Matsushika, A., and Mizuno, T. (1999) Repression of the gene encoding succinate dehydrogenase in response to glucose is mediated by the EIICBGlc protein in *Escherichia coli*. *J Biochem* 126, 354–360
5. Kumari, S., Tishel, R., Eisenbach, M., and Wolfe, A. J. (1995) Cloning, characterization, and functional expression of acs, the gene which encodes acetyl coenzyme A synthetase in *Escherichia coli*. *J Bacteriol* 177, 2878–2886
6. Keseler, I. M., Mackie, A., Peralta-Gil, M., Santos-Zavaleta, A., Gama-Castro, S., Bonavides-Martínez, C., Fulcher, C., Huerta, A. M., Kothari, A., Krummenacker, M., Latendresse, M., Muñiz-Rascado, L., Ong, Q., Paley, S., Schröder, I., Shearer, A. G., Subhraveti, P., Travers, M., Weerasinghe, D., Weiss, V., Collado-Vides, J., Gunsalus, R. P., Paulsen, I., and Karp, P. D. (2013) EcoCyc: fusing model organism databases with systems biology. *Nucleic Acids Res.* 41, D605–12
7. Berg, P. (1956) Acyl adenylates enzymatic mechanism of acetate activation. *J Biol Chem* 222, 991–1013
8. Kumari, S., Simel, E. J., and Wolfe, A. J. (2000) σ70 is the principal sigma factor responsible for transcription of acs, which encodes acetyl coenzyme A synthetase in *Escherichia coli*. *J Bacteriol* 182, 551–554
9. Gimenez, R., Badia, J., Aguilar, J., Baldoma, L., and Nunez, M. F. (2003) The gene yjcG, cotranscribed with the gene acs, encodes an acetate permease in *Escherichia coli*. *J Bacteriol* 185, 6448–6455
10. Serres, M. H., Gopal, S., Nahum, L. A., Liang, P., Gaasterland, T., and Riley, M. (2001) A functional update of the *Escherichia coli* K-12 genome. *Genome Biol* 2, 31–35
11. Wolfe, A. J. (2005) The acetate switch. *Microbiol Mol Biol Rev* 69, 12–50
12. Brown, T. D. K., Jonesmortimer, M. C., and Kornberg, H. L. (1977) Enzymic interconversion of acetate and acetyl-Coenzyme-A in *Escherichia coli*. *J Gen Microbiol* 102, 327–336
13. Wanner, B. L., and Wilmesriesenberg, M. R. (1992) Involvement of phosphotransacetylase, acetate kinase, and acetyl phosphate synthesis in control of the phosphate regulon in *Escherichia coli*. *J Bacteriol* 174, 2124–2130
14. Shalel-Levanon, S., San, K. Y., and Bennett, G. N. (2005) Effect of ArcA and FNR on the expression of genes related to the oxygen regulation and glycolysis pathway in *Escherichia coli* under growth conditions. *Biotechnol Bioeng* 92, 147–159
15. McCleary, W. R., Stock, J. B., and Ninfa, A. J. (1993) Is acetyl phosphate a global signal in *Escherichia coli*? *J Bacteriol* 175, 2793–2798
16. Dailey, F. E., and Berg, H. C. (1993) Change in direction of flagellar rotation in *Escherichia coli* mediate by acetate kinase. *J Bacteriol* 175, 3236–3239
17. McCleary, W. R., and Stock, J. B. (1994) Acetyl phosphate and the activation of 2-component response regulators. *J Biol Chem* 269, 31567–31572
18. Shi, I. Y., Stansbury, J., and Kuzminov, A. (2005) A defect in the acetyl coenzyme A-acetate pathway poisons recombinational repair-deficient mutants of *Escherichia coli*. *J Bacteriol* 187, 1266–1275

19. Eiteman, M. A., and Altman, E. (2006) Overcoming acetate in *Escherichia coli*recombinant protein fermentations. *Trends Biotechnol* 24, 530–536
20. Bernal, V., Masdemont, B., Arense, P., Canovas, M., and Iborra, J. L. (2007) Redirecting metabolic fluxes through cofactor engineering: Role of CoA-esters pool during L(-)-carnitine production by *Escherichia coli*. *J Biotechnol* 132, 110–117
21. Tsuruta, H., Paddon, C. J., Eng, D., Lenihan, J. R., Horning, T., Anthony, L. C., Regentin, R., Keasling, J. D., Renninger, N. S., and Newman, J. D. (2009) High-Level Production of Amorpha-4,11-Diene, a Precursor of the Antimalarial Agent Artemisinin, in *Escherichia coli*. *PLoS One* 4, e4489
22. San, K. Y., Bennett, G. N., Berrios-Rivera, S. J., Vadali, R. V, Yang, Y. T., Horton, E., Rudolph, F. B., Sariyar, B., and Blackwood, K. (2002) Metabolic engineering through cofactor manipulation and its effects on metabolic flux redistribution in *Escherichia coli*. *Metab Eng* 4, 182–192
23. Vadali, R. V, Bennett, G. N., and San, K. Y. (2004) Applicability of CoA/acetyl-CoA manipulation system to enhance isoamyl acetate production in *Escherichia coli*. *Metab Eng* 6, 294–299
24. Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K. A., Tomita, M., Wanner, B. L., and Mori, H. (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* 2, 2006.0008
25. Datsenko, K. A., and Wanner, B. L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* 97, 6640–6645
26. Hartree, E. F. (1972) Determination of protein modification of Lowry method that gives a linear photometric response. *Anal Biochem* 48, 422–427
27. Aoshima, M., Ishii, M., Yamagishi, A., Oshima, T., and Igarashi, Y. (2003) Metabolic characteristics of an isocitrate dehydrogenase defective derivative of *Escherichia coli* BL21(DE3). *Biotechnol Bioeng* 84, 732–737
28. Lin, H., Castro, N., Bennett, G., and San, K.-Y. (2006) Acetyl-CoA synthetase overexpression in *Escherichia coli* demonstrates more efficient acetate assimilation and lower acetate accumulation: a potential tool in metabolic engineering. *Appl Microbiol Biotechnol* 71, 870–874
29. Peng, L., and Shimizu, K. (2003) Global metabolic regulation analysis for *Escherichia coli* K-12 based on protein expression by 2-dimensional electrophoresis and enzyme activity measurement. *Appl Microbiol Biotechnol* 61, 163–178
30. Brown, J. P., and Perham, R. N. (1976) Selective inactivation of transacylase components of 2-oxo acid dehydrogenase multienzyme complexes of *Escherichia coli*. *Biochem J* 155, 419–427
31. Park, S. J., Cotter, P. A., and Gunsalus, R. P. (1995) Regulation of malate dehydrogenase (mdh) gene expression in *Escherichia coli* in response to oxygen, carbon, and heme availability. *J Bacteriol* 177, 6652–6656
32. Abdel-Hamid, A. M., Attwood, M. M., and Guest, J. R. (2001) Pyruvate oxidase contributes to the aerobic growth efficiency of *Escherichia coli*. *Microbiology* 147, 1483–1498
33. Emmerling, M., Bailey, J. E., and Sauer, U. (2000) Altered regulation of pyruvate kinase or co-overexpression of phosphofructokinase increases glycolytic fluxes in resting *Escherichia coli*. *Biotechnol Bioeng* 67, 623–627
34. Snoep, J. L., Demattos, M. J. T., Postma, P. W., and Neijssel, O. M. (1990) Involvement of pyruvate dehydrogenase in product formation in pyruvate-limite anaerobic chemostat cultures of *Enterococcus faecalis* NCTC-775. *Arch Microbiol* 154, 50–55
35. Sigala, J. C., Flores, S., Flores, N., Aguilar, C., de Anda, R., Gosset, G., and Bolivar, F. (2009) Acetate Metabolism in *Escherichia coli* Strains Lacking Phosphoenolpyruvate: Carbohydrate Phosphotransferase System; Evidence of Carbon Recycling Strategies and Futile Cycles. *J Mol Microbiol Biotechnol* 16, 224–235

36. Chang, D. E., Shin, S., Rhee, J. S., and Pan, J. G. (1999) Acetate metabolism in a pta mutant of *Escherichia coli* W Importance of maintaining acetyl coenzyme a flux for growth and survival. *J Bacteriol* 181, 6656–6663
37. Zhu, H. F., and Shimizu, K. (2005) Effect of a single-gene knockout on the metabolic regulation in *Escherichia coli* for D-lactate production under microaerobic condition. *Metab Eng* 7, 104–115
38. Tarmy, E. M., and Kaplan, N. O. (1968) Kinetics of *Escherichia coli* B D-lactate dehydrogenase and evidence for pyruvate-controlled change in conformation. *J Biol Chem* 243, 2587–2596
39. Cunningham, L., Georgellis, D., Green, J., and Guest, J. R. (1998) Co-regulation of lipoamide dehydrogenase and 2-oxoglutarate dehydrogenase synthesis in *Escherichia coli*: characterisation of an ArcA binding site in the lpd promoter. *FEMS Microbiol Lett* 169, 403–408
40. Spencer, M. E., and Guest, J. R. (1985) Transcriptional analysis of the sucAB, aceEF and lpd genes of *Escherichia coli*. *Mol Gen Genet* 200, 145–154
41. Hansen, R. G., and Henning, U. (1966) Regulation of pyruvate dehydrogenase activity in *Escherichia coli* K12. *Biochim Biophys Acta* 122, 355–358
42. Schwartz, E. R., and Reed, L. J. (1970) Regulation of activity of pyruvate dehydrogenase complex of *Escherichia coli*. *Biochemistry* 9, 1434–1439
43. Quail, M. A., and Guest, J. R. (1995) Purification, characterization and mode of action of PdhR, the transcriptional repressor of *pdhR-aceEF-lpd* operon of *Escherichia coli*. *Mol Microbiol* 15, 519–529
44. Miller, S. P., Chen, R. D., Karschnia, E. J., Romfo, C., Dean, A., and LaPorte, D. C. (2000) Locations of the regulatory sites for isocitrate dehydrogenase kinase/phosphatase. *J Biol Chem* 275, 833–839
45. Lorca, G. L., Ezersky, A., Lunin, V. V, Walker, J. R., Altamentova, S., Evdokimova, E., Vedadi, M., Bochkarev, A., and Savchenko, A. (2007) Glyoxylate and pyruvate are antagonistic effectors of the *Escherichia coli* IclR transcriptional regulator. *J Biol Chem* 282, 16476–16491
46. Niersbach, M., Kreuzaler, F., Geerse, R. H., Postma, P. W., and Hirsch, H. J. (1992) Cloning and nucleotide sequense of *Escherichia coli* K-12 *ppsA* gene, enconding PEP synthase. *Mol Gen Genet* 231, 332–336
47. Kumari, S., Beatty, C. M., Browning, D. F., Busby, S. J. W., Simel, E. J., Hovel-Miner, G., and Wolfe, A. J. (2000) Regulation of acetyl coenzyme A synthetase in *Escherichia coli*. *J Bacteriol* 182, 4173–4179
48. Fischer, E., and Sauer, U. (2003) A novel metabolic cycle catalyzes glucose oxidation and anaplerosis in hungry *Escherichia coli*. *J Biol Chem* 278, 46446–46451
49. Fox, D. K., and Roseman, S. (1986) Sugar transport by the bacterial phosphotransferase system . Isolation and characterization of homogeneous acetate kinase from Salmonella typhimurium and *Escherichia coli*. *J Biol Chem* 261, 3487–3497
50. Keseler, I. M., Bonavides-Martinez, C., Collado-Vides, J., Gama-Castro, S., Gunsalus, R. P., Johnson, D. A., Krummenacker, M., Nolan, L. M., Paley, S., and Paulsen, I. T. (2009) EcoCyc: A comprehensive view of *Escherichia coli* biology. *Nucleic Acids Res* 37, D464 – D470
51. Clark, D. P., and Cronan, J. E. (1996) Two-Carbon Compounds and Fatty Acids as Carbon Sources. *Escherichia coli Salmonella Cell. Mol. Biol.*, 343–357
52. Bledig, S. A., Ramseier, T. M., and Saier, M. H. (1996) FruR mediates catabolite activation of pyruvate kinase (*pykF*) gene expression in *Escherichia coli*. *J Bacteriol* 178, 280–283
53. Cunningham, L., Gruer, M. J., and Guest, J. R. (1997) Transcriptional regulation of the aconitase genes (acnA and acnB) of *Escherichia coli*. *Microbiology* 143, 3795–3805

54. Perrenoud, A., and Sauer, U. (2005) Impact of global transcriptional regulation by ArcA, ArcB, Cra, Crp, Cya, Fnr, and Mlc on glucose catabolism in *Escherichia coli*. *J Bacteriol* 187, 3171–3179
55. Rahman, M., Hasan, M. R., Oba, T., and Shimizu, K. (2006) Effect of *rpoS* gene knockout on the metabolism of *Escherichia coli* during exponential growth phase and early stationary phase based on gene expressions, enzyme activities and intracellular metabolite concentrations. *Biotechnol Bioeng* 94, 585–595
56. Resnik, E., Pan, B., Ramani, N., Freundlich, M., and LaPorte, D. C. (1996) Integration host factor amplifies the induction of the *aceBAK* operon of *Escherichia coli* by relieving IclR repression. *J. Bacteriol.* 178, 2715–2717
57. Oh, M. K., Rohlin, L., Kao, K. C., and Liao, J. C. (2002) Global expression profiling of acetate-grown *Escherichia coli*. *J Biol Chem* 277, 13175–13183
58. Gupta, S., and Clark, D. P. (1989) *Escherichia coli* derivatives lacking both alcohol dehydrogenase and phosphotransacetylase grow anaerobically by lactate fermentation. *J Bacteriol* 171, 3650–3655
59. Sauer, U., and Eikmanns, B. J. (2005) The PEP-pyruvate-oxaloacetate node as the switch point for carbon flux distribution in bacteria. *FEMS Microbiol Rev* 29, 765–794

Appendix

Primers used in this study.

Group	Gene name	Foward primer	Reverse primer
Internal control	*pola*	GCTGAACGTGCAGCCATTAA	CAATCATCGCCCGTTTGATAA
	dnaa	TGGCGAAAGAGCTGACTAACC	ACGGCAGGCATGAAGCA
	rrsa	CCTTACGACCAGGGCTACACA	CACTTTATGAGGTCCGCTTGCT
Sigma factors	*rpod*	TGCGTATGCGTTTCGGTATC	GCGGGTAACGTCGAACTGTT
	rpos	CGCCGCCGGATGATC	CAGACCACGATTGCCATAACG
Transcription factors	*ihfa*	CGAAAACGGGCGAGGATA	CGACCCGGCTTTTTAACTTCT
	crp	TGTTTGAAGAGGGCCAGGAA	CCACTTCACAGGCGGTTTTC
	pdhr	CATCGCCATCTGGCCTTTAT	CACGGCGGCTCTCTTCA
	frur	GGTTGGTGCCGATCAGGAT	TCTCGGCGGGAAACTTACG
PPP	*zwf*	GGCGCTGCGTTTTGCTAA	TTCTGCCACGGTAATCTCAACA
Glucose transport	*ptsg*	ACTGGCGTTGTGCTGTCCTT	GGTAAGCAGCCCACTGAGAGA
Glucolysis	*pykf*	CGAGTTCAACAATGACAACCGTAA	ATCAGCGGAGCATCCAGTTT
	pyka	GCGCTGACCGAAAAAGACAA	CAGCCAGGTAATCTACGCCAAT
	acee	CCAAAGGCAAAGCGACAGTAAT	CCTGGTGCGCGATGTTTT
Fermentations	*ldha*	ACGGAAAAAACCGCTAAAACTG	ATATTTAACGCCGTGCTTTTTCA
	lldd	GGGCTTGATGTCGTGCGTAT	AGAAAGCACGACCCAGCAGTA
	dld	TCGTGGCTGGTGGATTATTTC	AACGCTTTGCTGCCTTCCT
	adhe	GTGGTCCGGGCATGGTT	TTGCCCGCGCCTACAC
	pflb	ACTGAATACCGTAAAACTCACAACCA	AGATTTACGGCAACGCAGGAT
Acetate metabolism	*acs*	ACACACCATTCCTGCCAACA	TGTTGATACATCGCCTCGTACTG
	actp	ACCGGGTTTATGGGCTACTTCTA	CGGATTCGCACCAACCA
	acka	CGCGCAATGGACGTTTACT	TCCAGACGACCATCCATCAG
	poxb	AGCGTGCTGGGCTTTGTG	TAGTTCGGTGCCGTCAGTCA
TCA	*icda*	AAGTTCACCGAAGGAGCGTTTA	GCCACCGTCGATCAGTTCA
	suca	GTCTCGCGTTGCCAAGATTTAT	GTGGCGTAAGCGAGGTTTTC
	sdhc	CACATGATGATGGATTTTGGCTAT	GCACGACAGTAATAACAAAGGAGATTT
	mdh	AGGCGCTTGCACTACTGTTAAAA	CGGGAGTCACTGGAGCGATA
Glyoxilate shunt	*acea*	TGCACGGTGAGTCGAAAAAA	TAGACTGCTTCAATACCCGCTTT
	aceb	TGGCGTGGTGAGGCAAT	GGAAGAAATAGAGCGCAAAATCA
Gluconeogenesis and Anaplerosis	*maeb*	GAAGAGCTGGCGGAGATCAC	GCGGCTCAATACCAAAACGA
	sfca	TGCGGTTGGCAAAATGG	AGGGCTTCGGCAGAGGTT
	pck	CGTCTTTCCGTCCGTTTCAT	GCTCGGGCGAATAAACATGT
	ppc	GGAAGAGAACCTCGGCTACAAA	CCGCCCATCCACGAAGT
	pps	CCTCCCTGGGTGAAATGATTACTA	GGCGGTTGTGGCGAAA

Chapter 4

cAMP-CRP co-ordinates the expression of the protein acetylation pathway with central metabolism in *Escherichia coli*

The results presented in this chapter are based in the publication:

Castaño-Cerezo S, Bernal V, Blanco-Catalá J, Iborra JL, Cánovas M.
"cAMP-CRP co-ordinates the expression of the protein acetylation pathway with central metabolism in *Escherichia coli*."
Molecular Microbiology 2011;82:1110–28.

Abstract

Lysine acetylation is a well-established post-translational modification widely conserved and distributed in bacteria. Although multiple regulatory roles have been proved, little is known about its regulation. Here, we present evidence that the transcription of the Gcn5-like acetyltransferase YfiQ of *Escherichia coli* (proposed name: PatZ) is regulated by cAMP-CRP and its implications on acetate metabolism regulation. The acetate scavenging acetyl-CoA synthetase (Acs) is regulated at the transcriptional and post-translational levels. Post-translational regulation depends on a protein acetyltransferase (*yfiQ*) and a NAD^+-dependent deacetylase (*cobB*). We have studied their expression under different environmental conditions. *cobB* is constitutively expressed from a promoter located upstream *nagK*. The expression of *yfiQ* occurs from its own promoter; it is up regulated in the stationary phase and in the presence of non-PTS carbon sources and is positively regulated by cAMP-CRP. Two putative CRP binding sites are necessary for its full activity. Gene deletion revealed that *cobB* is essential for growth on acetate, *yfiQ* deletion restoring growth of the *cobB* mutant. The fine-tuning of metabolic enzymes results from the integration of multiple mechanisms, and redundant systems may exist. Despite the existence of divergent catabolite repression systems, this may be a conserved strategy common to both Gram-positive and -negative bacteria.

Introduction

Traditionally, post-translational modification (PTM) of proteins has been associated almost exclusively with eukaryotic cells. However, the recent advances of MS-based proteomics have contributed to uncovering a growing number of bacterial PTMs, which are lately receiving increasing attention of the scientific community. PTMs are an efficient mechanism to control the activity of structural proteins, gene expression regulators and enzymes in response to rapidly changing conditions. Such modifications may be involved in hitherto unknown or underappreciated regulatory mechanisms of metabolic and signalling networks.

Acetylation of lysine residues is a conserved PTM mechanism affecting many proteins. Acetyl-CoA-dependent acetyltransferases, Gcn5-like protein N-acetyltransferases (GNATs) and NAD^+-dependent (Sir2-like) protein deacetylases (sirtuins) are conserved in all domains of life (1, 2). In eukaryotes, acetylation of proteins regulates DNA-protein interactions, subcellular localization, transcriptional activity, protein stability and activity of enzymes and transcription factors (3–5). Its role in bacteria is starting to be uncovered. Despite some pioneering works, so far, the molecular actors in the protein acetylation-deacetylation system of *E. coli* have not been fully described, the relevance of such modifications in metabolic control has not been investigated and the regulation of these mechanisms and their coordination with other regulation strategies (such as transcriptional regulation) has not been described. So far, the Pat/CobB system of *S. enterica* is the prokaryotic paradigm of this PTM system (6).

Prokaryotic protein acetyltransferases involved in metabolic regulation have been poorly described. In *S. enterica* and *B. subtilis*, the genes encoding the major protein acetyltransferase enzymes have been identified. The *S. enterica* protein acetyltransferase (Pat) is a Gcn5-like N-acetyltransferase involved in the post-translational regulation of acetyl-CoA synthetase (Acs) and many other central metabolic enzymes. In *Rhodopseudomonas palustris*, four acyl-CoA synthetases are inactivated upon acetylation by a protein acetyltransferase (7). The architecture of

protein acetylation-deacetylation PTM systems in Gram-positive (*B. subtilis*) and Gram-negative (*E. coli* and *S. enterica*) are different (2, 8–10).

Sirtuins are a family of proteins with NAD^+-dependent deacetylase activity, well characterized in mammals, where they are key players in signalling related to stress resistance and energy metabolism (11–13). So far, bacterial sirtuin proteins have been identified and studied in few model organisms, such as *S. enterica*, *B. subtilis* and *E. coli* (7, 14, 15). The best-known bacterial sirtuin is CobB from *S. enterica*. This microorganism synthesizes two biologically active isoforms of CobB, one of which contains a 37-residue N-terminal extension: $CobB_S$ and $CobB_L$. Moreover, in *S. enterica* the *cobB* gene is expressed from two promoters which are independent of its neighbouring gene (*ycfX*, located only 18 bp apart from *cobB*), each one located within *ycfX* and *cobB*, respectively. It has been proposed that the expression of two CobB isoforms with independent transcription start sites would allow *S. enterica* to differentially regulate these isoforms (16). In *B. subtilis*, the control of protein deacetylation is more complex since two deacetylases are involved: SrtN, an NAD^+-dependent protein deacetylase (sirtuin) and AcuC, a deacetylase that does not require NAD^+ as co-substrate (9).

The role of the acetylation of prokaryotic proteins is poorly characterized. Extensive acetylation has been recently reported in *S. enterica* and *E. coli* and it has been proposed as a general control/regulatory mechanism (4, 17, 18). Interestingly, all three studies concluded that more than 50% of the acetylome is composed of metabolic enzymes and translation regulators, suggesting that the primary role of Nε-Lys acetylation may be controlling the metabolic fate of carbon and energy sources, and, particularly, the metabolism of fatty acids and nucleotides (2, 10, 19). Some transcriptional regulators (such as RcsB, the response regulator of a complex signal transduction system involved in diverse processes including cell division, and capsule and flagellum synthesis) are modulated by acetylation (5). In *Salmonella*, the expression of protein acetyltransferases and deacetylases is an important mechanism of regulation of central metabolism (17). In *E. coli*, despite N-acetylation of several metabolic proteins has been described, its relevance for metabolic regulation has not been studied (4, 18).

One of the best known metabolic targets of protein acetylation is the AMP-forming acetyl-coenzyme A synthetase (Acs), a ubiquitous enzyme important for the conversion of acetate to its high energy intermediate Acetyl-CoA. The activity of Acs is regulated by acetylation of a lysine residue conserved from bacteria to humans. This is a necessary step for processes such as lipid synthesis and energy generation. In *E. coli*, Acs is considered an anabolic, high affinity pathway for acetate assimilation, serving mainly to scavenge acetate at low concentrations (<10 mM) (20). The activity of Acs is controlled by acetylation, and the deacetylase activity of CobB has been demonstrated on Acs *in vitro* (15) (so far, *in vivo* activity of CobB has only been demonstrated on CheY (21)). Additionally, Acs has been proposed to be responsible for the acetylation of many proteins, even in autoacetylation (21). The coordinated transcriptional and post-translational control of Acs activity reflects the importance of maintaining a balance between acylated and free coenzyme A pools and to avoid AMP accumulation (1, 22). Both gram-positive and gram-negative bacteria control the activity of Acs, although relevant differences exist between the PTM systems in *B. subtilis* and *S. enterica* regarding the acetyltransferases involved, the organization and regulation of their corresponding operons and the mechanisms of their protein deacetylases (1, 9, 23).

It has been proposed that protein acetylation in response to environmental changes might be involved in regulating the activities of enzymes involved in critical metabolic processes (17, 18). However, the regulation of protein acetylation in concert with general mechanisms of metabolic control has not been explored so far. It is well known that the presence of readily metabolizable carbon sources in the culture medium generally results in the repression of genes for the utilization of secondary carbon sources and the activation of pathways involved in the excretion of carbon excess. This phenomenon is known as catabolite repression and is governed by different molecular mechanisms in Gram-positive and negative bacteria (24, 25).

In *E. coli*, *cobB* encodes the main NAD^+-dependent deacetylase (15). Although several putative N-acetyltransferases exist in the genome of *E. coli*, *yfiQ* has a high homology with the gene *pat* from *S. enterica* (unpublished results). The relevance of these genes in metabolic regulation in *E. coli* has not been explored so

far. In this work, we have studied the regulation of the protein acetylation/deacetylation system in *E. coli* along different phases of culture and in the presence of various carbon sources, mainly focussing on the regulation of Acs. Single and double deletion mutants were characterized. This work represents an initial contribution to the elucidation of the role of *cobB* and *yfiQ* gene products in the control of the metabolism of *E. coli*, describing for the first time how their expression is regulated and coordinated by central metabolism regulator CRP and the concentration cAMP.

Materials and methods

Bacterial strains used in this work

E. coli BW25113 strains (wild-type and *cobB* and *yfiQ* deletion mutants) were used throughout this study (**Table 1**). The *E. coli* BW25113 derivatives carry complete gene deletions and belong to the KO-collection (Baba et al., 2006). The *cobB*, *yfiQ* and *cyaA* deletion mutants were obtained from National BioResource Project (National Institute of Genetics, Mishima, Japan); the *rpoS* deletion mutant was obtained from the Coli Genetic Stock Center (Yale University, New Haven, CT). The *cobB/yfiQ* double deletion mutant was constructed using the phage lambda Red recombinase method (26). Primers used for gene knockout are shown in **Appendix**.

In the case of the kanamycin resistant strains, antibiotic resistance cassette was eliminated by site specific recombination using the lambda recombinase encoded in the temperature-sensitive pCP20 plasmid (**Table 1**) (26). Plasmid was curated by giving cultures a passage at 42°C.

Growth media and conditions

The standard minimal media (pH 7.4) contained: 2.6 g/L $(NH_4)_2SO_4$, 1.0 g/L NH_4Cl, 0.5 g/L NaCl, 15.0 g/L $Na_2HPO_4 \cdot 12\ H_2O$, 3.0 g/L KH_2PO_4, 50.0 mg/L $FeCl_3 \cdot 6\ H_2O$, 65.0 mg/L EDTA Na_2, 1.8 mg/L $ZnSO_4 \cdot 7\ H_2O$, 1.8 mg/L $CuSO_4 \cdot 5\ H_2O$, 1.2 mg/L $MnSO_4 \cdot H_2O$, 1.8 mg/L $CoCl_2 \cdot 6\ H_2O$, 2.0 mM $MgSO_4$, 0.2 mM $CaCl_2$, and 0.3 µM thiamine·HCl. As carbon source, 20 mM glucose or 60 mM acetate were used. Aerobic 100 mL batch cultures were grown in 1 L flasks at 37°C on a rotary shaker at 250 rpm. Frozen 20% glycerol stock cultures were used to inoculate precultures in minimal media using glucose or acetate as carbon source. Cultures were inoculated to an optical density (OD_{600nm}) of 0.05 units with exponentially growing precultures.

Analytical procedures

Growth analysis. To estimate cell concentration, cells were resuspended in 0.9% NaCl and absorbance was measured at 600 nm (Pharmacia Biotech NovaspecPlus Spectrophotometer, Amersham Biosciences, GE Healthcare Europe GmbH, Barcelona, Spain). A_{600} values and dry cell weight were correlated for each strain.

HPLC analysis of metabolites. Extracellular metabolites (mainly acetate and glucose) were analyzed by HPLC (Shimadzu Scientific Instruments, Columbia, MD), equipped with differential refractive (Shimadzu Scientific Instruments, Columbia, MD) and UV (Waters, Milford, MA) detectors, using a cation-exchange column (HPX-87H, BioRad Labs, Hercules, CA). The mobile phase was 5 mM H_2SO_4 at 0.4 ml·min^{-1} flow rate and 65ºC.

cAMP analysis. Extracellularly produced cAMP was analysed using an ELISA based kit from Sigma-Aldrich (St. Louis, MO) according to manufacturer's recommendations.

Acetyl-coenzyme A synthetase (Acs) assay. The method used was that established by Lin et al. (Lin *et al.*, 2006). The measurement buffer was 100 mM Tris-HCl (pH 7.8). The reaction mixture contained 5 mM D,L-Malate, 1 mM ATP, 2.5 mM $MgCl_2$, 0.1 mM coenzyme A, 3 mM NAD^+, 2.5 U/mL malate dehydrogenase, 1.25 U/mL citrate synthase and 100 mM sodium acetate. The acetyl-CoA synthetase activity was followed as the increase in NADH absorbance at 340 nm (ε_{NADH}=6.220 M^{-1}cm^{-1}). Enzyme activity unit was defined as the enzyme generating 1 μmol of NADH per min.

Relative Gene Expression: preparation of RNA and RT-PCR

Total RNA was isolated from $3 \cdot 10^8$ cells by Vantage Total RNA purification kit (ORIGENE, MD, USA) according to the manufacturer's recommendations. Additionally, Dnase I digestion of the isolated RNA was performed using the Rnase-Free Dnase Set (QIAGEN Ibérica, Madrid, Spain) to avoid DNA interferences during PCR steps. Isolated RNA purity and concentration were assessed in a NanoDrop® ND-1000 spectrophotometer (NanoDrop

Technologies, Wilmington, DE). RNA quality was evaluated by microfluidic capillary electrophoresis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) using Agilent RNA 6000 Pico kit. Chips were prepared and loaded according to the manufacturer's instructions. Isolated RNA was stored at -80ºC for no longer than three days.

One microgram of high quality RNA (rRNA ratio [23S/16S] ≈ 1.6, RNA integrity number [RIN] > 9.0, and A^{260}/A^{280} ratio > 2.0) was reverse transcribed with TaqMan® Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol and stored at -20ºC prior to use. Briefly, a 25 µL reaction mixture was incubated in a Peltier Thermal Cycler 200 (MJ Research Inc., Boston, MA) for 10 min at 25ºC, 30 min at 48ºC and 5 min at 95ºC.

The primers used in this work (see Additional file 1) were designed using the Primer Express® Software v3.0 (Applied Biosystems, Foster City, CA) and ordered from Applied Biosystems (Cheshire, UK). The *polA*, *dnaA* and *rrsA* genes (encoding DNA polymerase I, transcriptional dual regulator and 16S ribosomal RNA, respectively) were used as internal control for relative quantification.

Quantitative PCR was performed in a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) using Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Briefly, 25 µL reactions mixtures, with 5 ng template cDNA and 7.5 pmol of each primer, were incubated for 2 min at 50ºC, 10 min at 95ºC and 40 PCR cycles (15 s at 95ºC and 1 min at 60ºC). An additional dissociation step (15 s at 95ºC, 30 s at 60ºC and 15 s at 95ºC) was added to assess non-specific amplification. PCRs were run in triplicate. Raw data were transformed into threshold cycle (C_t) values. Relative gene expression for each mutant compared to wild type, was calculated by the comparative C_t Method ($\Delta\Delta C_t$).

Table 1. Strains and plasmids used in this work

Strain	Relevant genotype/phenotype	Source
***Escherichia coli* K12 BW25113**	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR*514 Δ(araBAD)$_{AH33}$ Δ(rhaBAD)$_{LD78}$	Keio Collection
BW25113 derivatives		
Δ*cobB*	[BW25113] *cobB:kan*	Keio Collection
Δ*yfiQ*	[BW25113] *yfiQ:kan*	Keio Collection
Δ*acs*	[BW25113] *acs:kan*	Keio Collection
Δ*rpoS*	[BW25113] *rpoS:kan*	Keio Collection
Δ*cyaA*	[BW25113] *cyaA:kan*	Keio Collection
SCC1	[BW25113] *yfiQ:frt cobB:kan*	This study
***Escherichia coli* K12 MG1655**	Host for pUA66 plasmid propagation	Thermo Scientific
***Escherichia coli* K12 BW25141**	Host for pKD13 plasmid propagation	Yale *E. coli* Genomic Resource Center
***Escherichia coli* K12 BW25113**	Host for pKD46 plasmid propagation	Yale *E. coli* Genomic Resource Center
***Escherichia coli* DH10b**	F^{-} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *recA*1 *endA*1 *araD*139 Δ(*ara, leu*)7697 *gal*U *gal*K λ^{-} *rps*L *nup*G. Host for cloning and plasmid propagation	Invitrogen
Plasmids	**Cloned region**	**Source**
pCP20	Amp^{R}/Cm^{R} plasmid that shows temperature-sensitive replication and thermal induction of FLP synthesis	Yale *E. coli* Genomic Resource Center
pKD46	Amp^{R} plasmid that shows temperature-sensitive replication and thermal induction of FLP synthesis	Yale *E. coli* Genomic Resource Center
pKD13	Kan^{R} /Amp^{R} plasmid	Yale *E. coli* Genomic Resource Center
pUA66	None	Thermo Scientific
pUA*acs*p	-290 to +50 bp of *acs* gene	This study
pUA*nagK*p	-300 to +50 bp of *nagK* gene	This study
pUA*cobB*p	-400 to +151 bp of *cobB* gene	This study
pUA*yfiP*p	-250 to +49 bp of *yfiP* gene	This study
pUA*yfiQ174*p	-75 to +100 bp of *yfiQ* gene	This study
pUA*yfiQ214*p	-115 to +100 bp of *yfiQ* gene	This study
pUA*yfiQ285*p	-186 to +100 bp of *yfiQ* gene	This study
pUA*yfiQ350*p	-250 to +100 bp of *yfiQ* gene	This study
pUA*yfiQ375*p	-377 to +100 bp of *yfiQ* gene	This study
pUA*yfiQ500*p	-400 to +100 bp of *yfiQ* gene	This study

Construction of pUA66-derived promoter probe plasmids

The pUA66 plasmid was used for the analysis of promoter activity. The plasmid was obtained from Thermo Scientific Open Biosystems (Huntsville, AL). For the construction of the five promoter constructs, the corresponding regions were PCR amplified from the genomic DNA of *E. coli* BW25113 strain (**Table 1**) as earlier reported (27). Forward and reverse primers were designed for the introduction of *Xho*I and *Bam*HI sites respectively (**Appendix**). The amplified sequences were cloned into the pUA66 plasmid (**Table 1**), which possesses a strong ribosome binding sequence (RBS), upstream of the *gfpmut2* gene.

For the analysis of the *yfiQ* promoter, the sequences of interest were PCR amplified using primers annealing at specific positions within the sequence. The truncated constructs were selected in order to include/exclude the putative CRP binding sites identified *in silico* using PATSER. Forward and reverse primers were designed to introduce *Xho*I and *Bam*HI sites respectively (**Appendix**) to be used for directed cloning into the pUA66 plasmid, as explained above.

All molecular biology procedures were performed using standard protocols (28).All molecular biology enzymes, plasmid extraction and PCR clean-up kits were purchased from Fermentas (Fermentas Gmbh, St. Leon-Rot, Germany). Genomic DNA was purified using a kit from Sigma-Aldrich (St. Louis, MO). All constructions were verified by PCR, restriction analysis and DNA sequencing.

***In vivo* analysis of promoter activity**

For the promoter activity analysis experiments, cultures were performed as shown before but using 10 mM glucose or 30 mM acetate as carbon source. Samples were taken at given cultivation times and analysed offline.

The EGFP and OD measurements were performed in 96-well plates using a Synergy HT spectrophotometer from Bio-Tek (Winooski, VT). In order to avoid cross talk between contiguous wells, black plates with clear bottom (Nunc, Roskilde, Denmark) were used. Working volume was 100 µL. Eight measurements were

performed and averaged for each sample. Whenever necessary, samples were diluted to meet the linearity range of the reader.

Data analysis was performed as reported by Zaslaver and col. (27). The raw data of EGFP and OD measurements were background subtracted. First, OD data points of a well containing medium with no cells were subtracted from the OD data points of the reporter strains. Next, autofluorescence of cells was corrected. For that aim, the EGFP fluorescence data of cells carrying the promoter-less plasmid (background fluorescence) were averaged and subtracted from the EGFP fluorescence data of the cells carrying the different reporter plasmids constructed (**Table 1**). Each background EGFP fluorescence value was subtracted from EGFP fluorescence value of the reporter strain at the same OD. For background subtraction, control experiments using the promoter-less plasmid pUA66 were performed using all three strain genetic backgrounds (wild type, Δ*cyaA* and Δ*rpoS*) and conditions (0-10 mM cAMP and glucose/acetate as carbon source).

Expression profiles were calculated by dividing background subtracted EGFP fluorescence by corrected OD. For promoter activity the time derivative of the EGFP/OD ratio [d(GFP/OD)/dt] was taken.

In silico analysis of promoters: PATSER and WCONSENSUS

For promoter prediction the WCONSENSUS and PATSER online programs were used (ftp://www.genetics.wustl.edu/pub/stormo/Consensus/) (29). In order to identify the putative CRP binding sites, the DNA regions comprising 400 bp upstream and 200 bp downstream the ATG of each gene were scanned, using PATSER. For these screenings, a Frequency Alignment Matrix was constructed as described before (30) using the WCONSENSUS program. For that aim, the nucleotide sequences of 311 CRP binding sites from *E. coli* promoters annotated in RegulonDB were utilized (31).

Results

Expression of *cobB* and *yfiQ* genes in glucose and acetate cultures in *E. coli* K12

E. coli possesses only one NAD^+-dependent protein deacetylase (sirtuin) gene, *cobB*, which has a high homology with the well described sirtuin from *S. enterica* (*cobB*). Sequence homology with the non-sirtuin protein deacetylase encoded by the *srtN* gene from the Gram positive *B. subtilis* is much lower. The same situation is found when comparing the putative protein acetyltransferase *yfiQ* with their homologues in *S. enterica* and *B. subtilis* (**Table 2**).

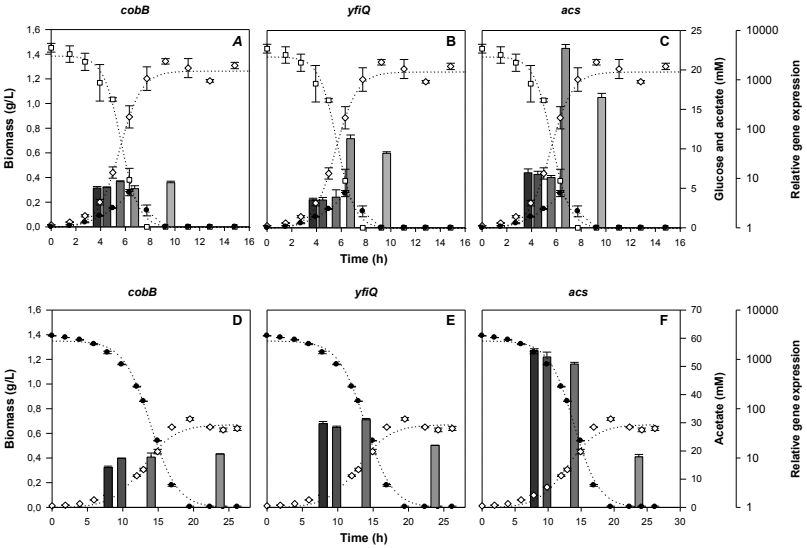


Figure 1. Relative expression of *yfiQ*, *cobB* and *acs* in *E. coli* BW25113 in minimal medium with glucose (**A-C**) or acetate (**D-F**) as the sole carbon source. Bacterial growth, glucose consumption and acetate production in the experiments is shown as reference of the metabolic phase of culture.

In order to determine if these genes are expressed or not and in which phases of culture, *E. coli* BW25113 (wild type) was grown in glucose and acetate minimal media and the expression of *cobB* and *yfiQ* was followed. Since it is known that the activity of acetyl-CoA synthetase is regulated by acetylation, the expression of *acs* was also analysed. In glucose cultures, the relative expression levels of *cobB*

Table 2. Comparison of *acs*, *nagK*, *cobB*, *yfiP* and *yfiQ* orthologs in *E. coli*, *S. enterica* and *B. subtilis*. Homology is referred to the ortholog in *E. coli*.

Function	***Escherichia coli* K12 MG1655**	***Salmonella enterica serovar typhymurium* LT2**		***Bacillus subtilis***	
	Gene/protein length	**Gene/protein length**	**Gene/protein homology (%)**[a]	**Gene/protein length**	**Gene protein homology (%)**[a]
Acetyl-coenzyme A synthetase (AMP-forming)	*Acs* 1959 bp (653 aa)	*acs* 1959 bp (653 aa)	86/95.2	*acsA* 1719 bp (573 aa)	*N.D.*[c]*/34.8%*
N-acetyl-D-glucosamine kinase (murein recycling)	*nagK* 912 bp (303 aa)	*ycfX* 912 bp (303 aa)	74.3/92.7	*N.A.*[a]	*N.A.*[a]
Protein deacetylase	*cobB* (NAD^+-dependent sirtuin) 840 bp (279 aa)	*cobB* (NAD^+-dependent sirtuin) 822 bp (274 aa)	79.5/89	*srtN* (NAD^+-dependent sirtuin)744 bp (248 aa) *acuC* (NAD^+-indep. protein deacetylase) 1164 bp (388 aa)	*N.D.*[c] /27.3% *N.D.*[c] /*N.D.*[c]
Conserved protein (Unknown function)	*yfiP* 699 bp (232 aa)	*yfiP* 681 bp (227 aa)	76.3/85.8	*N.A.*[b]	*N.A.*[b]
Protein acetyl transferase	*yfiQ* 2661 bp (886 aa)	*pat* 2661 bp (886 aa)	81.3/92.1	acuA 633 bp (211 aa)	*N.D.*[c]

[a]Homology is referred to the corresponding gene/protein of *E. coli*.
[a]*N.A.:* Not applicable. Gene ortholog not found in this organism.
[c]*N.D.:* Not detected. No significant homology was found.

remained constant and low during all the culture phases analysed. On the other hand, the expression levels of *yfiQ* and *acs* genes were low during exponential phase and were up-regulated 10 and 100-fold, respectively, upon arrival to stationary phase (**Fig. 1A** to **1C**). When the *E. coli* cells were grown in acetate as the sole carbon source, the expression of *yfiQ* and *acs* was high during all culture phases and similar to that observed in the stationary phase of glucose cultures. The expression level of both genes decreased only upon arrival to stationary phase (**Fig. 1D to 1F**). It should be noted that while the expression of *cobB* was similar for all culture phases, that of *yfiQ* and *acs* genes was co-regulated with the presence/absence of glucose and with acetate production/consumption phases. When the cells were using acetate as carbon source (stationary phase of glucose cultures and exponential phase of acetate cultures), the expression level of *yfiQ* and *acs* was high (**Fig. 1**). Therefore, it seems feasible that both *yfiQ* and *acs* genes may respond to similar transcriptional regulatory signals.

Effect of *cobB* and *yfiQ* genes knock-out on growth in glucose and acetate cultures

In order to get a further insight into the roles of *cobB* and *yfiQ*, the phenotypes of single and double deletion mutants were analysed. In glucose cultures, single or double deletion of *yfiQ* and *cobB* had only slight effects on the growth kinetics. Phenotypic differences arose when cells were grown in acetate minimal cultures. According to the acetate concentration selected, acetate-metabolizing pathways can be distinguished. At high acetate concentration acetate is mainly consumed through the high capacity and low affinity Pta-AckA pathway, while at low concentration it is scavenged by the high affinity Acs pathway (22, 32). The growth of the *yfiQ*- strain in acetate minimal media was similar to that of the wild type, independently of its concentration. However, the behaviour of the *cobB*- strain was dependent on acetate concentration, being unable to grow in 10 mM acetate minimal medium while growth at 60 mM acetate was severely impaired. Interestingly, growth was restored in the double *cobB- yfiQ*- mutant at high and low acetate concentrations. Acs is known to be essential for growth on low acetate concentrations. In order to assess whether the observed phenotypes could be related

to covalent inactivation of this enzyme, an *acs* deletion mutant was grown for comparison. Interestingly, the behaviour of the *acs*⁻ and *cobB*⁻ mutants was similar in all three experimental conditions (**Fig. 2**). Therefore, it can be hypothesized that, similarly to what occurs in *S. enterica*, Acs is acetylated by YfiQ and deacetylated by CobB in *E. coli* and the acetylated protein would be inactive.

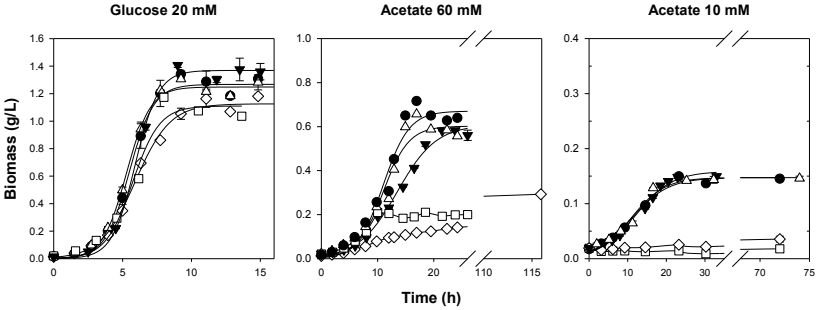


Figure 2. Effect of the deletion of *cobB* and *yfiQ* on the growth of *E. coli* BW25113. The wild type (●) and *acs* (□) deletion mutant (controls) and the *yfiQ* (Δ), *cobB* (◊) and *yfiQ*/*cobB* (▼) deletion mutants were grown in minimal medium with 20 mM glucose (A), 60 mM acetate (B) or 10 mM acetate (C) as the sole carbon source.

The deletion of *cobB* and *yfiQ* had a strong effect on the production and consumption of acetate. In glucose cultures, the *cobB* mutant showed increased acetate accumulation, reaching 10 mM in this mutant (compared to 4.5 mM in the wild type strain and 7 mM in the *yfiQ* mutant) and acetate uptake was also slower (**Fig. 3A to 3C**). Glucose uptake rate was higher in the *cobB*⁻ strain, which is in accordance with a lower glucose to biomass yield (**Table 3**). In acetate cultures, consumption of acetate was severely impaired in the *cobB*⁻ strain, while almost no effect was observed in the *yfiQ* mutant (**Fig. 3D to 3F, Table 3**).

Table 3. Effect of the deletion of *cobB* and *yfiQ* on the growth and metabolism of *E. coli* BW25113 in minimal medium with glucose (20 mM) or acetate (60 mM) as the sole carbon source.

	Glucose (20 mM)			**Acetate (60 mM)**	
	Specific growth rate (h^{-1})	***Glucose consumption*** (mmol·(g·h)$^{-1}$)	***Acetate production*** (mmol·(g·h)$^{-1}$)	***Specific growth rate*** (h^{-1})	***Acetate consumption*** (mmol·(g·h)$^{-1}$)
Wild type	0.73±0.03	-11.87±2.10	3.83±0.97	0.28±0.03	-19.59±0.89
Δ*cobB*	0.68±0.03	-15.22±1.36	8.95±1.34	0.18±0.01	-17.81±2.15
Δ*yfiQ*	0.70±0.01	-11.57±1.23	6.74±0.49	0.24±0.01	-16.42±1.28
Δ*acs*	0.74±0.03	-11.31±1.21	8.60±0.49	0.27±0.01	-29.10±0.51

The activity of acetyl-coenzyme A synthetase (Acs) was quantified in cell extracts of the three strains. Deletion of *yfiQ* meant increased Acs activity in the exponential phase of culture ($p<0.05$), while almost no effect was observed upon deletion of *cobB*. Differences in all other conditions were not statistically significant. No significant changes were observed in *acs* expression (quantified using RT-PCR) in either of the mutant strains.

Table 4. Acetyl-CoA synthetase in *E. coli* BW25113 (wild type), Δ*cobB* and Δ*yfiQ* strains**.** Enzyme activity, and relative gene expression was determined in the exponential and stationary phases of glucose batch cultures. See the text for details.

		Wild type	**Δ*cobB***	**Δ*yfiQ***
Acs Enzyme Activity (mU mg prot^{-1})	Exponential phase	35.47±12.39*	43.72±17.09	63.53±24.69*
	Stationary phase	50.46±17.25	59.70±24.37	72.92±22.18
Relative[a] *acs* expression(ΔΔCt)	Exponential phase	-	-0.8±0.10	-0.40±0.20
	Stationary phase	-	-0.04+0.18	-0.24±0.80

[a] The wild type strain in the same culture point was taken as reference.
* Difference is statistically significant ($p<0.05$; $n=8$).

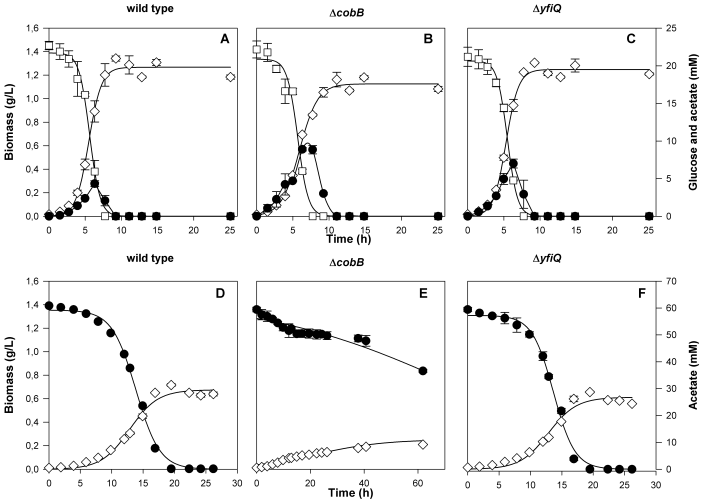


Figure 3. Effect of the deletion of *cobB* and *yfiQ* on the growth and acetate metabolism of *E. coli* BW25113. The wild type strain (control) and the *yfiQ* and *cobB* deletion mutants were grown in minimal medium with glucose (A-C) or acetate (D-F) as the sole carbon source. Bacterial growth (◊), glucose consumption (□) and acetate production/consumption (●) are shown in the different phases of the cultures.

Regulation of *cobB/yfiQ* expression

The *cobB* and *yfiQ* genes are annotated as belonging to two transcriptional units. Both genes are the second ORF (open reading frame) in operons made up of two genes. High degree of homology is also found when comparing these transcriptional units in *S. enterica* and *E. coli*. In both microorganisms, *cobB* is preceded by the gene encoding N-acetyl-glucosamine kinase (involved in murein recycling) and *yfiQ* is preceded by a gene encoding the hypothetical conserved protein *yfiP* of unknown function (31, 33). The degree of homology of these partners with their counterparts in *S. enterica* is similarly high (**Table 2**).

(i) Promoter activity

An operon is classically defined as a group of genes subjected to coordinate expression and regulation from a common promoter region. Nevertheless, there is increasing concern that in many operons, internal promoters are present, allowing for the precise regulation of individual genes in situations when the rest of the genes are not needed (27). In fact, in a recent genome-wide study, internal promoters have been *in silico* predicted in many *E. coli* operons (34). In order to determine if these operons are expressed as polycistronic mRNAs, or if the 5' upstream regions of *cobB* and/or *yfiQ* have transcription promoter activity, the 5' regions of all four genes were cloned into the pUA66 plasmid. This is a promoter-probe plasmid using EGFP as a reporter of expression (27) (**Table 1**). As a control, the 5' upstream region of *acs* was also cloned into pUA66. All five constructs were transformed into *E. coli* BW25113 and the resulting strains were grown in glucose and acetate minimal media. The empty plasmid was used as control.

Promoter activity experiments showed low and steady EGFP levels in the case of the *nagK*p and *yfiP*p constructs which, in addition, were not affected by the culture phase. Negligible promoter activity was observed in the case of the *cobB* 5' upstream region, at least under the conditions used in the assays. Finally, the 5' upstream regions of *yfiQ* and *acs* showed low fluorescence level during the exponential phase, this fluorescent signal recorded being highly enhanced upon entrance to the stationary phase (**Fig. 4**). These findings were further confirmed

using RT-PCR with primers specifically addressed to the ORFs and the intergenic regions (results not shown). In acetate cultures, the expression of *acs* and *yfiQ* was high from the beginning of the culture, indicating that under these conditions the protein acetylation/deacetylation *cobB/yfiQ* system is differentially regulated (results not shown).

Altogether, the RT-PCR and the promoter activity assays suggest that, in fact, *nagK-cobB* form a transcriptional unit which expression is directed from the *nagK* 5' upstream region while, in the case of the *yfiP-yfiQ* transcriptional unit, expression occurs from the 5' regions of both genes (**Fig. 4**).

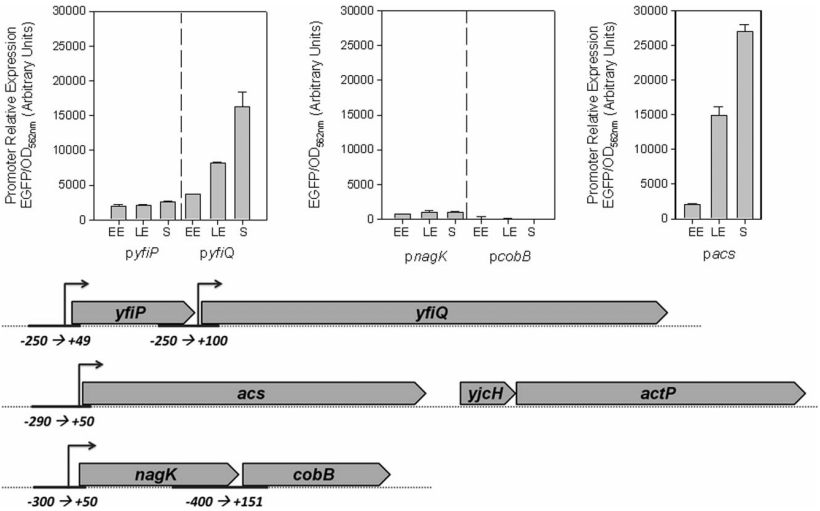


Figure 4. Promoter activity of the 5'-region of the genes *acs, nagK*, *cobB*, *yfiP* and *yfiQ* in the *E. coli* BW2513 strain. In the schematic, the architecture of the *yfiP-yfiQ*, *nagK-cobB* and *acs-yjcH-actP* transcriptional units is shown. Bent arrows indicate the approximate location of each promoter (as suggested by our own results) and its direction of transcription. Black horizontal lines indicate the cloned regions used for the analysis of the 5' regions of the genes under study that were cloned into the pUA66 promoter probe plasmid. Promoter activity was monitored by analysing cellular EGFP levels in three phases of the minimal medium glucose cultures: early (EE) and late (LE) exponential and stationary phase (S). The results shown are the average of four replicate cultures. See the Experimental Procedures section for details.

(ii) Bioinformatic analysis of promoter sequences

Since the expression of *yfiQ* is regulated with culture conditions, the 5' upstream region of this gene was *in silico* analysed using the online bioinformatic program PATSER (Hertz & Stormo, 1999). We found six putative CRP binding sites, each one located at -244, -188, -122, -117, -77, -52 and +22 bp from the ATG. Except for the last site, all of them lie within the *yfiP*-encoding sequence. Moreover, a putative σ^{70}-specific "extended" -10 box was located in this promoter region, at only 22 bp of the gene's ATG (34). The existence of an extended -10 box is typical of prokaryotic promoters lacking a -35 box. Additionally, when carefully comparing the positioning of these putative CRP-binding sites in the *yfiQ* promoter region, the two highest scoring sites were located at -41.5 (score 2.60) and -81.5 (score 2.66) bp from the RNA polymerase binding site. This means that CRP molecules interacting with these sites would bind duplex DNA upstream and at the same face as the RNA polymerase, therefore allowing for optimal interaction and recruiting of RNA polymerase, thus, activating transcription (35–37)

(iii) Effect of cAMP-CRP on the expression of *cobB*, *yfiQ* and *acs*

In order to confirm the role of CRP on the expression of *cobB*, *yfiQ* and *acs* genes, cAMP response experiments were performed. An adenylate cyclase deficient (Δ*cyaA*) strain was used in order to neglect endogenous cAMP synthesis. Growth of *E. coli* is highly affected by *cyaA* deletion, but the normal phenotype was recovered upon supplementation of glucose and acetate minimal media cultures with exogenous cAMP (0-10 mM) (**Fig. 5A**). As expected, the expression of *cobB* was not affected by cAMP in either glucose or acetate cultures, while *yfiQ* and *acs* were highly up-regulated, especially in the case of *acs* (**Fig. 5B-5D**).

To further confirm the activity of which promoter was regulated by cAMP, concentration response experiments were also performed using the Δ*cyaA* mutant strain transformed with the pUA66-derived constructs. As expected, no changes in the activity of the *nagK* promoter region were observed with cAMP, while no significant EGFP expression was detected with the *cobB* gene 5' upstream region construct (results not shown). The activity of the *yfiP* promoter was not affected by

cAMP concentration. On the other hand, the activity of the *yfiQ* and *acs* promoter regions was enhanced in the presence of cAMP. Interestingly, although the effect of cAMP was already observed in the exponential phase of the cultures, the highest changes occurred upon entry into stationary phase, suggesting that CRP is not the only factor necessary for gene expression (**Fig. 6A**). Moreover, the activation ratio in response to cAMP of the *acs* promoter was higher.

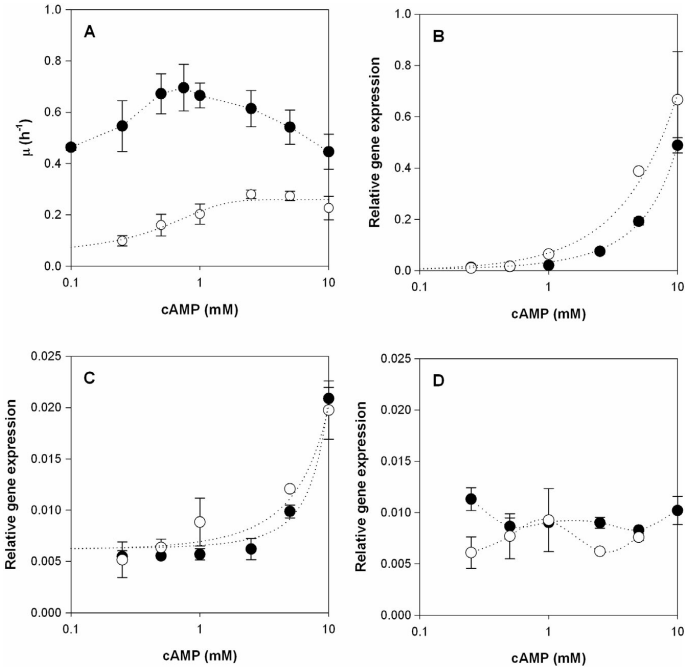


Figure 5. Growth (A) of *E. coli* Δ*cyaA* in response to medium supplementation with cAMP (0 to 10 mM). Relative expression of the *acs*(B), *yfiQ* (C) and, *cobB* (D) genes in response to medium supplementation with cAMP. Experiments were performed in glucose (black symbols) and acetate (white symbols) minimal medium, respectively. See the Experimental Procedures section for details.

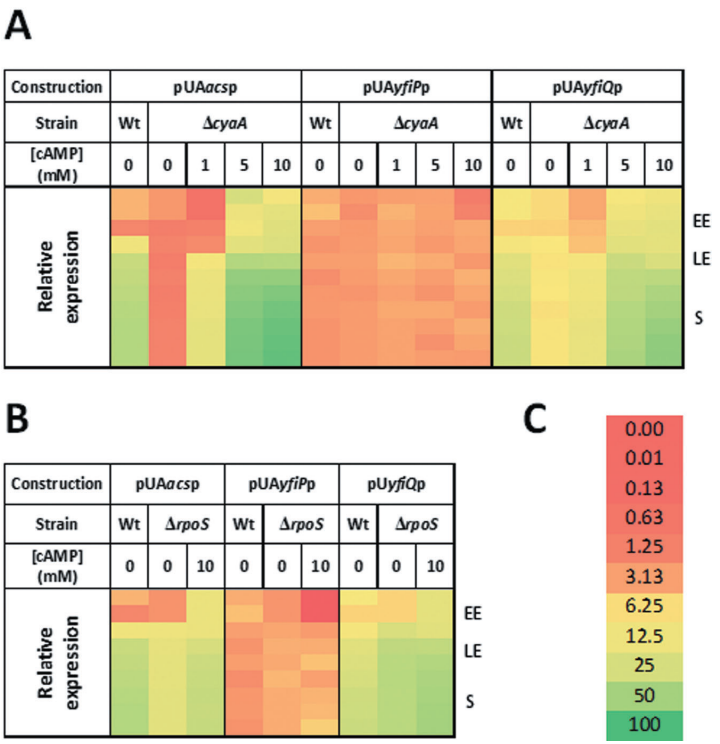


Figure 6. Promoter activity of the 5'-region of the genes *acs*, *yfiP* and *yfiQ* in *E. coli* mutant strains devoid of *cyaA* and *rpoS*. (A) Effect of cAMP on the promoter activity of the 5' regions of the *acs*, *yfiP* and *yfiQ* genes in *E. coli* Δ*cyaA*. (B) Promoter activity of the 5' regions of the *acs*, *yfiP* and *yfiQ* genes in *E. coli* Δ*rpoS*, in the absence and presence of externally added (10 mM) cAMP. For the sake of clarity, relative expression from exponential (top of figures) to stationary phase (bottom of figures) was normalized according to the maximum activity assessed. EE, LE and S stand for early exponential, late exponential and stationary phases of the growth curve. Results were presented in a colour map, according to the scale shown in (C).

In order to confirm the physiological relevance of cAMP levels on the onset of *acs* and *yfiQ* expression, we measured the promoter activity using the methodology proposed by Zaslaver and col. (27). For that aim, EGFP profiles were recorded during cultures at 30 minutes intervals and promoter activity was calculated as the time-derivative of the EGFP *per* OD plot. Both promoters showed maximal activity at 6.5-7 h of culture, coinciding with glucose depletion, growth arrest and the onset of cAMP synthesis (**Fig. 7**).

(iv) The promoter of *yfiQ*

To further confirm the relevance of 5'-upstream sequences for the expression of *yfiQ* and to identify the minimal promoter region, we constructed several subclones of this sequence and cloned them into the pUA66 plasmid. Specific sequences containing subsets of the putative CRP binding sites were PCR amplified and cloned (**Fig. 8A**). Promoter activity assays were performed in a wild type background. The constructs p*yfiQ*214, 285, 350, 375 and 500 exhibited the same transcriptional activity, while the expression level in the case of p*yfiQ*174 was only one third of the maximum (**Fig. 8B**). This confirms that the two highest scoring putative CRP-binding sites (located at -41.5 and -81.5 bp from the RNA Pol binding site, from now on named CRP I and CRP II) are essential for the complete induction of *yfiQ*.

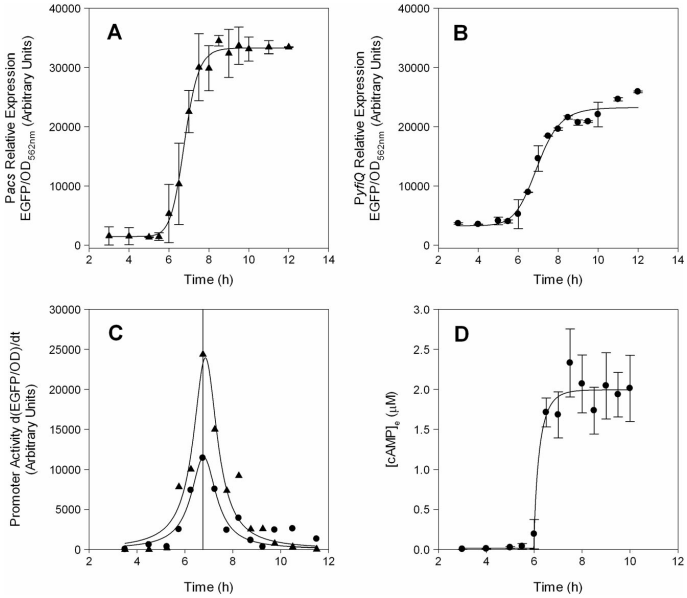


Figure 7. Correlation between extracellular cAMP concentration and relative expression from the 5'-upstream regions of *acs* (A) and (B) *yfiQ* genes. (C) Specific activity of the 5'-upstream regions of *acs* and *yfiQ*. (D) Extracellular concentration of cAMP. Experiments were performed in glucose minimal medium using the *E. coli* BW25113 strain (see Experimental Procedures section). The 5'-upstream regions of *acs* and *yfiQ* genes were cloned in the pUA66 plasmid (Thermo Scientific, USA), upstream of *gfpmut2* reporter gene.

(v) Effect of RpoS on the expression of *cobB*, *yfiQ* and *acs*

Since expression of *yfiQ* was highly up regulated upon entrance into the stationary phase, we hypothesized that its expression might be dependent on RpoS, the stationary phase sigma factor.

Promoter activity assays were performed using a Δ*rpoS* background. Similar expression profiles were observed for the *nagK*, *cobB* and *yfiP* promoter constructs (results not shown). As previously reported, the deletion of *rpoS* had a high effect on the expression of *acs* (38). This is due to indirect effects, since σ^{70} is the main sigma factor for *acs* promoter (39). In the case of *yfiQ*, *rpoS* deletion had no effect, the expression profile being quite similar to that of the wild type strain even in cAMP-supplemented cultures (**Fig. 6B**).

***In silico* comparison of gene, protein and promoter region sequences of protein acetyltransferases in Enterobacteria.**

In order to further understand if the regulation of *yfiQ* expression found in *E. coli* might also be relevant in other bacteria, the sequences of YfiQ protein, *yfiQ* gene, and the corresponding 5' upstream regions of several members of the genus Enterobacteria were analysed and compared (see **Appendix**). All the Enterobacteria used for these comparisons had an *yfiQ* ortholog with high degree of homology to that of *E. coli*. Additionally, all the Enterobacteria used for this comparison also had a *crp* ortholog. In all these bacteria, putative CRP binding sequences were found in the 5'-upstream sequences of *yfiQ* using the PATSER software and the CRP Frequency Alignment Matrix constructed for *E. coli* (**Fig. 8C**). For many of these putative CRP binding sequences, their positions relative to the ATG of *yfiQ* are similar to those found in *E. coli*. Similarity was higher in the case of *Shigella* and *Salmonella* species. In fact, the sequence of *Shigella* is almost identical to that of *E. coli* and, therefore, putative binding sites also have high scores. In *Salmonella*, three high scoring putative CRP consensus sequences are found, two of them at positions relative to the ATG of *yfiQ* similar to those encountered in *E. coli*. Nevertheless, the TSSs of *yfiQ* in these microorganisms have not been determined and, therefore, the real relevance of these sequences cannot be understood. The PATSER software also identifies the CRP binding sites in the sequence of the major promoter of *acs* (*acs*P2).

These sites are located at -69.5 and -112.55 bp of the TSS of this gene (located at -19 bp of the ATG), *i.e.* separated by an integer number of turns of the DNA helix and bound to duplex DNA from the same side as the RNA polymerase. Similar analyses were performed for the *cobB* sequences, although See the Appendix section for a thorough description of the bioinformatic analysis in these Enterobacteria species.

Altogether, this finding supports the idea that the co-regulation of protein acetylation with the central carbon metabolism may be a characteristic feature of microbial metabolism, which is conserved in many Enterobacteria. In fact, analysing similar co-regulation networks in other bacteria with cAMP-independent catabolite repression systems would answer the relevant question if this is a more general strategy followed by microorganisms.

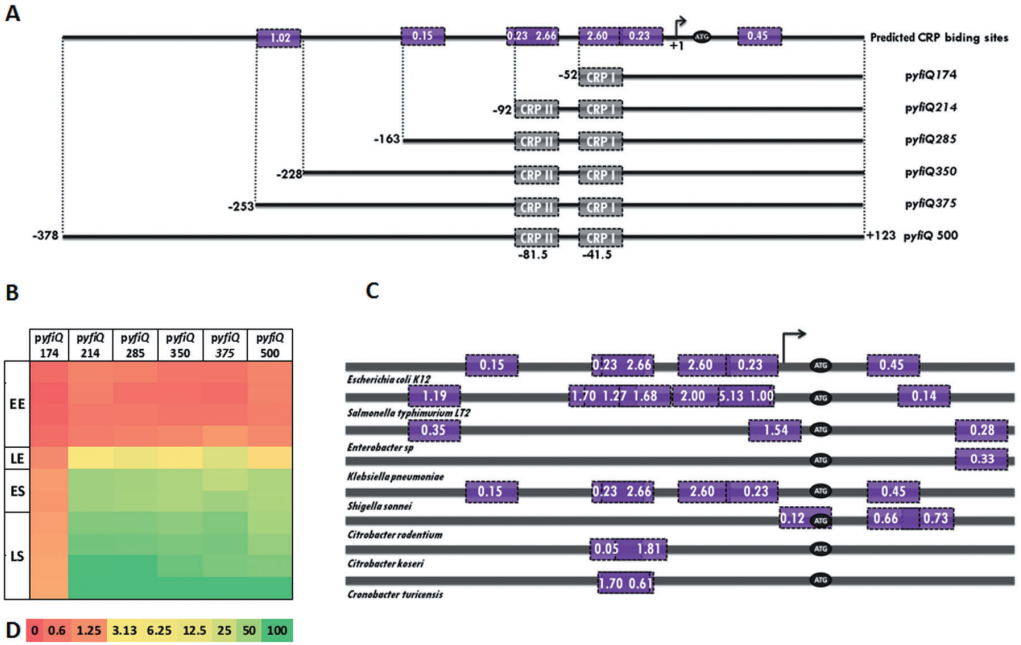


Figure 8. (A) Putative cAMP-CRP-binding sites in the 5'-upstream region of *yfiQ* gene in *E. coli* and other Enterobacteria. The putative cAMP-CRP binding sites were *in silico* predicted using PATSER on line software. (B) Scheme of subclone constructs of the *yfiQ* gene 5'-upstream region. All constructs were selected in order to include/exclude the predicted CRP binding sequences. (C) Promoter activity of the subclones of the 5'-region of the *yfiQ* gene in *E. coli* BW25113 (wild type). For the sake of clarity, relative expression from early exponential (top) to late stationary phase (bottom) was normalized according to the maximum activity assessed. EE, LE, ES and LS stand for early exponential, late exponential, early stationary and late stationary phases of the growth curve. Results were presented in a colour map, according to the scale shown in (D). Experiments were performed in glucose minimal medium, according to the Materials and Methods section.

Discussion

While post-translational modification of eukaryotic proteins has long been regarded as an important mechanism of control/regulation of physiological processes, these mechanisms are less known in bacteria. Among these, protein phosphorylation has focussed most attention. Although not as frequent as in eukaryotes, phosphorylation of proteins is also common in prokaryotes (40). Phosphorylation modulates the activity of the two component signalling systems and of a few metabolic proteins. This is the case of isocitrate dehydrogenase (Icdh), which regulation by a specific phosphatase/kinase expressed along with the glyoxylate pathway enzymes is crucial to distribute fluxes at the isocitrate node (41). Recently, it has been reported that phosphorylation and acetylation control mechanisms can concur, as in the case of CheY, a chemotaxis response regulator which is subjected to both phosphorylation and acetylation (21). The existence of many more cases of not yet characterized multiple simultaneous regulations is likely, given the high abundance and evolutionary conservation of this protein modification in bacteria, therefore complicating the global cellular regulatory network (2, 4, 10, 17, 18). So far, acetylation of prokaryotic proteins has been best described in *S. enterica*. In this work we have analysed the expression in *E. coli* of *cobB* and *yfiQ*, which are homologous to the protein acetylation/deacetylation system of *S. enterica*.

Acs allows the cells to scavenge small concentrations of acetate in the environment, delaying the entry into stationary phase and allowing them to successfully compete during carbon starvation periods (20, 42). In *S. enterica*, acetate metabolism is finely regulated by acetylation of Acs. The protein acetylation/ deacetylation system controls Acs activity in response to carbon metabolism and energy charge state of the cell, since the NAD^+/NADH and acetyl-CoA/CoA ratios modulate the activity of the molecular actors of this system, sirtuins and acetylases, respectively (22).

In this work, we have shown that YfiQ/CobB also modulate the acetate metabolism in E. coli. The protein acetylase YfiQ reduces Acs activity. Furthermore, the role of CobB on acetate metabolism in E. coli is well demonstrated since higher acetate production was observed in glucose cultures (**Fig. 3B**) and growth of the strain

was severely impaired in acetate cultures (**Fig. 2B and 2C**). Moreover normal growth phenotype was recovered in the double *yfiQ/cobB* mutant, suggesting that growth arrest was due to the YfiQ-specific acetylation of one or more proteins. However, despite deacetylation of Acs by CobB has already been observed in vitro using purified proteins (15), little effect was observed on Acs activity upon *cobB* deletion *in vivo* (**Table 4**). This suggests that, either this deacetylation reaction does not occur in vivo under these conditions or the activity of CobB is further regulated by any small molecule. Moreover, it has to be noted that CobB from *S. enterica* is able to deacetylate many central metabolism enzymes (17). Although a similar analysis has not been performed so far in *E. coli*, *in vivo* effects of *cobB* deletion might be due to the combination of multiple effects on different enzyme targets. In addition, the existence of a second deacetylase in *E. coli* has been suggested (8), which would help to explain these results (**Table 4**).

In this work we have demonstrated that the expression of the CobB/YfiQ system of protein PTM is differently regulated in E. coli (**Fig. 9**). The *cobB* gene is expressed jointly with *nagK* from the *nagKp* promoter (**Fig. 4**). The level of activity of this promoter is low as demonstrated both by RT-PCR and promoter probe plasmids (**Fig. 1 and 4**). In addition, the expression levels were similar at different growth phases and using either glucose or acetate as carbon source, which indicates that it is a constitutive promoter. Quite different was the behaviour of the *yfiP-yfiQ* transcriptional unit. In this case, expression occurs from both the *yfiPp* and *yfiQp* promoters. While the former behaves as a constitutive promoter, expression from the latter is highly dependent on the phase of culture and on the carbon source. In fact, the response to cAMP concentration was experimentally demonstrated, and putative CRP-binding sites were *in silico* predicted in the *yfiQp*, revealing that CRP regulates this promoter (**Fig. 5-8**). The minimal CRP-responsive promoter sequence was identified and consists of two proximal CRP binding sites (CRP I and CRP II) located at optimal distance from the -10 box (**Fig. 8**).

Interestingly, this behaviour was quite different to that previously reported in *S. enterica*. In this microorganism, expression of *cobB* and *pat* was maximum in the pre-log and mid-log growth phases, respectively (17), therefore indicating that expression of

cobB is not constitutive and that characteristic phases of *yfiQ* maximum expression are different to those in *E. coli*. In addition, the effects observed on the growth and expression profiles in cells grown in glucose or citrate (a gluconeogenic carbon source) minimal media are not at all comparable to those observed in acetate minimal media in the case of *E. coli*. In *S. enterica*, the Δ*cobB* mutant grows faster than the wild-type strain in glucose but slower than the wild-type in citrate minimal media, whereas the Δ*pat* mutant (with decreased acetylation) has the opposite behaviour (17). Altogether, these facts further indicate that, despite their high phylogenetic relatedness, differences may exist in the role and the regulation of the protein acetylation pathways in these two microorganisms probably having different target proteins.

Although *in vivo* regulation of protein acetyltransferase activity has previously been proposed in *S. enterica* and *B. subtilis* (17, 23), here we describe, for the first time in bacteria, how the protein acetylation/deacetylation pathway is regulated in concert with the metabolic state of cells. Most proteins involved in metabolic processes such as synthesis of proteins and building blocks and carbohydrate metabolism are active during the exponential phase but may be inactive during the stationary phase. Lysine acetylation may play a key role in controlling or affecting protein activities in response to environmental changes (18). The expression of the protein acetyltransferase YfiQ is regulated by cAMP-CRP. Catabolite repression is the best-characterized mechanism of carbon metabolism regulation in enteric bacteria. cAMP synthesis is triggered by low glucose availability and cAMP-CRP-regulated metabolic pathways are rearranged in order to increase metabolic yield, preparing the cells for carbon limitation or starvation, for instance, increasing the uptake of alternative (non-PTS) carbon sources (such as acetate) (35).

The regulation of the *nagK-cobB* and *yfiP-yfiQ* transcriptional units in *S. enterica* has not been described. However, cAMP-dependent catabolite repression in *Salmonella* is similar to that in *E. coli* (35), and the bioinformatic analysis of the 5'-upstream region of the pat gene from *S. enterica* revealed that putative CRP-binding sequences are also present with high degree of confidence (**Fig. 8**). Moreover, the putative CRP-binding sites found are located at distances from the ATG start codon which are compatible

with a regulatory role (35). Interestingly, many of these putative CRP binding sites were also found in the 5' regions of the *yfiQ* gene of many other Enterobacteria species, although those of *Salmonella* and, especially, *Shigella* resembled more the ones found in *E. coli* (both in terms of sequence conservation and position relative to the ATG start codon) (**Appendix**). Altogether, these findings further support the idea that CRP-mediated regulation of protein acetylation may be a conserved mechanism in this family.

Importantly, cAMP-CRP also activates the transcription of *acs* (38), which means that both genes are up-regulated at the same time. The implications of this redundant system for the fine-tuning of Acs activity are clear: acetylation by means of YfiQ would avoid reaching a too high Acs activity under conditions that induce *acs* expression. In *B. subtilis*, Gardner and col. reported that the AcuA acetylase is carefully maintained at a level allowing sufficient AcsA to remain unacetylated. The expression of protein acetylases during growth in low acetate media would have negative effects, since largely acetylated Acs could block the synthesis of acetyl-CoA. Moreover, excessive activity of AMP-forming Acs in a *pat* deficient *S. enterica* strain is deleterious to acetate grown cells, since leads to ATP depletion, AMP accumulation and to an unbalanced energy charge (1, 22). Therefore, protein deacetylases should contribute to maintaining cellular homeostasis.

This co-regulation could be a conserved metabolic feature in the networks of other less related bacteria. A similar scenario is found in *B. subtilis*, where catabolite repression occurs by a cAMP independent pathway. The complex of CcpA (catabolite control protein A, which exerts catabolite repression in Gram positive bacteria) and P-HPr (the serine phosphorylated form of the histidine containing protein H-Pr) trigger the expression of several genes involved in the formation of acetate and acetoin, major extracellular products in the glucose metabolism of this microorganism (43–45). The expression of *acsA* (encoding AMP-forming Acs) and its specific protein acetylation/deacetylation system (encoded by *acuABC* operon) in *B. subtilis* is also co-regulated with the metabolic state of cells in order to maintain a pool of free CoA that can satisfy the requirements of other processes. In fact, these two transcriptional units

(*acsA* and *acuABC*) are divergently transcribed, their -35 sequences being separated by only 20 bp. Similarly to what was observed with *acs* and *yfiQ* in *E. coli*, the expression of these genes was maximal in stationary phase and was repressed by glucose following a CcpA-dependent mechanism (24). It has been hypothesized that the putative post-translational modification AcuABC system might be responsive to unidentified metabolic signals (1).

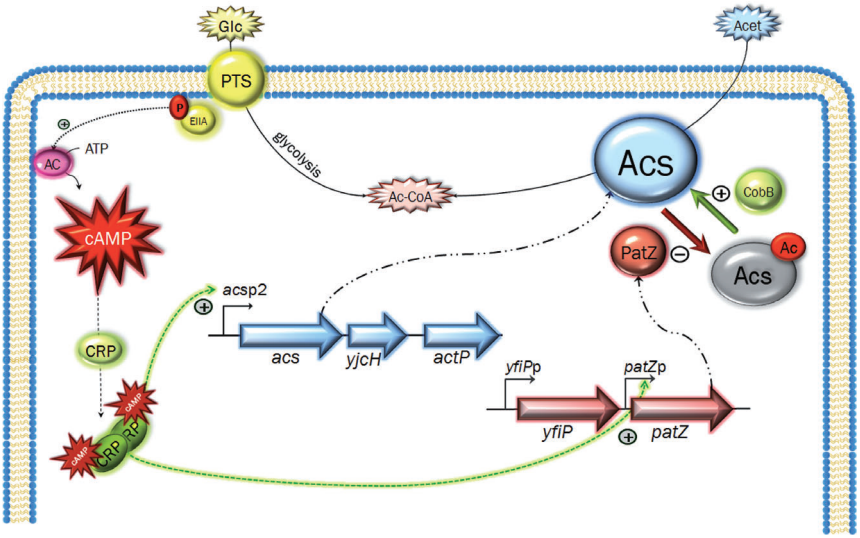


Figure 9. Scheme of the regulation of acetate metabolism in *E. coli*. Upon glucose limitation or entry into stationary phase, cellular cAMP levels rise and alter the transcription of many genes related to catabolism. The expression of *acs* (encoding AcCoA synthetase) and *yfiQ* (encoding a protein acetylase) is enhanced by cAMP. The *in vivo* activity of AcCoA synthetase is controlled by covalent modification. Acetylation of newly synthesized AcCoA synthetase prevents excessive activity that might lead to AMP accumulation and growth arrest. The expression of *cobB* (encoding an NAD^+-dependent protein deacetylase) is low and steady along all phases of culture. See the text for details.

Altogether, these data show that the concurrent regulation of cellular metabolic state and protein acetylation seems to be a prevalent and conserved feature in metabolic networks from both Gram-positive and negative bacteria, being crucial for the adaptation to different environments and, therefore, for successful survival and growth (**Fig. 9**). Further research is needed in order to answer the relevant question: is this a general strategy followed by microorganisms?

It should be noted that these concurring different regulation mechanisms (transcriptional and post-translational) indicate that the multiple regulatory networks of bacteria are interconnected, interdependent and, at least partially, redundant. This degree of redundancy is crucial for the fine-tuning of metabolic pathways. Recent papers reported on the concomitant regulation of CheY and RcsB by phosphorylation and acetylation. This suggests an even higher degree of complexity due to the close interrelation between components of regulatory networks in *E. coli*. The superposition of catabolite repression mechanisms to this system would further complicate the picture of the global regulatory network.

Summarizing, in this chapter, the coordinated regulation of protein acetylation/deacetylation systems and the general metabolism of bacteria is reported for the first time. Undoubtedly, the dissection of molecular-level interactions at the promoters of *yfiQ* and *yfiP*, and the identification of other metabolic targets regulated by the *yfiQ/cobB* system are crucial for a further understanding of the effect of protein acetylation/deacetylation, not only in Enterobacteria such as *S. enterica* and *E. coli*, but also in other microorganisms. Additionally, this report underlines the real importance of this post-translational regulation system for the regulation of bacterial metabolism.

Bibliography

1. Gardner, J. G., Grundy, F. J., Henkin, T. M., and Escalante-Semerena, J. C. (2006) Control of acetyl-coenzyme A synthetase (AcsA) activity by acetylation/deacetylation without NAD+ involvement in *Bacillus subtilis*. *J. Bacteriol.* 188, 5460–5468
2. Thao, S., and Escalante-semerena, J. C. (2011) Analyses of *Salmonella enterica* Pat, a Biochemical and Thermodynamic Multidomain, Multimeric Nε-Lysine Acetyltransferase Involved in Carbon and Energy Metabolism. *MBio* 2, 1–8
3. Kim, S. C., Sprung, R., Chen, Y., Xu, Y., Ball, H., Pei, J., Cheng, T., Kho, Y., Xiao, H., Xiao, L., Grishin, N. V, White, M., Yang, X.-J., and Zhao, Y. (2006) Substrate and Functional Diversity of Lysine Acetylation Revealed by a Proteomics Survey. *Mol Cell* 23, 607–18
4. Zhang, J., Sprung, R., Pei, J., Tan, X., Kim, S., Zhu, H., Liu, C.-F., Grishin, N. V, and Zhao, Y. (2009) Lysine acetylation is a highly abundant and evolutionarily conserved modification in *Escherichia coli*. *Mol Cell Prot* 8, 215–225
5. Thao, S., Chen, C.-S., Zhu, H., and Escalante-Semerena, J. C. (2010) Nε-lysine acetylation of a bacterial transcription factor inhibits Its DNA-binding activity. *PLoS One* 5, e15123
6. Starai, V. J., and Escalante-Semerena, J. C. (2004) Identification of the protein acetyltransferase (Pat) enzyme that acetylates acetyl-CoA synthetase in *Salmonella enterica*. *J Mol Biol* 340, 1005–1012
7. Crosby, H. A., Heiniger, E. K., Harwood, C. S., and Escalante-Semerena, J. C. (2010) Reversible N-ε-lysine acetylation regulates the activity of acyl-CoA synthetases involved in anaerobic benzoate catabolism in *Rhodopseudomonas palustris*. *Mol Microbiol* 76, 874–888
8. Hu, L. I., Lima, B. P., and Wolfe, A. J. (2010) Bacterial protein acetylation: the dawning of a new age. *Mol. Microbiol.* 77, 15–21
9. Gardner, J. G., and Escalante-Semerena, J. C. (2008) Biochemical and mutational analyses of AcuA, the acetyltransferase enzyme that controls the activity of the acetyl coenzyme a synthetase (AcsA) in *Bacillus subtilis*. *J. Bacteriol.* 190, 5132–5136
10. Thao, S., and Escalante-Semerena, J. C. (2011) Control of protein function by reversible Nε-lysine acetylation in bacteria. *Curr Opin Microbiol* 14, 200–204
11. Finkel, T., Deng, C.-X., and Mostoslavsky, R. (2009) Recent progress in the biology and physiology of sirtuins. *Nature* 460, 587–591
12. Lombard, D. B., Alt, F. W., Cheng, H.-L., Bunkenborg, J., Streeper, R. S., Mostoslavsky, R., Kim, J., Yancopoulos, G., Valenzuela, D., Murphy, A., Yang, Y., Chen, Y., Hirschey, M. D., Bronson, R. T., Haigis, M., Guarente, L. P., Farese Jr., R. V, Weissman, S., Verdin, E., and Schwer, B. (2007) Mammalian Sir2 Homolog SIRT3 Regulates Global Mitochondrial Lysine Acetylation. *Mol. Cell. Biol.* 27, 8807–8814
13. Schlicker, C., Gertz, M., Papatheodorou, P., Kachholz, B., Becker, C. F. W., and Steegborn, C. (2008) Substrates and Regulation Mechanisms for the Human Mitochondrial Sirtuins Sirt3 and Sirt5. *J Mol Biol* 382, 790–801
14. Starai, V. J., Celic, I., Cole, R. N., Boeke, J. D., and Escalante-Semerena, J. C. (2002) Sir2-dependent activation of acetyl-CoA synthetase by deacetylation of active lysine. *Science (80-.).* 298, 2390–2392
15. Zhao, K., Chai, X., and Marmorstein, R. (2004) Structure and substrate binding properties of CobB, a Sir2 homolog protein deacetylase from *Escherichia coli*. *J Mol Biol* 337, 731–741
16. Tucker, A. C., and Escalante-Semerena, J. C. (2010) Biologically Active Isoforms of CobB Sirtuin Deacetylase in Salmonella enterica and *Erwinia amylovora*. *J. Bacteriol.* 192, 6200–6208

17. Wang, Q., Zhang, Y., Yang, C., Xiong, H., Lin, Y., Yao, J., Li, H., Xie, L., Zhao, W., Yao, Y., Ning, Z.-B., Zeng, R., Xiong, Y., Guan, K.-L., Zhao, S., and Zhao, G.-P. (2010) Acetylation of metabolic enzymes coordinates carbon source utilization and metabolic flux. *Science* 327, 1004–7
18. Jo, B., Kim, J. A., Moon, J. H., Ryu, S. E., Pan, J. G., Yu, B. J., and Yu, Byung Jo, Jung Ae Kim, Jeong Hee Moon, Seong Eon Ryu, A. J.-G. P. (2008) The Diversity of Lysine-Acetylated Proteins in *Escherichia coli*. *J. Microbiol. Biotechnol.* 18, 1529–1536
19. Norris, K. L. K., Lee, J.-Y. J., and Yao, T.-P. T. (2009) Acetylation Goes Global: The Emergence of Acetylation Biology. *Sci. Signal.* 2, 1–7
20. Starai, V. J., and Escalante-Semerena, J. C. (2004) Acetyl-coenzyme A synthetase (AMP forming). *Cell Mol Life Sci* 61, 2020–2030
21. Li, R., Gu, J., Chen, Y.-Y., Xiao, C.-L., Wang, L.-W., Zhang, Z.-P., Bi, L.-J., Wei, H.-P., Wang, X.-D., Deng, J.-Y., and Zhang, X.-E. (2010) CobB regulates Escherichia coli chemotaxis by deacetylating the response regulator CheY. *Mol. Microbiol.* 76, 1162–1174
22. Chan, C. H., Garrity, J., Crosby, H. A., and Escalante-Semerena, J. C. (2011) In *Salmonella enterica*, the sirtuin-dependent protein acylation/deacylation system (SDPADS) maintains energy homeostasis during growth on low concentrations of acetate. *Mol Microbiol* 80, 168–183
23. Gardner, J. G., and Escalante-Semerena, J. C. (2009) In *Bacillus subtilis*, the sirtuin protein deacetylase, encoded by the *srtN* gene (formerly yhdZ), and functions encoded by the *acuABC* genes control the activity of acetyl coenzyme A synthetase. *J. Bacteriol.* 191, 1749–1755
24. Grundy, F. J., Turinsky, A. J., and Henkin, T. M. (1994) Catabolite regulation of *Bacillus subtilis* acetate and acetoin utilization genes by CcpA. *J. Bacteriol.* 176, 4527–4533
25. Saier, M. H., Chauvaux, S., Deutscher, J., Reizer, J., and Ye, J.-J. (1995) Protein phosphorylation and regulation of carbon metabolism in Gram-negative versus Gram-positive bacteria. *Trends Biochem Sci* 20, 267–271
26. Datsenko, K. A., and Wanner, B. L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* 97, 6640–6645
27. Zaslaver, A., Bren, A., Ronen, M., Itzkovitz, S., Kikoin, I., Shavit, S., Liebermeister, W., Surette, M. G., and Alon, U. (2006) A comprehensive library of fluorescent transcriptional reporters for *Escherichia coli*. *Nat. Methods* 3, 623–628
28. Sambrook, J., and Russell, D. W. (2001) *Molecular cloning: A laboratory manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York)Third Edit.
29. Hertz, G. Z., and Stormo, G. D. (1999) Identifying DNA and protein patterns with statistically significant alignments of multiple sequences. *Bioinformatics* 15, 563–577
30. Huerta, A. M., and Collado-Vides, J. (2003) Sigma70 Promoters in *Escherichia coli*: Specific Transcription in Dense Regions of Overlapping Promoter-like Signals. *J. Mol. Biol.* 333, 261–278
31. Gama-Castro, S., Salgado, H., Peralta-Gil, M., Santos-Zavaleta, A., Muñiz-Rascado, L., Solano-Lira, H., Jimenez-Jacinto, V., Weiss, V., García-Sotelo, J. S., López-Fuentes, A., Porrón-Sotelo, L., Alquicira-Hernández, S., Medina-Rivera, A., Martínez-Flores, I., Alquicira-Hernández, K., Martínez-Adame, R., Bonavides-Martínez, C., Miranda-Ríos, J., Huerta, A. M., Mendoza-Vargas, A., Collado-Torres, L., Taboada, B., Vega-Alvarado, L., Olvera, M., Olvera, L., Grande, R., Morett, E., and Collado-Vides, J. (2011) RegulonDB version 7.0: transcriptional regulation of *Escherichia coli* K-12 integrated within genetic sensory response units (Gensor Units). *Nucleic Acids Res* 39, D98–D105
32. Castaño-Cerezo, S., Pastor, J. M., Renilla, S., Bernal, V., Iborra, J. L., and Cánovas, M. (2009) An insight into the role of phosphotransacetylase (pta) and the acetate/acetyl-CoA node in *Escherichia coli*. *Microb. Cell Fact.* 8, 54

33. Keseler, I. M., Collado-Vides, J., Santos-Zavaleta, A., Peralta-Gil, M., Gama-Castro, S., Muñiz-Rascado, L., Bonavides-Martinez, Cã©., Paley, S., Krummenacker, M., Altman, T., Kaipa, P., Spaulding, A., Pacheco, J., Latendresse, M., Fulcher, C., Sarker, M., Shearer, A. G., Mackie, A., Paulsen, I., Gunsalus, R. P., and Karp, P. D. (2011) EcoCyc: a comprehensive database of *Escherichia coli* biology. *Nucleic Acids Res* 39, D583–D590
34. Mendoza-Vargas, A., Olvera, L., Olvera, M., Grande, R., Vega-Alvarado, L., Taboada, B., Jimenez-Jacinto, V., Salgado, H., Juárez, K., Contreras-Moreira, B., Huerta, A. M., Collado-Vides, J., and Morett, E. (2009) Genome-Wide Identification of Transcription Start Sites, Promoters and Transcription Factor Binding Sites in *E. coli*. *PLoS One* 4, e7526
35. Saier Jr., M. H., Ramseier, T. M., and Reizer, J. (1996) in *Escherichia coli and Salmonella: Molecular and Cellular Biology*, ed Neidhardt FC (American Society for Microbiology Press, Washington, D.C.), pp 1325–1343.
36. Watson, J. D., Baker, T. A., Bell, S. P., Gann, A., Levine, M. K., and Losick, R. (2004) *Molecular Biology of the Gene* (Benjamin Cummings & Cold Spring Harbor Laboratory Press, San Francisco). 5th Ed.
37. Ptashne, M., and Gann, A. (1997) Transcriptional activation by recruitment. *Nature* 386, 569–577
38. Kumari, S., Beatty, C. M., Browning, D. F., Busby, S. J. W., Simel, E. J., Hovel-Miner, G., and Wolfe, A. J. (2000) Regulation of acetyl coenzyme A synthetase in *Escherichia coli*. *J Bacteriol* 182, 4173–4179
39. Kumari, S., Simel, E. J., and Wolfe, A. J. (2000) σ70 is the principal sigma factor responsible for transcription of acs, which encodes acetyl coenzyme A synthetase in *Escherichia coli*. *J Bacteriol* 182, 551–554
40. Macek, B., Gnad, F., Soufi, B., Kumar, C., Olsen, J. V, Mijakovic, I., and Mann, M. (2008) Phosphoproteome Analysis of *E. coli* Reveals Evolutionary Conservation of Bacterial Ser/Thr/Tyr Phosphorylation. *Mol Cell Proteomics* 7, 299–307
41. LaPorte, D. C., and Koshland, D. E. (1982) A protein with kinase and phosphatase activities involved in regulation of tricarboxylic acid cycle. *Nature* 300, 458–460
42. Sclavi, B., Beatty, C. M., Thach, D. S., Fredericks, C. E., Buckle, M., and Wolfe, A. J. (2007) The multiple roles of CRP at the complex acs promoter depend on activation region 2 and IHF. *Sébastien Lemire* 65, 425–440
43. Henkin, T. M. (1996) The role of the CcpA transcriptional regulator in carbon metabolism in *Bacillus subtilis*. *FEMS Microbiol Lett* 135, 9–15
44. Warner, J. B., and Lolkema, J. S. (2003) CcpA-Dependent Carbon Catabolite Repression in Bacteria. *Microbiol. Mol. Biol. Rev.* 67, 475–490
45. Fujita, Y. (2009) Carbon Catabolite Control of the Metabolic Network in *Bacillus subtilis*. *Biosci Biotechnol Biochem* 73, 245–259

Appendix

Supplementary Table 1 Primers used in this work.

Primers used for cloning of the 5' regions upstream the *acs, nagK, cobB, yfiP* and *yfiQ* genes.

Primers	Forward primer (5´ to 3´)	Reverse primer (5´ to 3´)
p*acs*	GTTGTTCTCGAGAACGGTCTGCGATGTTGG	GTTGTTGGATCCGTAACAAATAACCACACTGTG
p*nagK*	GTTGTTCTCGAGTGGCAGGGCTGTTCGG	GTTGTTGGATCCACACGCCAAGCGCAATTT
p*cobB*	GTTGTTCTCGAGCTGCGGCTGTGGTCAGC	GTTGTTGGATCCTTCCTGCCCCTGTCAGT
p*yfiP*	GTTGTTCTCGAGAGCGTAGCGGTAAAGTGC	GTTGTTGGATCCCGCGCGCAATACGCTCG
p*yfiQ*	GTTGTTCTCGAGGTCGATCTTTCCCGGCTT	GTTGTTGGATCCTACGCATCATCAGGTAACC

Primers used for the quantification of gene expression using RT-PCR.

Primers	Forward primer (5´ to 3´)	Reverse primer (5´ to 3´)
polA	GCTGAACGTGCAGCCATTAA	CAATCATCGCCCGTTTGATAA
dnaA	TGGCGAAAGAGCTGACTAACC	ACGGCAGGCATGAAGCA
rrsA	CCTTACGACCAGGGCTACACA	CACTTTATGAGGTCCGCTTGCT
acs	AACACACCATTCCTGCCAACA	TGTTGATACATCGCCTCGTACTG
nagk	GTCATTGGTGGTGGCTTATCG	GGAACACGAGCTACAGGTAAGAGAT
Inter-*nagK-cobB*	GGTGATGCGGGAGGAATG	CGACGCGACAGCATAGCA
cobB	AGTGTGGTTTGGCGAAATGC	GCCATCGACAACGCCATATAA
yfiP	GCCTATCGCCTGCGTGAA	TGGCTACCTCGGCAGTACAAT
Inter-*yfiP-yfiQ*	CATCACAGCAGAACAGTTAGAAAGC	GCTATCGATTTTGGTCGCAGTA
yfiQ	CGGCGGCCCGTAGTG	CATTCCTGCCGTCGTGTTG

Supplementary Data: CRP binding site alignment matrix used for promoter analysis.
For the analysis of CRP-binding sites in the promoters analysed in this work, a CRP-binding sites alignment matrix was constructed specifically for this work. For that aim, the nucleotide sequences of 311 CRP binding sites from *E. coli* promoters annotated in RegulonDB were utilized.

A	67	68	105	138	143	142	101	22	24	16	20	220	46	48	59	120	65	107	17	40	219	49	191	71	102	97	64
C	67	74	59	35	16	16	29	39	11	30	3	39	53	107	71	35	58	53	25	221	10	203	18	51	32	38	50
G	71	72	60	42	25	35	36	28	219	6	244	18	49	71	47	71	88	56	26	8	41	10	52	46	9	25	23
T	86	77	67	76	107	98	125	202	37	239	24	14	143	65	114	65	80	75	223	22	21	29	30	123	148	131	154

This Frequency Alignment Matrix was calculated as previously described (30) using the WCONSENSUS program (ftp://www.genetics.wustl.edu/pub/stormo/Consensus/) (29).

Supplementary Data: Analysis of *crp*, *acs* and *yfiQ* genes and corresponding proteins in Enterobacteria.
In order to verify whether our findings in *E. coli* could be conserved in other Enterobacteria, the sequences of *crp*, *acs* and *yfiQ* genes (and their corresponding proteins) were retrieved and compared. Eight representative Enterobacteria species (*Salmonella typhimurium LT2*, *Enterobacter sp. Cronobacter turiciensis, Pantoae ananatis, Shigella sonnei Klebsiella pneumoniae, Citrobacter kaseri,* and *Citrobacter rodentium*) were analyzed.

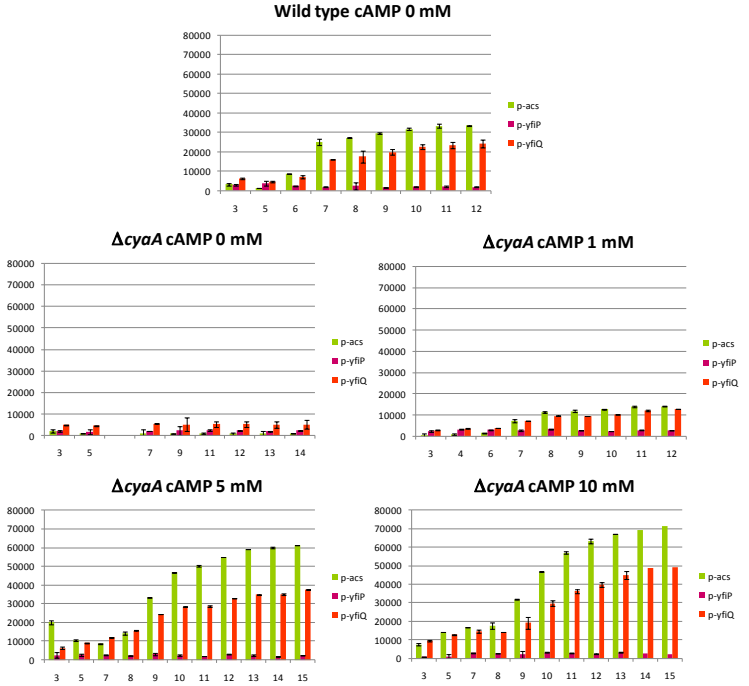
	CRP protein		***crp* gene**		**ACS protein**		***acs* gene**		**YfiQ protein**		***yfiQ* gene**	
	score	%	score	%	score	%	score	%	score	%	score	%
Escherichia coli K12	436	100,0	1170	100,0	1353	100,0	3618	100,0	1814	100,0	4915	100,0
Shigella sonnei	436	100,0	1158	99,0	1351	99,9	3557	98,3	1808	99,7	4771	97,1
Salmonella enterica	435	99,8	749	64,0	1293	95,6	2073	57,3	1686	92,9	2535	51,6
Citrobacter koseri	435	99,8	793	67,8	1297	95,9	2134	59,0	1694	93,4	2645	53,8
Citrobacter rodentium	435	99,8	743	63,5	1285	95,0	2089	57,7	1693	93,3	2663	54,2
Cronobacter turiciensis	435	99,8	715	61,1	1259	93,1	1901	52,5	1523	84,0	1862	37,9
Klebsiella pneumoniae	435	99,8	749	64,0	1261	93,2	1757	48,6	1536	84,7	1921	39,1
Pantoea ananatis	435	99,8	599	51,2	1104	81,6	1570	43,4	1372	75,6	1337	27,2
Enterobacter sp	432	99,1	754	64,4	1274	94,2	2058	56,9	1534	84,6	1815	36,9

The three genes were present in all eight species, although with a widely varying degree of conservation. The sequences of the CRP and Acs proteins were highly similar to those of *E. coli* (% homology over 99% for Acs and over 93% for CRP, except in the case of *Pantoea ananatis* which % homology was exceptionally lower). The degree of conservation of YfiQ protein sequence was lower but still significant, with % homology over 84% (except in the case of *Pantoea ananatis*, with a 75% of homology).

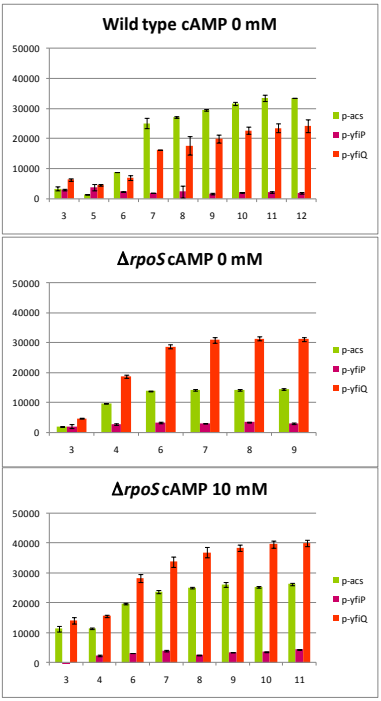
Supplementary Data: Analysis of *yfiQ* promoters in Enterobacteria.
The sequences of the 5'-region of *yfiQ* and its homologue counterparts in all eight Enterobacteria species were analyzed using the online software PATSER (ftp://www.genetics.wustl.edu/pub/stormo/Consensus/) (29). In order to identify the putative CRP binding sites, the DNA regions comprising 400 bp upstream and 200 bp downstream the ATG of each gene were scanned, using the previously shown Position Weight Matrix.
In the following Table, putative CRP binding sites identified for each sequence, location and corresponding scores and Ln(p-value) are shown. See the text of the article and Figure 4B for further discussion.

Microorganism	Location	score	Ln (p-value)	sequence
Escherichia coli K12	-174.5	0.15	-4.06	TACTGCCGAGGTAGCCATCGCACTGTT
	-108.5	0.23	-4.11	CGAGCATTTTACCCGCTTTAAAACACG
	-103.5	2.66	-5.64	ATTTTACCCGCTTTAAAACACGCTATC
	-63.5	2.60	-5.59	GCAACATCTGGGTAGCATCACAGCAGA
	-38.5	0.23	-4.10	GAACAGTTAGAAAGCGTTTAAAATCAT
	+35.5	0.45	-4.23	CACTACTGCGACCAAAATCGATAGCGG
Salmonella typhimurium LT2	-204.5	1.19	-4.67	GAAATCCATGCCGAAGGGCAATATTGT
	-120.5	1.70	-4.99	AGTTTAGGTGAACATTTCACCCGTTTC
	-112.5	1.27	-4.72	TGAACATTTCACCCGTTTCAAAACGCG
	-95.5	1.68	-4.98	TCAAAACGCGCTATCTGGCAGGAAAAA
	-67.5	2.00	-5.19	GCAACATCCGGGAAACGTCACAGCATA
	-42.5	5.13	-7.52	TAAAATAGCGAAAGCGTTTAAAATTAT
	-41.5	1.00	-4.56	AAAATAGCGAAAGCGTTTAAAATTATC
	+51.5	0.14	-4.05	ATCGATCGCGGTGATTGGCGCATCAAT
Enterobacter sp	-204.5	0.35	-4.17	GCGGCTCATGCTGAGGATCAATATTGC
	-24.5	1.54	-4.89	TAAAATCATCAGGTCACTCGTGCTTTC
	+44.5	0.28	-4.13	CCCGATCGTGCGGGATATTTAATGATG
Cronobacter turiciensis	-104.5	1.70	-4.99	TCACTTTGCGCTGTTTAAAACGCGTTA
	-102.5	0.61	-4.32	ACTTTGCGCTGTTTAAAACGCGTTATC
Pantoae ananatis	+41.5	0.04	-4.00	TGGAAGCGCTGTTAAGACCAAAATCAA
Shigella sonnei	-174.5	0.15	-4.06	TACTGCCGAGGTAGCCATCGCACTGTT
	-108.5	0.23	-4.11	CGAGCATTTTACCCGCTTTAAAACACG
	-103.5	2.66	-5.64	ATTTTACCCGCTTTAAAACACGCTATC
	-63.5	2.60	-5.59	GCAACATCTGGGTAGCATCACAGCAGA
	-38.5	0.23	-4.10	GAACAGTTAGAAAGCGTTTAAAATCAT
	+35.5	0.45	-4.23	CACTACTGCGACCAAAATCGATAGCGG
Klebsiella pneumoniae	+44.5	0.33	-4.16	CCGCAGCGCGCTGGCTATCTCATGATG
Citrobacter koseri	-108.5	0.05	-4.00	ACTTCTCACGCTTTAAAACGCGCTATC
	-96.5	1.81	-5.06	TTAAAACGCGCTATCTGGCAGGAAAAA
Citrobacter rodentium	-10.5	0.12	-4.04	CACTTCTGTGTAAGGGAAACCGATatg
	+35.5	0.66	-4.36	CGCTACTGCGACCAAAATCGATAGCCG
	+54.5	0.73	-4.40	GATAGCCGTGATCGGCGCATCGATGAA

Supplementary Figure 1. Promoter activity of the 5'-region of the genes *acs*, *yfiP* and *yfiQ* in *E. coli* mutant strains devoid of *cyaA*. Effect of cAMP on the promoter activity of the 5' regions of the *acs*, *yfiP* and *yfiQ* genes in *E. coli* Δ*cyaA*, in the absence and presence of externally added (10 mM) cAMP. The full data used for the construction of Figure 5A is here shown. *E. coli* BW25113 (wild type) and Δ*cyaA* strains were transformed with the pUA66 (control), pUApacs, pUAyfiP and pUApyfiQ plasmids. EGFP fluorescence is expressed in arbitrary units and corrected from autofluorescence using the promoterless construct. EGFP fluorescence was off-line measured and expressed as explained in the Experimental Procedures section.



Supplementary Figure 2. Promoter activity of the 5'-region of the *acs*, *yfiP* and *yfiQ* genes in *E. coli* wild type and the *rpoS* deficient mutant strains. The full data used for the construction of Figure 5B is here shown. *E. coli* BW25113 (wild type) and Δ*rpoS* strains were transformed with the pUA66 (control), pUApacs, pUAyfiP and pUApyfiQ plasmids. EGFP fluorescence is expressed in arbitrary units and corrected from autofluorescence using the promoterless construct. EGFP fluorescence was off-line measured and expressed as explained in the Experimental Procedures section. Experiments were performed in minimal medium with 20 mM glucose as the sole carbon source. The interplay of cAMP-CRP activation and *rpoS* deletion was investigated by analyzing the effect of the addition of 10 mM cAMP on the promoter activity of the pUA66-derived constructs.



Chapter 5

Characterizing protein acetylation and acetate overflow in *Escherichia coli* BL21 and K12

The results presented in this chapter are part of a manuscript under preparation

Abstract

One of the best-known differences between *E. coli* K12 and BL21 strains is their different acetate production. Many studies have tried to decipher the mechanisms that cause this difference. Besides, it has been demonstrated that acetate metabolism is regulated by reversible protein lysine acetylation in many organisms. Based on these evidences, the aim of this study was to understand the role of lysine acetylation in the different acetate metabolism shown in both in K and B *E. coli* strains.

Higher protein lysine acetylation was observed in K12 compared with BL21 strains, especially in glucose stationary phase and acetate cultures. The lower protein acetylation and the higher acetyl-CoA synthetase activity in BL21 might contribute to the faster growth rate in acetate cultures. Moreover, the expression of protein acetyltransferase (*patZ*) and acetyl-CoA synthetase (*acs*) genes showed slighter catabolite repression in BL21 than in K12. This fact indicates that in BL21 the acetyl-CoA synthetase activity is partially inactivated by acetylation. The phenotypic changes of the Δ*cobB* and Δ*patZ* BL21 knockout strains were more severely affected than the K12 mutants. Acetate production was enhanced in the *cobB* mutant, while no growth was observed in low acetate cultures. On the other hand, the Δ*patZ* mutant exhibited no acetate production in glucose cultures and showed higher growth rate in acetate cultures.

Acetate overflow is tightly regulated by lysine acetylation in BL21 strain. Despite this, the small differences in *patZ* and *cobB* gene expression between both B and K strains are not sufficient for explaining the role of this post-translational modification in the different acetate overflow observed. Key metabolites involved in the acetylation/deacetylation reactions will be essential to complete the picture of this differential post-translational regulation.

Introduction

Escherichia coli is one of the best-known microbial cell factories used in Biotechnology. Although the K12 strain is the best characterized from the physiological point of view, several other strains are known which possess specific features which make them better candidates for the development of industrial processes. For instance, the BL21 strain is used in the industry and the academia for heterologous production of proteins that do not require post-translational processing (1).

The BL21 strain differs from the K12 strain in several aspects. Genome sequencing has revealed the reasons for many of these differences (2, 3). DNA mobile elements (IS) are scattered at different locations in the genomes of both strains, interrupting in occasions the sequences of specific ORFs. The BL21 strain lacks certain genes, *e.g.*, the flagella cluster, due to an IS1-associated 41-kbp deletion that is characteristic of the B lineage. One of its most prevalent characteristics, the absence of two proteases (*lon*, *ompT*), is also due to insertion of IS elements (2). Another well-known characteristic of the BL21 strain is the low acetate production during growth on high glucose concentration cultures, which cannot be directly related to the observed genomic differences. Acetate accumulation is determinant for industrial applications of microorganisms, especially for the production of recombinant proteins, since it inhibits growth and decreases protein production yield (4, 5). Moreover, inefficient glucose metabolization limits overall bioprocess yields (6, 7).

Many efforts have been devoted to reveal why the acetate metabolism in *E. coli* BL21 is different from the K12 strain (6–13). Two metabolic pathways could be the responsible for the differences in acetate overflow between the two strains. The first one is the superpathway of acetate producing and consuming routes. There are two major pathways for acetate production in *E. coli*: the irreversible oxidative decarboxylation of pyruvate catalysed by pyruvate oxidase (PoxB) and the reversible phosphotransacetylase-acetate kinase pathway (Pta-AckA) (14). On the other hand, there are two pathways for acetate assimilation that differ in their affinity towards the substrate. *E. coli* uses the Pta-AckA pathway when the concentration of acetate in the

medium is high. At low acetate concentration, this bacterium uses acetyl-CoA synthetase, which has a high affinity towards its substrate (K_m=200 μM for acetate) (14). Moreover, acetate metabolization involves the glyoxylate shunt and the TCA cycle (15–18). The glyoxylate shunt is an anabolic pathway which is essential for growth on acetate as the sole carbon source, while the major function of the TCA cycle is the production of metabolic energy (18). These two pathways compete for a common metabolite, isocitrate, which is substrate of both isocitrate dehydrogenase (TCA cycle) and isocitrate lyase (which catalyses the first step of the glyoxylate shunt). The fluxes through the isocitrate node are tightly regulated by reversible phosphorylation of isocitrate dehydrogenase (18–20).

It has been claimed that BL21 acetate metabolism might be the result of several overlapping effects including different levels of activity of major acetate pathways and also general transcriptional regulators (21). One of the most prevalent reasons for lower acetate overflow in the BL21 is the higher expression of *acs* during growth (11, 22, 23). This might be attributed to a less tight *acs* transcriptional regulation. Actually, BL21 strains can co-consume glucose and acetate but they are unable to do it with other combinations of carbon sources, such as glucose and lactose (24). Additionally, other authors have shown that the levels of cAMP in BL21 cultures are higher than in K12, enabling higher *acs* gene expression due to a more active CRP (22). Simultaneous acetate production and consumption has been also demonstrated in glucose-limited chemostat cultures at low dilution rates in the K12 strain, caused by the high *acs* expression due to the higher cAMP concentration (18, 25). Moreover, it has been demonstrated that the higher flux though the glyoxylate shunt in the B strain contributes to lower acetate production, but recent studies have shown that transcription levels of the glyoxylate operon (*aceBAK*) are lower in the B strains than in the K (22).

It the past years, the knowledge about post-translational modifications in bacteria has increased (26). Several reports have evidenced that lysine acetylation of proteins contributes to the regulation of bacterial metabolism (27, 28). The activity of acetyl-CoA synthetase (Acs) is regulated by acetylation of a specific lysine residue in the

vicinity of the substrate binding pocket in *Salmonella enterica*, *Bacillus subtilis* and *Rhodopseudomonas palustris* (29–31). The best-known protein acetyltransferases in prokaryotes belong to the Gcn5-like acetyltransferase family (in *E. coli*, PatZ), but there exists more putative N-acetyltransferases annotated in *E. coli* genome (31–35). Two different types of deacetylases can be found in bacteria: the NAD^+-dependent deacetylases (also known as sirtuins), and the NAD^+-independent deacetylases. Almost all microorganisms own a gene encoding for a sirtuin in their genome (named normally as *cobB*), while the NAD^+-independent deacetylases have been described only in few organisms, such us *Bacillus subtilis* and *Mycobacterium smegmatis* (29, 30, 36–38).

It has been demonstrated that lysine acetylation regulates acetate metabolism in *E. coli* K12 by the protein acetyltransferase PatZ and the sirtuin CobB (28, 39). Last year it was described that the deletion of the main acetyltransferase *patZ* in *E. coli* did not alter protein acetylation levels in this strain. However, chemical acetylation by acetyl-phosphate was reported to be the main mechanism of protein acetylation in *E. coli*. The same authors described that protein acetylation was more abundant in K12 strain than in BL21 because of a higher acetate production in the K12 strain (39), even though its contribution to metabolism has not been described.

The main objective of this study is to shed light on the role of lysine acetylation in the metabolic differences observed between both *E. coli* K and B strains, especially, trying to unravel how they contribute to the acetate metabolism. The occurrence of protein acetylation in *E. coli* K and B strains will be analysed under different environmental conditions and responses to the deletion of the acetyltransferase *patZ* and the NAD^+-dependent deacetylase *cobB* will be described.

Material and methods

Strains and culture conditions

The *E. coli* K12 (BW25113) and BL21 (DE3) strains and their knockout derivatives used in this study are listed in **Suppl. Table 1** (Appendix). The K12 strains were supplied by the KO-collection (Keio University, Japan) (40). *E. coli* BL21 (DE3) was purchased from Sigma Aldrich (St. Louis, MO) and its knockout strains were constructed using the phage lambda Red recombinase method (41). The primers used for the construction of mutants are listed in **Suppl. Table 2 (Appendix).**

For the characterization experiments bacteria were grown using standard M9 minimal medium (pH 7.4) containing: 2.6 g l^{-1} $(NH4)_2$ SO_4, 1.0 g l^{-1} NH_4Cl, 0.5 g l^{-1} NaCl, 15.0 g l^{-1} $Na_2HPO_4 \cdot 12$ H_2O, 3.0 g l^{-1} KH_2PO_4, 50.0 mg l^{-1} $FeCl_3 \cdot 6$ H_2O, 65.0 mg l^{-1} EDTA Na_2, 1.8 mg l^{-1} $ZnSO_4 \cdot 7$ H_2O, 1.8 mg l^{-1} $CuSO_4 \cdot 5$ H_2O, 1.2 mg l^{-1} $MnSO_4 \cdot H_2O$, 1.8 mg l^{-1} $CoCl_2 \cdot 6$ H_2O, 2.0 mM $MgSO_4$, 0.2 mM $CaCl_2$, and 0.3 mM thiamine·HCl. As carbon source, 20 mM glucose, or 10-60 mM acetate were used. Aerobic 100 ml batch cultures were grown in 1 l flasks at 37°C on a rotary shaker at 250 r.p.m. Cultures were inoculated to an initial optical density (OD600) of 0.05 units with exponentially growing precultures.

Enzyme activities

Approximately 20 mg of *E. coli cells* were harvested from each condition (glucose exponential phase, glucose stationary phase or acetate exponential phase) and centrifuged for 10 minutes at 4ºC (7000xg). Supernatant was removed and cells were resuspended in 50 mM phosphate buffer pH 7.5. For lysis, cells were sonicated on ice (3 cycles, 20 s each), with a probe of 3 mm diameter of Vibra Cell VC 375 ultrasonic processor (Sonics Materials, Danbury, CT). Protein extract was clarified by centrifugation for 15 min at 20.000xg at 4ºC to remove cell debris. Supernatant was collected after centrifugation and kept at -80ºC until use. Protein concentration was determined by Bicinchoninic acid method (BCA) using a commercial kit (Pierce BCA Kit Pierce, Thermo Fisher Scientific, Rockford, IL).

The enzyme activity assays were optimized for the conditions and media. All measurements were carried out in a microplate spectrophotometer Synergy HT (BioTek, Winooski, VT). Enzyme activity was defined as μmol of substrate consumed per minute and mg of protein (U/ mg). All enzyme activities were measured at 37°C.

Isocitrate dehydrogenase (Icdh) The method was described by Aoshima et al. (42). The measurement buffer was 65 mM potassium phosphate (pH 7.5). The reaction components were 5 mM $MgCl_2$, 2 mM $NADP^+$ and 2.5 mM D, L-isocitrate. The enzyme activity was followed by the increase in NADPH absorbance at 340 nm (ε_{NADPH} = 6.220 $M^{-1}cm^{-1}$). One unit of enzyme activity was that required for the generation of 1 μmol of NADPH per min.

Isocitrate lyase (AceA) The assay was that described by Aoshima et al. (42) using the same buffer as above. The reaction mixture was composed of 5 mM $MgCl_2$, 20 mM phenylhydrazine and 5 mM D, L-sodium isocitrate. The enzyme activity was followed by the increase in absorbance at 324 nm due to the reaction of the glyoxylate produced with phenylhydrazine ($\varepsilon_{glyoxylate\text{-}phenylhydrazone}$ = 16,8 $M^{-1}cm^{-1}$). One unit of enzyme activity was taken as that needed to generate 1 μmol of adduct per min.

Acetyl-CoA synthetase (Acs) The method used was that established by Lin et al. (43). The measurement buffer was 100 mM Tris-HCl (pH 7.8). The reaction mixture contained 5 mM D, L-Malate, 1 mM ATP, 2.5 mM $MgCl_2$, 0.1 mM coenzyme A, 3 mM NAD^+, 2.5 U/mL malate dehydrogenase, 1.25 U/mL citrate synthase and 100 mM sodium acetate. The acetyl-CoA synthetase activity was followed as the increase in NADH absorbance at 340 nm (ε_{NADH} = 6.220 $M^{-1}cm^{-1}$). Enzyme activity unit was defined as the enzyme generating 1 μmol of NADH per min.

Phosphotransacetylase (Pta) The assay was carried out as in Peng et al. (44). The measurement buffer was 250 mM Tris-HCl, pH 7.8. The reaction components were 1 mM $MgCl_2$, 10 mM D, L-malic acid, 3 mM NAD^+, 0.5 mM coenzyme A, 2.5 U/mL malate dehydrogenase, 1.25 U/mL citrate synthase and 10 mM acetyl-phosphate. The enzyme activity was followed as the increase in NADH absorbance at 340 nm (ε_{NADH} = 6.220 $M^{-1}cm^{-1}$), one unit being taken as the enzyme required for the generation of 1

μmol of NADH per min.

Acetate kinase (AckA) The assay was carried as described by Bergmeyer and col. with minor modifications (45). The measurement buffer was 250 mM Tris-HCl, pH 7.8. The reaction components were 10 mM $MgCl_2$, 2 mM ADP, 4 mM of $NADP^+$, 10 mM glucose, 20 U/mL hexokinase, 10 U/mL glucose 6-phosphate dehydrogenase, and 10 mM acetyl-phosphate. The enzyme activity was followed as the increase in NADPH absorbance at 340 nm (ε_{NADH} = 6.220 $M^{-1}cm^{-1}$), one unit being taken as the enzyme required for the generation of 1 μmol of NADPH per min.

Relative gene expression

Total RNA was isolated from $3x10^8$ cells by Vantage Total RNA purification kit (ORIGENE, MD, USA) according to the manufacturer's recommendations. Additionally, DNase I digestion of the isolated RNA was performed using the RNase-Free DNase Set (QIAGEN Ibérica, Madrid, Spain) to avoid DNA interferences during PCR steps. Isolated RNA purity and concentration were assessed in a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA quality was evaluated by microfluidic capillary electrophoresis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) using Agilent RNA 6000 Pico kit. Chips were prepared and loaded according to the manufacturer's instructions. Isolated RNA was stored at -80°C for no longer than 3 days.

One microgram of high quality RNA [rRNA ratio (23S/ 16S) a 1.6, RNA integrity number (RIN) > 9.0, and A^{260}/A^{280} ratio > 2.0) was reverse-transcribed with TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol and stored at -20°C before use. Briefly, a 25 ml reaction mixture was incubated in a Peltier Thermal Cycler 200 (MJ Research Inc., Boston, MA) for 10 min at 25°C, 30 min at 48°C and 5 min at 95°C.

The primers used in this work (**Suppl. Table 3, Appendix**) were designed using the Primer Express Software v3.0 (Applied Biosystems, Foster City, CA) and ordered from Sigma Aldrich (St.Louis, MO). The *polA*, *dnaA* and *rrsA* genes (encoding DNA polymerase I, transcriptional dual regulator and 16S ribosomal RNA,

respectively) were used as internal control for relative quantification.

Quantitative PCR was performed in a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Briefly, 25 μl reactions mixtures, with 5 ng template cDNA and 7.5 pmol of each primer, were incubated for 2 min at 50°C, 10 min at 95°C and 40 PCR cycles (15 s at 95°C and 1 min at 60°C). An additional dissociation step (15 s at 95°C, 30 s at 60°C and 15 s at 95°C) was added to assess non-specific amplification. PCRs were run in triplicate. Raw data were transformed into threshold cycle (C_t) values. Relative gene expression for each condition compared with the control was calculated by the comparative C_t method ($\Delta\Delta C_t$).

Analytical methods

Growth analysis. To estimate cell concentration, cells were resuspended in 0.9% NaCl and absorbance was measured at 600 nm (Pharmacia Biotech NovaspecPlus Spectrophotometer, Amersham Biosciences, GE Healthcare Europe GmbH, Barcelona, Spain). A^{600} values and dry cell weight were correlated.

HPLC analysis of metabolites. Extracellular metabolites (mainly acetate and glucose) were analysed by HPLC (Shimadzu Scientific Instruments, Columbia, MD), equipped with differential refractive (Shimadzu Scientific Instruments, Columbia, MD) and UV (Waters, Milford, MA) detectors, using a cation-exchange column (HPX- 87H, Bio-Rad Labs, Hercules, CA). The mobile phase was 15 mM H_2SO_4 at 0.5 ml min^{-1} flow rate and 65°C.

Western blotting

20 μg of cell crude protein extract obtained as described above were resolved by SDS-PAGE in 10% acrylamide gels. Proteins were blotted onto PVDF membranes, which were proofed against a rabbit anti acetyl-lysine antibody (InmuneChem, Burnaby, Canada) according to the manufacturer instructions. A goat anti-rabbit antibody conjugated with HPR (Santa Cruz Biotechnology, Heidelberg, Germany) was used for development.

Results

Differences in the acetate metabolism in *E. coli* K and B strains

Phenotypic changes

Several differences between the B and K strains were observed during glucose and acetate batch cultures. The K strain grows faster in glucose cultures compared with the BL21, while the growth of the B strain was faster on acetate (**Tables 1** and **2**). The maximum biomass yield of the K strain was higher in both culture conditions (**Tables 1** and **2**). Acetate production in glucose cultures was faster in the K strain, reaching a higher concentration (**Table 1**).

Table 1. Physiological parameters of the B and K *E. coli* strains and their knockout mutants in glucose minimal medium batch cultures

	Specific growth rate ($\mu=h^{-1}$)	Glucose consumption rate [mmol·(g·h)$^{-1}$]	Acetate production rate [mmol·(g·h)$^{-1}$]	Biomass yield ($g \cdot g^{-1}$)
	BL21			
Wild type	0.67±0.01	-12.69±0.38	2.37±0.03	0.50±0.01
Δ*cobB*	0.74±0.01	-15.12±0.89	13.85±0.80	0.39±0.02
Δ*patZ*	0.73±0.02	-11.26±0.03	N.D.	0.50±0.01
	K12			
Wild type	0.73±0.03	-11.87±2.10	3.83±0.97	0.52±0.03
Δ*cobB*	0.68±0.03	-15.22±1.36	8.95±1.34	0.52±0.02
Δ*patZ*	0.70±0.01	-11.57±1.23	6.74±0.49	0.56±0.01

Lysine Acetylation differences

The global pattern of protein acetylation of both strains was compared under different growth conditions using western blotting with anti-acetyl-lysine antibodies (**Fig. 1**). This post-translational modification was more abundant in the K12 strain compared that of the BL21. The levels of acetylation were especially higher in those conditions where the cells were consuming acetate (glucose stationary phase and acetate cultures).

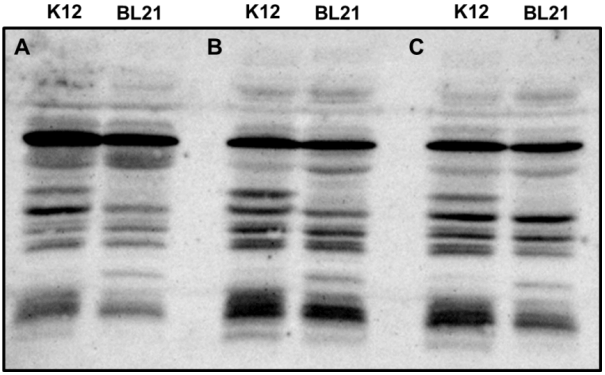


Fig. 1. Western blots showing protein lysine acetylation in protein crude cell extracts in *E. coli* K12 and BL21 strains. Glucose batch exponential phase (**A**), glucose batch stationary phase (**B**) and acetate batch exponential phase (**C**).

Table 2. Specific growth rate of the B21 and K12 *E. coli* strains and their knockout mutants in acetate (60 and 10 mM) minimal medium batch cultures. *N.D.*: not detected.

	BL21	**K12**	**BL21**	**K12**
	Specific growth rate (h^{-1})		*Biomass yield ($g\ g^{-1}$)*	
	60 mM acetate			
Wild type	0.32±0.01	0.28±0.03	0.107±0.004	0.124±0.002
Δ*cobB*	0.23±0.02	0.18±0.01	0.041±0.005	0.043±0.004
Δ*patZ*	0.33±0.01	0.24±0.01	0.096±0.001	0.113±0.001
	10 mM acetate			
Wild type	0.15±0.01	0.10±0.01	0.114±0.001	0.152±0.003
Δ*cobB*	N.D.	0.02±0.01	N.D.	0.001±0.001
Δ*patZ*	0.19±0.01	0.11±0.01	0.112±0.001	0.149±0.002

Gene expression differences

In order to assess if the differences in protein acetylation were due to differences in the expression of the genes involved in this post-translational modification, the expression of acetyl-CoA synthetase (*acs*), the protein acetyltransferase (*patZ*) and the sirtuin type deacetylase (*cobB*) was measured in the B and K strains during growth on glucose and acetate.

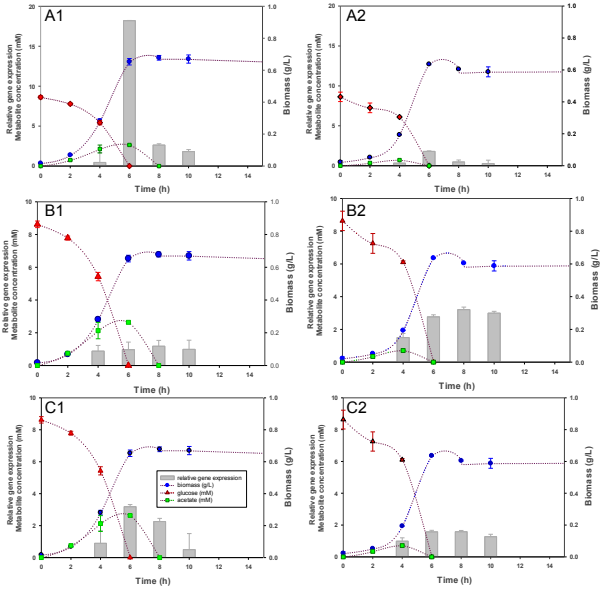


Fig. 2. Differential gene expression of *acs* (A), *cobB* (B) and *patZ* (C) during glucose cultures in the K12 (1) and BL21(2) strains.

In glucose cultures, the expression of the *acs* gene was higher in the BL21 than in the K12 at the beginning of the culture, differences being less pronounced at the onset of the stationary phase. In acetate cultures, the expression of *acs* was similar in both strains during the exponential phase of cultures. A similar behaviour was observed in *patZ* gene expression in glucose cultures (**Fig. 2** and **suppl. Table 4.**), whereas in acetate cultures *patZ* expression increased after the first 10 hours of culture in the BL21 strain (**Table 3**).

The expression pattern of *cobB* was similar between both strains, although its expression increased slightly in the stationary phase in the BL21 strain growing in glucose (**Fig. 2**). Interestingly, the analysis of the genomic environment of these genes revealed that *nagK* gene (which forms a transcriptional unit together with *cobB*) is interrupted by an IS element in the BL21 strain.

Table 3. Relative gene expression of the genes involved in protein lysine acetylation in *E. coli* during acetate batch cultures.

		time n		
Log_2 8h /time **n**		**10 hours**	**14 hours**	**23 hours**
acs	**K12**	-0.30±0.36	-0.32±0.18	-4.33±0.22
	BL21	1.98±1.01	1.54±1.24	0.38±1.06
cobB	**K12**	0.43±0.09	0.48±0.28	0.63±0.08
	BL21	-0.84±1.32	-0.53±1.64	-0.24±1.16
patZ	**K12**	-0.17±0.17	0.18±0.17	-1.01±0.12
	BL21	0.76±0.28	0.60±0.28	-0.24±1.16

Enzyme activities differences

Enzymes related with acetate metabolism in *E. coli* were measured in glucose (exponential and stationary phase) and acetate (exponential phase) cultures (**Fig. 3**).

In the case of the isocitrate node, the highest differences were shown in isocitrate lyase (**Fig. 3B**). In all the conditions assayed the specific activity was significantly higher in the K12 strain than in the BL21, especially in acetate cultures, which is in contrast with previous reports of fluxes in the glyoxylate shunt and expression of the *aceBAK* operon. Differences in the isocitrate dehydrogenase activity were almost negligible in glucose cultures, although its activity was higher in the K12 strain in acetate cultures (**Fig. 3A**).

In the case of the acetate consuming/producing pathways, all the enzymes measured showed different activity between the strains and conditions. The activity of the main acetate-producing pathway in glucose cultures, the Pta-AckA pathway, was clearly higher in *E. coli* BL21 compared with the K12 strain (**Fig. 3 C-D**). At exponential phase, both the Pta and AckA enzyme activities were higher in the BL21 strain. In acetate cultures, both activities did not show big significant differences. The activity of Acs was higher in the BL21 strain in acetate cultures, which is in agreement with its higher growth rate under these conditions (**Fig. 3 E**).

Is lysine acetylation responsible for these metabolic differences?

Differences in the metabolism and the expression of the genes related to lysine acetylation (*cobB* and *patZ*) between the B and K strains have been described above. The physiological effect of the deletion of the genes related with this post-translational modification in *E. coli* B and K strains was shown for the first time.

Phenotype differences

The phenotypic differences between the Δ*cobB* and Δ*patZ* mutants of the K12 strain in glucose and acetate batch cultures have been previously described (28). The phenotypic changes in the BL21 mutants under these conditions were more dramatic than in the K12. In glucose cultures, the acetate production rate increased more than 6-fold in BL21 Δ*cobB*, while in K12 Δ*cobB* it was only twice. Acetate production was not detected in the BL21 Δ*patZ* mutant (**Table 1**).

In order to further understand the effects of the mutations on acetate metabolism, the mutants were tested to grow on acetate as the sole carbon source. Experiments were performed at two different acetate concentrations in order to distinguish between the two major acetate consumption pathways, since at low concentrations, only the high affinity Acs pathway is active (46). The most evident change was the higher growth rate of the BL21 Δ*patZ* mutant compared to the wild type in both acetate concentrations. This effect was especially evident at 10 mM acetate, where the acetyl-CoA synthetase is the only enzyme capable of acetate assimilation **(Table 2)**. This differs from what was observed in the K12 strain, were the growth rate was slightly slower in the Δ*patZ* mutant in both acetate concentrations (28)

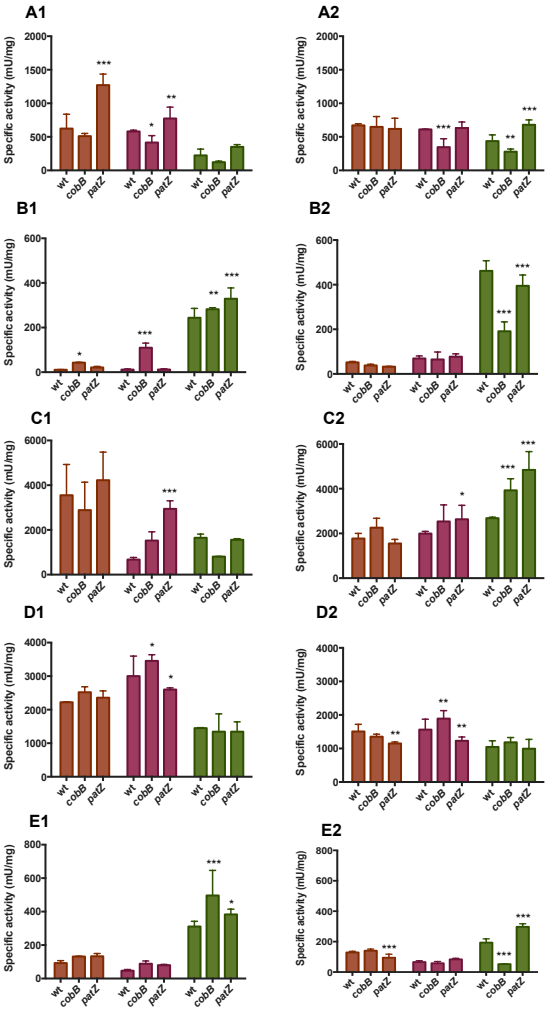


Fig 3. Enzyme activities related to acetate metabolism measured in cell crude extracts of the *E. coli* strains BL21 (1) and K12 (2) and their knockout mutants Δ*cobB* and Δ*patZ*. Cells were cultured under different conditions: glucose batch culture exponential phase (**orange**), stationary phase (**pink**) and acetate cultures exponential phase (**green**). The enzymes measured were isocitrate dehydrogenase (A), isocitrate lyase (B), phosphotransacetylase (C), acetate kinase (D) and acetyl-CoA synthetase (E).

Represented values are the average of the enzyme activities measured (n=8). Error bars indicate the standard deviation of the measurements (n=8). Statistical test (two-way Anova) was performed in order to calculate differential significance between mutants and their corresponding parent strains in each condition (p-value < 0.0001 (***) or <0.001 (**) or <0.01(*)).

Lysine Acetylation differences

Deletion of *cobB* and *patZ* affected protein acetylation levels in both *E. coli* strains. In exponential phase the differences of acetylation between all strains were almost negligible (**Fig. 4A**). More differences were observed in the stationary phase. The most noticeable change was the higher protein acetylation in both K and B Δ*cobB* mutants (**Fig. 4B**). In acetate cultures, the acetylation levels were different between mutants and strains. Although it has been previously described that protein acetylation levels are higher in the *cobB* mutant under almost all conditions, the protein acetylation levels also increased surprisingly in the *patZ* mutant. If the two pairs of mutants are compared, acetylation levels are always higher in the K12 background. Interestingly, these differences reflected the different growth behaviour of the strains, especially the higher growth rate in the BL21 *patZ* mutant (**Table 1 and 2**).

Effect of the deletion of cobB and patZ on enzyme activities related to acetate metabolism

The activities of enzymes related with acetate metabolism were measured in the Δ*cobB* and Δ*patZ* mutants. The changes assessed in the *cobB* and *patZ* mutants were highly dependent on the genomic background, which further evidenced the differences between both strains.

The isocitrate dehydrogenase activity was similar for all the strains in glucose batch cultures. The activity was higher in glucose cultures in the BL21 Δ*patZ* mutant, while this mutation had no effect in the K12 genetic background. In acetate cultures the differences were more noticeable, with higher activity in the K12 strain and its mutants (**Fig. 3 A1-2**). The activity of the first enzyme of the glyoxylate shunt, isocitrate lyase, was always higher in the K12 strain, except in the case of the BL21 Δ*cobB* mutant, that showed higher activity in the stationary phase of glucose cultures. In acetate cultures the presence of this enzyme is essential for cell growth; consequently, the activity was much higher than in glucose cultures in both strains. The most striking difference observed was the opposite effect of the deletion of *cobB* in both genetic backgrounds. In the BL21 Δ*cobB* mutant isocitrate lyase activity was higher than in the BL21 strain (**Fig. 3 B 1-2**), which can contribute to its better growth compared to the corresponding K12 mutant.

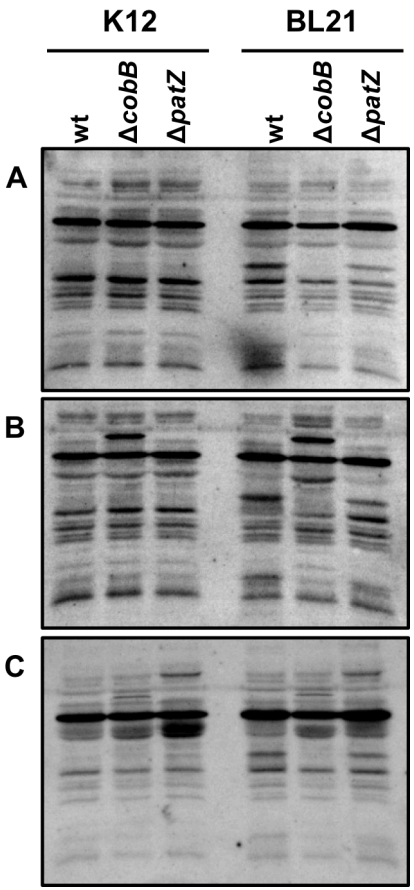


Fig 4. Western blot of acetylated proteins under different genomic backgrounds and culture conditions, glucose batch cultures exponential phase (**A**), stationary phase (**B**) and acetate batch cultures exponential phase (**C**)

The acetyl-CoA synthetase activity was higher in acetate compared with glucose cultures, but was also higher in the BL21 strains compared with the K12 in this condition. The differences between the BL21 knockouts and their parent strain were almost not noticeable in glucose cultures, whereas in acetate cultures the BL21 Δ*cobB* mutant showed a higher activity compared with its parent strain. On the other hand, higher differences were observed in K12 mutants in acetate cultures. The Δ*cobB* mutant

showed half of the parent strain activity while the Δ*patZ* mutant almost doubled the wild type activity (**Fig. 3 E1-2**).

On the other hand, the reversible Pta-AckA is the main responsible for the acetate production in the exponential phase of glucose cultures. Under this condition, differences in the Pta and AckA enzyme activities between the knockouts and their wild type were not significant, but the activity level was higher in the BL21 compared with the K12 (**Fig. 3 D-E**). The activities of these enzymes at the stationary phase are not very relevant due to the inability to consume the low acetate concentrations present in the media. In high concentration acetate cultures this pathway is responsible for acetate consumption. The AckA activity was similar between both strains and knockouts, while the Pta enzyme activity was higher in the K12 strain and their knockouts and also higher compared with the AckA activity (**Fig. 3D**).

Discussion

During many years, numerous studies have tried to unravel why the acetate metabolism of the K and B strains is different (2, 3, 7, 9–11, 24, 47, 48). Although genome sequencing allowed understanding some specific features, it is not enough to explain metabolic differences between both strains, especially in what refers to acetate. It has been recently proposed that regulatory mechanisms could be responsible for their differences on acetate production/consumption (21, 39).

Different behaviour between K and B strains were observed depending on the carbon source. The biomass level in the K12 strain was smaller than in the BL21 (49). This result does not agree with previous studies, probably caused by the lower glucose concentration used in our experiments, since detrimental effects of acetate production on growth of the K12 strain are more evident in high glucose cultures (9). The faster growth of the BL21 strain (especially at low acetate concentrations) can be explained mostly because of the higher activity of acetyl-CoA synthetase (**Fig 3 E**). This hypothesis is the more consistent if we take into account that the higher differences in growth rate were observed in the low acetate concentration cultures, where acetyl-CoA synthetase is the only enzyme responsible for acetate consumption.

The results regarding enzymes of the isocitrate node are somewhat contradictory. One of the classic hypothesis explaining why these two strains have a different acetate metabolism is based on the higher activity of the enzymes related to the glyoxylate shunt and, therefore, to the higher flux through this pathway. The glyoxylate shunt flux has been recently measured in both strains; in glucose batch cultures in exponential phase net flux through the glyoxylate shunt is only observed in the BL21 strain (24, 49). Classic studies in the late 90´s showed that the RNA and protein levels of the first enzyme of this pathway, isocitrate lyase, were higher in BL21 than in K12 (10). Another study using northern blot showed that the expression of *aceA* is constitutive during glucose batch cultures (11). In contrast, our results showed that the activity of isocitrate lyase in the BL21 strain was lower than in the K12 in all the conditions assayed. In fact, in a recent transcriptomic and proteomic study the levels of expression

of the glyoxylate shunt operon (*aceBAK*) genes were lower in BL21 at the exponential phase compared with the K12 (22). We confirmed that the *aceA* gene expression was 4 fold lower in the BL21 strain than in the K12, although no changes were observed at the stationary phase (**Appendix, suppl. Table 4**). Marisch and collaborators also found lower levels of expression of the isocitrate dehydrogenase gene (*icd*) in BL21. Altogether, this indicates that there is no direct correlation between the higher flux through the glyoxylate shunt and the levels of the transcripts and proteins of this pathway. It seems reasonable that flux partitioning at the isocitrate node in BL21 is the result of other processes not described yet.

Different protein acetylation patterns could account for this observation. A recent study showed that on average, lysine acetylation is more abundant in the K12 strain than in the BL21 in the glucose exponential phase (39). They stated that this higher protein acetylation in K12 is caused by the higher acetate production in the K12 strain (39). In our experiments, we also observed a higher acetate production in the K12 during exponential phase on glucose cultures (**Table 1**) but differences in protein lysine acetylation in both strains under the same condition were lower than those observed in the proteomic study (**Fig. 1**) (39). The different results obtained could be caused by the lower sensitivity of western blotting compared to mass spectrometry based proteomics. Moreover, we have observed that even higher differences in protein acetylation levels occur when *E. coli* is using acetate as carbon source (glucose stationary phase and acetate cultures) **(Fig. 1**).

Gene expression of *acs*, *cobB* and *patZ* was assessed in order to understand if their differential gene expression are responsible for the different acetate metabolism in the BL21 and K12 strains. We observed that the expression profile of *acs* and *patZ* genes was higher in the B strain at the early exponential phase of glucose cultures. Previous studies reported that the levels of cAMP are higher in the BL21 strain (22) and it has also been described that acetate consumption in BL21 is not so intensively regulated by catabolite repression (24). In order to assess whether differences in the promoter region upstream *acs* and *patZ* could be affecting the affinity for cAMP-CRP binding and being related to the differences observed in expression, the sequences of the CRP binding sites

were analysed. The CRP binding sites were compared to the consensus sequence and scores were calculated as described previously (28). Although both regions were slightly different between strains, the scores of the CRP binding sites were the same for both strains (Results not shown). We have observed that the adenylate cyclase from both strains differ in the amino acid sequence (99.3% of identity). This could suggest that the different cAMP levels in both strains could be the major reason for the lower catabolite repression of *acs* and *patZ* in the BL21 strain. The higher gene expression (this study) and protein levels (personal communication Brian T. Weinert) of *patZ* in the BL21 strain could suggest a higher protein acetylation in this strain, at least at the exponential phase of glucose cultures. However the deletion of the acetyltransferase in both strains did not decrease the acetylation in any condition (**Fig. 4**), which correlates with previous studies (39). Therefore, which is the reason of the different phenotype observed between the BL21 and K12 Δ*patZ* mutants compared with their parent strains? Probably this protein acetyltransferase is not responsible for most of the acetylation events. The phenotype observed in the Δ*patZ* mutants in all the conditions, especially in the BL21 strain, indicates that probably it is responsible for some of the acetylation events. An interesting result was the higher acetylation of proteins observed in acetate cultures in both *patZ* mutants, an effect being more obvious in the K12 mutant (**Fig. 4**). These results showed that the role of *patZ* could be more important than previously described (39), probably being involved in controlling the occurrence of chemical acetylation. In fact, the different phenotypes of the Δ*patZ* mutants in acetate cultures could be due to the higher "unspecific" acetylation of proteins in the K12, which might slow down its growth.

The phenotypes of both Δ*cobB* and Δ*patZ* mutants were differentially affected in acetate cultures in the B strain. In high acetate concentration cultures all of the BL21 mutants grew faster than the K12, probably caused by a higher acetyl-CoA synthetase activity. The slower BL21 Δ*cobB* growth rate might be triggered by the lower phosphotransacetylase activity. On the other hand, in low concentration acetate cultures the phenotypes were even more affected, no growth was observed in the Δ*cobB*

mutant and faster in the Δ*patZ*. Altogether these results indicate that the acetyl-CoA synthetase enzyme has a more tight regulation in the B strain.

Probably the differences at the gene and protein expression level of the sirtuin *cobB* and the protein acetyltransferase *patZ* are not enough to explain the phenotypes observed in the K and B strains and their knockout mutants. In fact, it is currently accepted that phenotypic differences between the K and B strains are due to the sum of several factors. However, it is clear that the absence of *cobB* and *patZ* in the BL21 strain provoked sharp phenotypic effects especially on acetate consumption. This indicates that protein lysine acetylation, modulated by these two proteins, contributes to the different acetate metabolism regulation in the K12 and BL21 strains, being this regulation even more tightly exerted in the B strain.

Protein acetylation contributes to the differences in the acetate metabolism in *E. coli* BL21 and K12. In previous reports it was proposed that the differential lysine acetylation between the K12 and BL21 strains is caused by a higher acetate overflow in the K12 strain due to a higher acetyl phosphate production. We have confirmed that global levels of protein acetylation are lower in the BL21 strain, and that it is highly affected by the phase of the culture and the carbon source. Deletion of *patZ* in BL21 further reduced acetate overflow and improved growth, indicating that acetate overflow and acetate consumption are tightly regulated by protein acetylation in *E. coli* BL21. Altogether, differential expression of *acs* and *patZ* in the exponential phase of cultures can account for differences in acetate overflow between strains.

Due to the subtle differences in the expression of the proteins involved in this PTM, several efforts should be carried out in order to know the real behaviour of this system in both strains. Metabolites involved in chemical and enzymatic acetylation and deacetylation reactions could be the clue for unravelling the differences in acetate metabolism between both strains.

Bibliography

1. Baneyx, F. (1999) Recombinant protein expression in *Escherichia coli*. *Curr. Opin. Biotechnol.* 10, 411–21
2. Studier, F. W., Daegelen, P., Lenski, R. E., Maslov, S., and Kim, J. F. (2009) Understanding the differences between genome sequences of *Escherichia coli* B strains REL606 and BL21(DE3) and comparison of the *E. coli* B and K-12 genomes. *J. Mol. Biol.* 394, 653–80
3. Jeong, H., Barbe, V., Lee, C. H., Vallenet, D., Yu, D. S., Choi, S.-H., Couloux, A., Lee, S.-W., Yoon, S. H., Cattolico, L., Hur, C.-G., Park, H.-S., Ségurens, B., Kim, S. C., Oh, T. K., Lenski, R. E., Studier, F. W., Daegelen, P., and Kim, J. F. (2009) Genome sequences of *Escherichia coli* B strains REL606 and BL21(DE3). *J. Mol. Biol.* 394, 644–52
4. Kleman, G. L., and Strohl, W. R. (1994) Acetate metabolism by *Escherichia coli* in high-cell-density fermentation. *Appl. Environ. Microbiol.* 60, 3952–3958
5. De Mey, M., De Maeseneire, S., Soetaert, W., and Vandamme, E. (2007) Minimizing acetate formation in *E. coli* fermentations. *J. Ind. Microbiol. Biotechnol.* 34, 689–700
6. Lara, A. R., Caspeta, L., Gosset, G., Bolívar, F., and Ramírez, O. T. (2008) Utility of an *Escherichia coli* strain engineered in the substrate uptake system for improved culture performance at high glucose and cell concentrations: An alternative to fed-batch cultures. *Biotechnol. Bioeng.* 99, 893–901
7. Negrete, A., Majdalani, N., Phue, J.-N., and Shiloach, J. (2013) Reducing acetate excretion from *E. coli* K-12 by over-expressing the small RNA SgrS. *N. Biotechnol.* 30, 269–73
8. Luli, G. W., and Strohl, W. R. (1990) Comparison of growth, acetate production, and acetate inhibition of *Escherichia coli* strains in batch and fed-batch fermentations. *Appl. Environ. Microbiol.* 56, 1004–1011
9. Shiloach, J., Kaufman, J., Guillard, A. S., and Fass, R. (1996) Effect of glucose supply strategy on acetate accumulation, growth, and recombinant protein production by *Escherichia coli* BL21 (lambda DE3) and *Escherichia coli* JM109. *Biotechnol. Bioeng.* 49, 421–428
10. Van de Walle, M., and Shiloach, J. (1998) Proposed mechanism of acetate accumulation in two recombinant *Escherichia coli* strains during high density fermentation. *Biotechnol. Bioeng.* 57, 71–78
11. Phue, J.-N., and Shiloach, J. (2004) Transcription levels of key metabolic genes are the cause for different glucose utilization pathways in *E. coli* B (BL21) and *E. coli* K (JM109). *J. Biotechnol.* 109, 21–30
12. Phue, J. N., Noronha, S. B., Hattacharyya, R., Wolfe, A. J., and Shiloach, J. (2005) Glucose metabolism at high density growth of *E. coli* B and *E. coli* K: Differences in metabolic pathways are responsible for efficient glucose utilization in *E. coli* B as determined by microarrays and northern blot analyses. *Biotechnol. Bioeng.* 90, 805–820
13. El-Mansi, M., Cozzone, A. J., Shiloach, J., and Eikmanns, B. J. (2006) Control of carbon flux through enzymes of central and intermediary metabolism during growth of *Escherichia coli* on acetate. *Curr Opin Microbiol* 9, 173–179
14. Wolfe, A. J. (2005) The acetate switch. *Microbiol Mol Biol Rev* 69, 12–50
15. Maharjan, R. P., Yu, P.-L. L., Seeto, S., Ferenci, T., and Prasad Maharjan, R. (2005) The role of isocitrate lyase and the glyoxylate cycle in *Escherichia coli* growing under glucose limitation. *Res Microbiol* 156, 178–183
16. Walsh, K., and Koshland, D. E. (1984) Determination of flux through the branch point of two metabolic cycles. The tricarboxylic acid cycle and the glyoxylate shunt. *J Biol Chem* 259, 9646–9654

17. Fischer, E., and Sauer, U. (2003) A novel metabolic cycle catalyzes glucose oxidation and anaplerosis in hungry *Escherichia coli*. *J Biol Chem* 278, 46446–46451
18. Renilla, S., Bernal, V., Fuhrer, T., Castaño-Cerezo, S., Pastor, J. M., Iborra, J. L., Sauer, U., and Cánovas, M. (2012) Acetate scavenging activity in *Escherichia coli*: interplay of acetyl-CoA synthetase and the PEP-glyoxylate cycle in chemostat cultures. *Appl Microbiol Biotechnol* 95, 2109–2124
19. LaPorte, D. C., and Chung, T. (1985) A single gene codes for the kinase and phosphatase which regulate isocitrate dehydrogenase. *J Biol Chem* 260, 15291–15297
20. Chung, T., Klumpp, D. J., and LaPorte, D. C. (1988) Glyoxylate bypass operon of *Escherichia coli*: cloning and determination of the functional map. *J. Bacteriol.* 170, 386–392
21. Son, Y.-J., Phue, J.-N., Trinh, L. B., Lee, S. J., and Shiloach, J. (2011) The role of Cra in regulating acetate excretion and osmotic tolerance in *E. coli* K-12 and *E. coli* B at high density growth. *Microb. Cell Fact.* 10, 52
22. Marisch, K., Bayer, K., Scharl, T., Mairhofer, J., Krempl, P. M., Hummel, K., Razzazi-Fazeli, E., and Striedner, G. (2013) A comparative analysis of industrial *Escherichia coli* K-12 and B strains in high-glucose batch cultivations on process-, transcriptome- and proteome level. *PLoS One* 8, e70516
23. Phue, J.-N., Kedem, B., Jaluria, P., and Shiloach, J. (2007) Evaluating microarrays using a semiparametric approach: application to the central carbon metabolism of *Escherichia coli* BL21 and JM109. *Genomics* 89, 300–5
24. Waegeman, H., Maertens, J., Beauprez, J., De Mey, M., and Soetaert, W. (2012) Effect of *iclR* and *arcA* deletions on physiology and metabolic fluxes in *Escherichia coli* BL21 (DE3). *Biotechnol. Lett.* 34, 329–37
25. Valgepea, K., Adamberg, K., Nahku, R., Lahtvee, P.-J., Arike, L., and Vilu, R. (2010) Systems biology approach reveals that overflow metabolism of acetate in *Escherichia coli* is triggered by carbon catabolite repression of acetyl-CoA synthetase. *BMC Syst Biol* 4, 166
26. Mischerikow, N., Spedale, G., Maarten Altelaar, A. F., Marc Timmers, H. T., Pim Pijnappel, W. W. M., and Heck, A. J. R. (2009) In-depth profiling of post-translational modifications on the related transcription factor complexes TFIID and SAGA. *J. Proteome Res.* 8, 5020–5030
27. Wang, Q., Zhang, Y., Yang, C., Xiong, H., Lin, Y., Yao, J., Li, H., Xie, L., Zhao, W., Yao, Y., Ning, Z.-B., Zeng, R., Xiong, Y., Guan, K.-L., Zhao, S., and Zhao, G.-P. (2010) Acetylation of metabolic enzymes coordinates carbon source utilization and metabolic flux. *Science* 327, 1004–7
28. Castaño-Cerezo, S., Bernal, V., Blanco-Catalá, J., Iborra, J. L., and Cánovas, M. (2011) cAMP-CRP co-ordinates the expression of the protein acetylation pathway with central metabolism in *Escherichia coli*. *Mol. Microbiol.* 82, 1110–1128
29. Starai, V. J., Celic, I., Cole, R. N., Boeke, J. D., and Escalante-Semerena, J. C. (2002) Sir2-dependent activation of acetyl-CoA synthetase by deacetylation of active lysine. *Science (80-.).* 298, 2390–2392
30. Gardner, J. G., Grundy, F. J., Henkin, T. M., and Escalante-Semerena, J. C. (2006) Control of acetyl-coenzyme A synthetase (AcsA) activity by acetylation/deacetylation without NAD+ involvement in *Bacillus subtilis*. *J. Bacteriol.* 188, 5460–5468
31. Crosby, H. a, Rank, K. C., Rayment, I., and Escalante-Semerena, J. C. (2012) Structural insights into the substrate specificity of the *Rhodopseudomonas palustris* protein acetyltransferase RpPat: identification of a loop critical for recognition by RpPat. *J. Biol. Chem.* 287, 41392–404
32. Thao, S., and Escalante-semerena, J. C. (2011) Analyses of *Salmonella enterica* Pat, a Biochemical and Thermodynamic Multidomain, Multimeric Nε-Lysine Acetyltransferase Involved in Carbon and Energy Metabolism. *MBio* 2, 1–8

33. Gardner, J. G., and Escalante-Semerena, J. C. (2008) Biochemical and mutational analyses of AcuA, the acetyltransferase enzyme that controls the activity of the acetyl coenzyme a synthetase (AcsA) in *Bacillus subtilis*. *J. Bacteriol.* 190, 5132–5136
34. Nambi, S., Basu, N., and Visweswariah, S. (2010) cAMP-regulated Protein Lysine Acetylases in Mycobacteria. *J Biol Chem* 285, 24313–24323
35. Lima, B. P., Thanh Huyen, T. T., Bäsell, K., Becher, D., Antelmann, H., and Wolfe, A. J. (2012) Inhibition of acetyl phosphate-dependent transcription by an acetylatable lysine on RNA polymerase. *J. Biol. Chem.* 287, 32147–60
36. Gardner, J. G., and Escalante-Semerena, J. C. (2009) In *Bacillus subtilis*, the sirtuin protein deacetylase, encoded by the *srtN* gene (formerly yhdZ), and functions encoded by the *acuABC* genes control the activity of acetyl coenzyme A synthetase. *J. Bacteriol.* 191, 1749–1755
37. Hayden, J. D., Brown, L. R., Gunawardena, H. P., Perkowski, E. F., Chen, X., and Braunstein, M. (2013) Reversible acetylation regulates acetate and propionate metabolism in *Mycobacterium smegmatis*. *Microbiology* 159, 1986–99
38. Zhao, K., Chai, X., and Marmorstein, R. (2004) Structure and substrate binding properties of CobB, a Sir2 homolog protein deacetylase from *Escherichia coli*. *J Mol Biol* 337, 731–741
39. Weinert, B. T. T., Iesmantavicius, V., Wagner, S. A. A., Schölz, C., Gummesson, B., Beli, P., Nyström, T., Choudhary, C., Scho, C., and Nystro, T. (2013) Acetyl-Phosphate Is a Critical Determinant of Lysine Acetylation in *E . coli*. *Mol. Cell* 51, 1–8
40. Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K. A., Tomita, M., Wanner, B. L., and Mori, H. (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* 2, 2006.0008
41. Datsenko, K. A., and Wanner, B. L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* 97, 6640–6645
42. Aoshima, M., Ishii, M., Yamagishi, A., Oshima, T., and Igarashi, Y. (2003) Metabolic characteristics of an isocitrate dehydrogenase defective derivative of *Escherichia coli* BL21(DE3). *Biotechnol Bioeng* 84, 732–737
43. Lin, H., Castro, N., Bennett, G., and San, K.-Y. (2006) Acetyl-CoA synthetase overexpression in *Escherichia coli* demonstrates more efficient acetate assimilation and lower acetate accumulation: a potential tool in metabolic engineering. *Appl Microbiol Biotechnol* 71, 870–874
44. Peng, L., and Shimizu, K. (2003) Global metabolic regulation analysis for *Escherichia coli* K-12 based on protein expression by 2-dimensional electrophoresis and enzyme activity measurement. *Appl Microbiol Biotechnol* 61, 163–178
45. Bergmeyer, H. U., Forster, G., and Bernt, E. (1974) in *Methods of Enzymatic Analysis*, pp 784–793.
46. Starai, V. J., and Escalante-Semerena, J. C. (2004) Acetyl-coenzyme A synthetase (AMP forming). *Cell Mol Life Sci* 61, 2020–2030
47. Han, M.-J., Lee, S. Y., and Hong, S. H. (2012) Comparative analysis of envelope proteomes in *Escherichia coli* B and K-12 strains. *J. Microbiol. Biotechnol.* 22, 470–8
48. Meier, S., Jensen, P. R., and Duus, J. Ø. (2012) Direct observation of metabolic differences in living *Escherichia coli* strains K-12 and BL21. *Chembiochem* 13, 308–10
49. Waegeman, H., Beauprez, J., Moens, H., Maertens, J., De Mey, M., Foulquie-Moreno, M., Heijnen, J., Charlier, D., and Soetaert, W. (2011) Effect of *iclR* and*arcA* knockouts on biomass formation and metabolic fluxes in *Escherichia coli* K-12 and its implications on understanding the metabolism of Escherichia coli BL21 (DE3). *BMC Microbiol.* 11, 70

Appendix

Suppl. Table 1. List of strains and mutants of *E. coli* used in this study.

Strains	Relevant genotype	Source
***E. coli* BW25113 (K12)**	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR*514 Δ(araBAD)$_{AH33}$ Δ(rhaBAD)$_{LD78}$	Keio University (Japan)
Δ*cobB*	[BW25113] *cobB* deficient	Keio University (Japan)
Δ*patZ*	[BW25113] *patZ* deficient	Keio University (Japan)
***E. coli* BL21 DE3**	*F- ompT hsdSB(rB- mB-) gal dcm λ(DE3) tonA*	Sigma Aldrich (St. Louis, MO)
Δ*cobB*	[BL21] *cobB* deficient	This study
Δ*patZ*	[BL21] *patZ* deficient	This study

Suppl. Table 2 List of plasmids used in this study

Plasmids	Description	Source
pCP20	Amp^{R}/Cm^{R} , this plasmid is temperature sensitive and under high temperatures the transcription of FLP is induced.	Yale Stock Center (USA)
pKD13	Kan^{R} /Amp^{R}	Yale Stock Center (USA)
pKD46	Amp^{R} , the replication is temperature- sensitive and the induction of FLP is temperature induced.	Yale Stock Center (USA)

Suppl. Table 3 List of primers used in this study

	Sequence 5´→3´
***cobB* KO fwd**	GTGGTGCGGCCTTCCTACATCTAACCGATTAAACAACAGAGGTTGCTATGATTCCGGGGATCCGTCGACC
***cobB* KO rev**	CCCCTTGCAGGCCTGATAAGCGTAGTGCATCAGGCAATGCTTCCCGCTTTTGTAGGCTGGAGCTGCTTCG
***patZ* KO fwd**	GTTAGAAAGCGTTTAAAATCATTCGGTCACTTCTGCGGGAGACCGGTATGATTCCGGGGATCCGTCGACC
***patZ* KO rev**	TTAAGTGGTCAACATTTCCAGTACCTTACTCATGATTCCTCGCGCTGGGCTGTAGGCTGGAGCTGCTTCG
***cobB* check fwd**	TGTTCCGCGCATTGAACGC
***cobB* check rev**	AACGCCTTATCCGGCCCAC
***patZ* check fwd**	GCTATCTGGCAGGAAAAACG
***patZ* check rev**	GCAGACGACATAAGCGGGCA
K1	CAGTCATAGCCGAATAGCCT
K2	CGGTGCCCTGAATGAACTGC
***polA*-RT fwd**	GCTGAACGTGCAGCCATTAA
***polA*-RT rev**	CAATCATCGCCCGTTTGATAA
***dnaA*-RT fwd**	TGGCGAAAGAGCTGACTAACC
***dnaA*-RT rev**	ACGGCAGGCATGAAGCA
***rrsA*-RT fwd**	CCTTACGACCAGGGCTACACA
***rrsA*-RT rev**	CACTTTATGAGGTCCGCTTGCT
***acs*-RT fwd**	AACACACCATTCCTGCCAACA
***acs*-RT rev**	TGTTGATACATCGCCTCGTACTG
***cobB*-RT fwd**	AGTGTGGTTTGGCGAAATGC
***cobB*-RT rev**	GCCATCGACAACGCCATATAA
patZ-RT fwd	CGGCGGCCCGTAGTG
patZ_K-RT rev	CATTCCTGCCGTCGTGTTG
patZ_B-RT rev	TCCTGCCGTGGTGTTCAA

Supl. Table 4. Differential gene expression (Log_2 K12/BL21) of *acs*, *aceA*, *cobB*, *icdA*, *patZ* and *pta* between the K and B strains in glucose batch cultures at exponential and stationary phase. .

Gene	**Glc exp phase**	**Glc sta phase**
aceA	2.13±0.36	-0.88±0.98
acs	-2.21±0.28	0.35±0.41
icdA	-1.11±0.22	-0.90±1.25
pta	-1.31±0.22	0.38±0.20

Chapter 6

Protein deacetylation regulates acetate metabolism, motility and acid stress response in *Escherichia coli*

The results presented in this chapter are part of a manuscript under preparation

"In order to discover something truly new, at least one of your basic assumptions has to change"
Uri Alon

Abstract

Several different types of reversible post-translational modifications, such as phosphorylation or acetylation of proteins are known to play an important regulatory role in cells and tissue. Although protein acetylation is widely observed, it has only been associated with specific regulatory functions in few cases and its function is generally poorly understood. To investigate functionality, we analysed the acetylome of the knockout mutants of *cobB*, the only known sirtuin deacetylase in *Escherichia coli*, and *patZ* (formerly *yfiQ*), the best-known protein acetyltransferase. In four different conditions, more than 2,000 unique acetylated peptides, belonging to 809 proteins, were identified and quantified. Nearly 65% of these proteins are related to metabolism.

The deacetylase activity of CobB is global and contributes to the deacetylation of a large number of substrates and has a major impact on bacterial physiology. Apart from the well-demonstrated regulation of the acetyl-CoA synthetase, CobB controlled acetylation levels of Isocitrate lyase that contributes to the fine-tuning regulation of the glyoxylate shunt. Acetylation of lysine 154 of the transcription factor RcsB prevents DNA binding, activating flagella biosynthesis and motility, and increases susceptibility to acid stress. Surprisingly, deletion of the acetyltransferase *patZ* increased acetylation, especially in acetate cultures. These results suggest that the role of patZ could be related to regulating the acetylating agents levels in the cell.

The proteomic and physiological results presented offered new insights into the roles of lysine acetylation and its regulation in *E. coli*, underlining the impact of this post-translational modification in the global regulatory mechanisms of the cell and providing a valuable resource to the community.

Introduction

From bacteria to higher animals and plants, organisms need to adapt to their environment. Physiological processes are regulated at several levels, for example transcriptional control of gene expression to allosteric effects. Reversible post-translational modification is a fast mechanism for controlling the activity of proteins, and in particular in metabolism they are expected to have key relevance in controlling the use of competing pathways (1, 2). Almost 200 different types of protein modifications have been described, with lysine harbouring most types of modifications in its side chain is lysine (3–6).

Although protein acetylation at lysine residues has been known since the 70s, it has emerged in the past 10 years as a highly prominent post-translational modification, widely spread in all domains of life. Conventionally, this reversible protein modification was mainly related to transcriptional regulation: increased acetylation of histones decreases its interaction with DNA, thereby decreasing nucleosome compactness (7). Recent studies have shown that a high percentage of proteins related to metabolism are lysine acetylated (8–15). The bacterial paradigm of this regulation is *Salmonella enterica* where acetyl-coenzyme A synthetase was the first enzyme whose activity was described to be reversibly regulated by lysine acetylation (16, 17). In this microorganism, almost 200 proteins are acetylated, and almost half of these targets were metabolic enzymes. However, a thorough characterization of the functional implications that protein acetylation has on bacterial physiology and, particularly, metabolism is lacking.

The proteins involved in protein acetylation and deacetylation have already been described in several bacteria (16, 18–27) . A Gnc5-like acetyltransferase, Pat, that uses acetyl-CoA as substrate, was discovered in *S. enterica* (17). The deacetylase CobB, the first bacterial sirtuin, is capable of deacetylating acetyl-lysine residues using NAD^+ as a substrate (28). It has been described that cyclic AMP is an allosteric activator of the only known lysine acetyltransferase in *Mycobacterium*, and this metabolite is the main activator of virulence in these species (21). It remains unclear if other acetyltransferases

and deacetylases exist in these microorganisms (18, 21, 24, 29). Four different proteomic studies have revealed that lysine acetylation is abundant in *E. coli*, the most recent one reported over 1000 acetylated proteins (11–13, 30). Physiological implications of lysine acetylation in *E. coli* are subject of intense study and include altered activity of the acetyl CoA synthetase, RNA polymerase, the chemotaxis response regulator (CheY), the regulator of capsule synthesis (RcsB), ribonuclease R (RNase R) and N-hydroxyarylamine O-acetyltransferase (31–36).

The sirtuin CobB is the only deacetylase known in *E. coli*. Interestingly, the expression of the best known acetyltransferase PatZ of *E. coli* (formerly, YfiQ) is affected by metabolic signals (31). Here we set out to understand how the protein acetylation state impacts on the physiology of *E. coli*. Pathways affected by protein acetylation were identified in CobB and PatZ mutant strains taking a systems approach utilizing high throughput proteomic and transcriptomic tools. Metabolic, fluxomic and molecular biology studies were focused on regulation of the central carbon metabolism (especially the acetate overflow and glyoxylate shunt routes) and signalling pathways related to chemotaxis and stress response.

Materials and methods

Escherichia coli strains and culture conditions.

E. coli wild type BW25113 and its knockout strains (**Suppl. Table 1**) were grown in minimal media in batch mode with glucose and acetate as described in Castaño-Cerezo et al. (31) and in glucose-limited chemostat at a dilution rate of 0.2 h^{-1}(37, 38).

Proteomics.

Sample preparation for lysine acetylation mapping.

Cells were harvested at exponential and stationary phase in glucose cultures, exponential phase in acetate cultures and in steady state in glucose-limited chemostats. Cell pellets were washed three times with PBS and then resuspended in lysis buffer containing 8 M urea, 50 mM ammonium bicarbonate, 1 tablet of complete mini EDTA-free Cocktail (Roche, Boehringer Mannhein), supplemented with 10 mM nicotinamide and 10 µM trichostatin in order to inhibit deacetylases. Cells were sonicated on ice for three cycles (20 secs each) with a probe of 3 mm of diameter in a Vibra Cell VC 375 ultrasonic processor (Sonics Materials, Danbury, CT). The lysate was clarified by centrifugation for 20 min at 20,000xg at 4ºC.

Three mg of protein of each condition and strain were reduced with 2 mM dithiotreitol for 30 min at 56ºC and alkylated with 4 mM iodoacetamide during 20 minutes in the dark, followed by LysC (1:75) digestion during 4 hours at 37ºC. Samples were diluted 4-fold in 50 mM ammonium bicarbonate buffer and digested with trypsin (Promega, Madison, WI) (1:100) during 16 hours at 37ºC.

For quantitative analysis of peptide lysine acetylation, stable isotope dimethyl labeling was used as described in Boersema et al (39). Labelled peptides from each strain were mixed in a 1:1:1 proportion. Acetylated peptides were immunoprecipitated as described (40). Nine milligrams of peptides were resuspended in immunoprecipitation buffer (50 mM MOPS, 10 mM sodium phosphate and 50 mM NaCl pH 7.4). The peptide solution was mixed with 100 µL of anti-acetyl lysine antibody beads

(InmuneChem, Burnaby, Canada) and incubated for 16 hours at 4ºC. Beads were washed 4 times with immunoprecipitation buffer, twice with water and eluted with 0.1% trifluoracetic acid. Lysine acetylated peptides were desalted using C_{18} *StageTips.*

MS specifications

Samples were resuspended in 10% formic acid (FA) / 5% DMSO and 40% of the sample was analysed using a Proxeon Easy-nLC100 (Thermo Scientific) connected to an Orbitrap Q-Exactive mass spectrometer. Samples were first trapped (Dr Maisch Reprosil C18, 3 um, 2 cm x 100 µm) before being separated on an analytical column (Agilent Zorbax SB-C18, 1.8 µm, 40 cm x 75 µm), using a gradient of 60 min at a column flow of 150 nl min^{-1}. Trapping was performed at 8 µL/min for 10 min in solvent A (0.1 M acetic acid in water) and the gradient was as follows: 7- 30% solvent B (0.1 M acetic acid in acetonitrile) in 91 min, 30-100% in 3 min, 100% solvent B for 2 min, and 7% solvent A for 18 min. Nanospray was performed at 1.7 kV using a fused silica capillary that was pulled in-house and coated with gold (o.d. 360 µm; i.d. 20 µm; tip i.d. 10 µm). The mass spectrometers were used in a data-dependent mode, which automatically switched between MS and MS/MS. Full scan MS spectra from *m/z* 350 – 1500 were acquired at a resolution of 35.000 at *m/z 400* after the accumulation to a target value of 3e6. Up to ten most intense precursor ions were selected for fragmentation. HCD fragmentation was performed at normalised collision energy of 25% after the accumulation to a target value of 5e4. MS2 was acquired at a resolution of 17.500 and dynamic exclusion was enabled (exclusion size list 500, exclusion duration 30 s).

Data analysis

Raw data was analysed by MaxQuant (version 1.3.0.5) (41) Andromeda (42) was used to search the MS/MS data against the Uniprot *E.coli* MG1655 database (version v2012-09, 4431 sequences), including a list of common contaminants and concatenated with the reversed version of all sequences. Trypsin/P was chosen as cleavage specificity allowing three missed cleavages. Carbamidomethylation (C) was set as a fixed modification, while Oxidation (M), Acetyl (Protein N-term) and Acetyl (K)

were used as variable modifications. For dimethyl labelling DimethylLys0 and DimethylNter0 were set as light labels, DimethylLys4 and DimethylNter4 were set as medium labels, and DimethylLys8 and DimethylNter8 were set as heavy labels. The database searches were performed using a peptide tolerance of 20 ppm for the first search and 6 ppm for the main search. HCD fragment ion tolerance was set to 20 ppm. Data filtering was carried out using the following parameters: Peptide FDR was set to 1%; max peptide PEP was set to 1; minimum peptide length was set to 5; minimum razor peptides were set to 1; peptides used for protein quantification was set to razor and unique peptides; Protein quantification was performed by using only unmodified peptides and Oxidation (M) and Acetyl (Protein N-term); the re-quantify option was enabled. Further data processing was performed using the Perseus tool (version 1.3.0.4) available in the MaxQuant environment.

***In vitro* enzyme activities.**

Acetyl-CoA synthetase (Acs), phosphotransacetylase (Pta), isocitrate lyase (AceA) and isocitrate dehydrogenase (Icd) were assayed as previously described (43).

DNA microarray.

Global gene expression was assessed in glucose exponential phase and chemostat cultures. RNA was purified using Vantage RNA purification kit (Origene, MD, USA). Purity and concentration of isolated RNA were assessed in a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington DE). Quality was evaluated by microfluidic capillary electrophoresis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) using Agilent RNA 6000 Pico kit. GeneChip *E. coli* Genome 2.0 arrays (Affymetrix, Santa Clara, CA) were prepared and loaded according to the manufacturer's instructions. Signal extraction and normalization was performed using GeneChip Expression Console and RMA algorithm was applied (44). Log_2 signals were loaded into Babelomics and Class Comparison analysis were performed using Limma method (FDR 0.05) (45). Hierarchical clustering and matrix annotation was performed using Perseus (41).

Protein purification and *in vitro* deacetylation assays.

Protein purification.

The acetyl-CoA synthetase (Acs), isocitrate lyase (AceA) and the NAD^+-dependent deacetylase (sirtuin) CobB from *E. coli* BW25113 were expressed using ASKA clone plasmids (GFP-) (46). In order to obtain hyper acetylated proteins, plasmids were transformed into *E. coli* BL21 Δ*cobB* for protein expression. The transformants were grown for 14 hours at 28ºC with 0.1 mM of IPTG induction. Cells were harvested by centrifugation and washed three times with 0.9% NaCl and 10 mM $MgSO_4$. Cell pellets were resuspended in binding buffer (15.5 mM Na_2HPO_4, 4.5 mM NaH_2PO_4, 500 mM NaCl and 20 mM imidazole, pH 7.4) and lysed by sonication (3x30'' cycles) on ice. Cell debris was removed by centrifugation and protein extract was loaded onto His GraviTrap columns (GE healthcare, Buckinghamshire, UK). His-tagged proteins were purified according to the manufacturer protocol. Purified proteins containing imidazole were cleaned using Amicon ultra 4 centrifugal filters (Millipore, Country Cork, Ireland).

In vitro *protein deacetylation assays*.

10 µg of CobB and 10 µg of either Acs, AceA were incubated in deacetylation buffer (50 mM HEPES, 6 mM $MgCl_2$, 2 mM NAD^+, 1 mM DTT and 5% glycerol, pH 7.0) during one hour at 37ºC with NAD^+ 5 mM as substrate.

After incubation, aliquots of the reaction were used for enzyme activity assays, western blotting or mass spectrometry for lysine acetylation mapping (**Supplementary material**).

Metabolic flux ratio analysis and ^{13}C-constrained metabolic flux analysis

Metabolic flux ratio analysis in *E. coli* BW25113 and its knockout mutants Δ*cobB*, Δ*patZ*, Δ*cobB*Δ*aceK*, Δ*patZ*Δ*aceK* in glucose batch exponential phase and chemostat cultures ($D=0.2h^{-1}$) was performed as previously described (47). One milligram of cells was washed twice with 1 mL of 0.9% NaCl and 10 mM $MgSO_4$, hydrolysed in 300 µL 6 M HCl at 105°C for 15 h in sealed 1.5 mL tubes. The hydrolysates were dried in a heating block at 85°C under a stream of air and then

derivatized at 85°C for 60 min in 30 µL of dimethylformamide (Fluka, ciudad, país) and 30 µL of N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide with 1% (v/v) tert-butyldimethylchlorosilane (Fluka, ciudad, pais) with slight shaking (Zamboni et al. 2009). One microliter of the derivatized sample was injected into a 6,890 N Network GC system, combined with a 5,975 Inert XL Mass Selective Detector (Agilent Technologies). The gas chromatography–mass spectrometry-derived mass isotope distributions of proteinogenic amino acids were corrected for naturally occurring isotopes (48), and nine ratios of fluxes through converging reactions were determined. Calculations were performed using the Matlab®-based software FiatFlux 1.65 (49).

Intracellular net carbon fluxes were estimated by using the stoichiometric model previously described (47) that included all major pathways of central carbon metabolism, including the glyoxylate shunt and the Entner–Doudoroff (ED) pathway. The matrix consisted of 25 reactions and 21 metabolites. Net fluxes were then calculated using (1) the stoichiometric reaction matrix, (2) the relative metabolic flux ratios, (3) physiological data, and (4) precursor requirements for biomass synthesis. Specifically, the following flux ratios were used: serine derived through the EMP pathway, pyruvate derived through the ED pathway, oxaloacetate (OAA) originating from PEP, PEP originating from OAA, OAA originated from glyoxylate, the lower and upper bounds of pyruvate originating from malate, and the upper bound of PEP derived through the PP pathway.

Molecular biology

The *rcsB* gene was PCR-amplified from *E. coli* BW25113 genomic DNA and cloned into the *pBAD24* plasmid (50) using *Xba*I and *Hind*III enzymes. Single amino acid mutants K153R, K153Q and K153E were obtained by site directed mutagenesis. The Stratagene kit for mutagenesis was used according to the manufacturer instructions. Mutagenesis primers are listed in **Supplementary Table 2**.

Electron microscopy for flagella observation

E. coli strains were grown in glycerol minimal medium. The cells were harvested at mid-exponential phase and fixed with 3% glutaraldehyde. After 3 washes

with PBS, each cell suspension was placed on electron microscopy grids and stained for 15 seconds with 2% uranyl acetate before flagella examination in a JEM-1011 Electron Microscope (Jeol, Tokyo, Japan) operating at 90 kV.

Mobility assays

All strains were grown until mid-exponential phase. 5 µL of these cultures were inoculated into semisolid agar (10 g/L tryptone, 5 g/L NaCl and 0.25% agar). Plates were checked for mobility after 16 h incubation at 30ºC.

Acid stress resistance test

All tested strains were grown overnight in glycerol minimal medium (pH 5.5) supplemented with 1.5 mM glutamic acid. A 1:1000 dilution of the overnight culture was inoculated in glycerol minimal medium pH 2.5 supplemented with 1.5 mM glutamic acid. Cell survival was measured after two hours in acidic media as previously described (51).

Glutamate decarboxylase activity (GAD)

For Gad enzyme activity determination, approximately $3 \cdot 10^8$ stationary phase cells were grown in glycerol minimal media at pH 5.5 were harvested by centrifugation 4000 g 4 ºC and washed twice with 0.9% NaCl. Cells were mixed one to one with the Gad reagent (1 g/L L-glutamic acid, 0.05 g/L bromocresol green, 90 g/L NaCl 0.3% (v/v) Triton-X100) and incubated for 30 minutes at 35ºC. The presence of this enzyme was monitored by the color shift of bromocresol green, which turns from yellow to blue (52).

Results

To further understand protein acetylation in *E. coli*, we analysed the effects of the deletion of the only lysine deacetylase known (*cobB*) and the best characterized lysine acetyltransferase (*patZ*). This study was performed under physiologically relevant conditions, selecting those where large changes in protein acetylation were expected. We have previously demonstrated that the expression of *patZ* is up-regulated by cAMP (*e.g.* upon glucose limitation or during growth on non-PTS carbon sources) (31). The growth of the three *E. coli* strains was compared in glucose batch (non-carbon limited) and chemostat (carbon-limited) cultures and in acetate (non-PTS carbon source) batch cultures. It was precisely under gluconeogenic conditions, such as acetate batch and glucose limited chemostat cultures, that the phenotype of the Δ*cobB* mutant was especially affected (**Suppl. Fig. 1**). The severe growth impairment of the *cobB* mutant during conditions of high expression of *patZ* led us to hypothesize that this effect was caused by increased protein lysine acetylation of proteins crucial for optimal growth.

Mapping Lysine acetylated proteins in *E. coli*.

The profound phenotypic effects observed in the mutants indicated that the pattern of lysine acetylation was altered (**Table 1**) (31). In order to prove it, a proteomic study was carried out to identify and quantify acetylated peptides and relate them to physiological changes. Immunoprecipitation of acetylated peptides followed by MS-based proteomics was performed: stable isotope dimethyl labelling was used for the relative quantification of peptide acetylation ratios between strains (39).

For each condition, four biological replicates were analysed. Overall, 2502 acetylated peptides were detected belonging to 809 different proteins (**Suppl. Table 3**). Approximately half of these proteins were acetylated on one single residue, but over 20% of these proteins were highly acetylated (*i.e.*, modified in more than three sites) (**Suppl. Fig. 2**).

We quantified the relative ratio of peptide acetylation in *cobB* and *patZ* mutants compared to the wild type under four different environmental conditions. As

expected, the phenotypes of mutants mirrored altered peptide acetylation ratios of proteins as detailed below.

Table 1. Physiological characterization of *Escherichia coli* and its knockout mutants grown in glucose batch and glucose limited chemostat cultures.

Physiology	wt	Δ*cobB*	Δ*patZ*	Δ*cobB*Δ*aceK*	Δ*patZ*Δ*aceK*
Glucose batch cultures					
μ^{max} (h^{-1})	0.74±0.02	0.68±0.03	0.70±0.01	0.65±0.01	0.67±0.01
q_{glc} [mmol · (g h)$^{-1}$]	-8.87±1.19	-10.51±1.74	-8.20±0.16	-8.21±0.44	-8.36±1.79
q_{acet} [mmol · (g h)$^{-1}$]	4.42±0.17	5.98±0.22	3.97±1.03	6.07±0.15	4.49±0.11
$Y_{cel/glc}$ (g · g^{-1})	0.52±0.03	0.52±0.02	0.56±0.01	0.50±0.02	0.46±0.04
Glucose Chemostat cultures					
μ^{max} (h^{-1})	0.23±0.01	0.19±0.00	0.23±0.01	0.19±0.01	0.23±0.01
q_{glc} [mmol · (g h)$^{-1}$]	-2.70±0.17	-3.68±0.23	-2.64±0.09	-2.55±0.01	-2.85±0.28
q_{acet} [mmol · (g h)$^{-1}$]	0.00±0.00	0.63±0.14	0.00±0.00	0.62±0.03	0.00±0.00
$Y_{cel/glc}$ (g · g^{-1})	0.42±0.02	0.27±0.02	0.44±0.03	0.37±0.01	0.41±0.03

The mutant Δ*patZ*, deficient in the best-known lysine acetyltransferase, did not show many changes in acetylation ratios in glucose cultures (**Fig. 1A, B and C**). Although a lower acetylation status was expected in this mutant, this was only the case in chemostat cultures, where the abundance of almost 7% of the acetylated peptides found was at least half compared to the wild type (**Fig. 1C**). However, none of the proteins with decreased acetylation levels have been demonstrated previously to be PatZ substrates. The acetylation ratios of the Δ*patZ* mutant in exponential phase glucose cultures were hardly altered compared with the wild type (**Fig. 1A**), nor was its growth phenotype in this condition. It could be argued that, since in exponential phase the *patZ* gene expression is low, there should not be many differences in acetylation ratios in the Δ*patZ* mutant. However, also in glucose-limited chemostat cultures the acetylation ratios did not show the expected trend. This might be explained by the presence of at least 25 putative acetyltransferases existing in *E. coli* (32), that could potentially take over the function of PatZ. Surprising changes in the ratio of peptide acetylation were observed in acetate cultures and, to lesser extent, in the stationary phase glucose cultures in the Δ*patZ* mutant (**Fig.1 B and D**). Deletion of *patZ* gene led to an overall over-acetylation of the whole proteome: the acetylation ratio of 75% of peptides was more than twice that of the wild type in acetate cultures **Fig. 1D**). Despite the high

impact of *patZ* deletion on protein acetylation, no evident phenotypic effects were observed.

Deletion of *cobB* caused bigger phenotypic changes. The growth of this mutant was altered in all conditions assayed (**Table 1**). Deletion of the only deacetylase known in *E. coli* should increase the degree of acetylation of proteins. Our results confirmed that CobB has a major role as deacetylase in *E. coli*. Over 17% of the acetylated peptides quantified showed increased acetylation under each of the conditions (at least 2-fold in the *cobB* mutant compared to the wild type) (**Fig. 1E-H**). Moreover, in the conditions where the change in phenotype was more profound, *i.e.* acetate and chemostat cultures, the number of peptides observed with increased acetylation status was also higher (30% and 21% respectively).

The analysis of the consensus motif of acetylated sites in *E. coli* is shown in **Fig. 2A**. The glycine residue at position -1 is known to be conserved (29) and, as previously reported, acetylated lysines are more likely found near other lysines, thus decreasing the length of the tryptic peptides (40, 53). Another characteristic of the acetylation motif in *E. coli* is the high abundance of aspartic and glutamic residues close to the acetylated lysine, which has also been observed in the acetylome of rat and *Thermus thermophilus* (8, 54).

The analysis of the functions of acetylated proteins sheds light on the major biological processes affected. In our study, 64% of the modified proteins detected have a metabolic function, and almost 80% of these are involved in primary metabolism, such as the pathways related to the metabolism of nitrogenated compounds (*e.g.* nucleotides and amino acids biosynthesis), and carbohydrates (**Fig. 2B**). Other functions are also over-represented in our data set (*e.g.* functions related to sensing and stimulus response). Additionally, almost 7% of the acetylated proteins have a role in transcription, showing that lysine acetylation can have multiple biological functions, besides metabolism, like cell communication, stress survival and the transcriptional machinery (**Suppl. Table 5**).

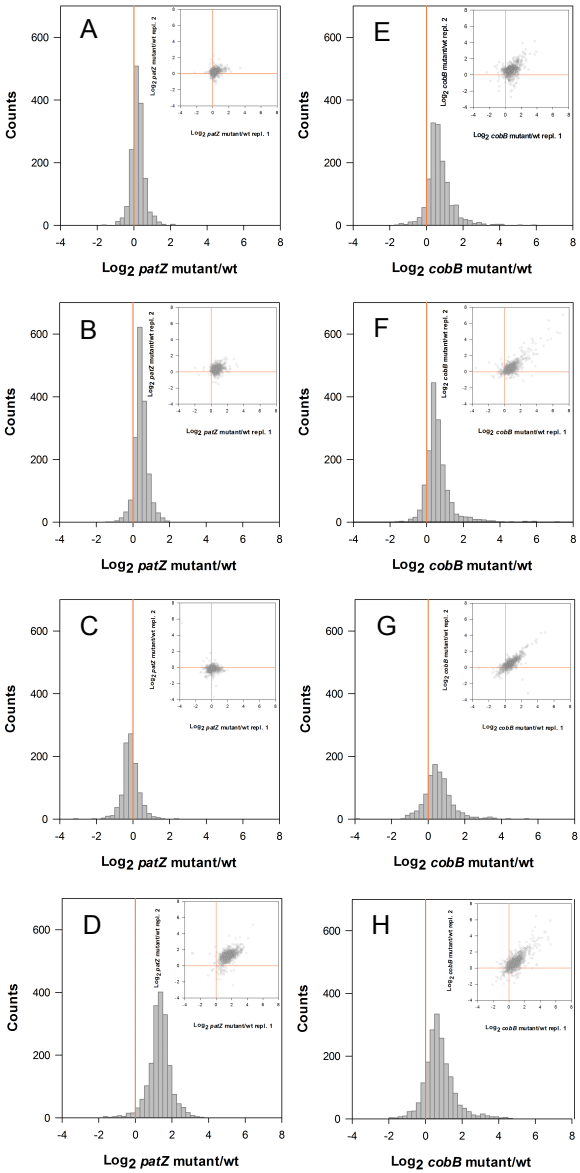


Fig. 1. Frequency histogram of the acetylated sites ratio and acetylated peptides Log_2 ratio for Δ*patZ*/wt (A-D) and Δ*cobB*/wt (E-I) in glucose exponential phase (A, E), glucose stationary phase (B, F), glucose limited cultures (C, G) and acetate cultures (D, H).

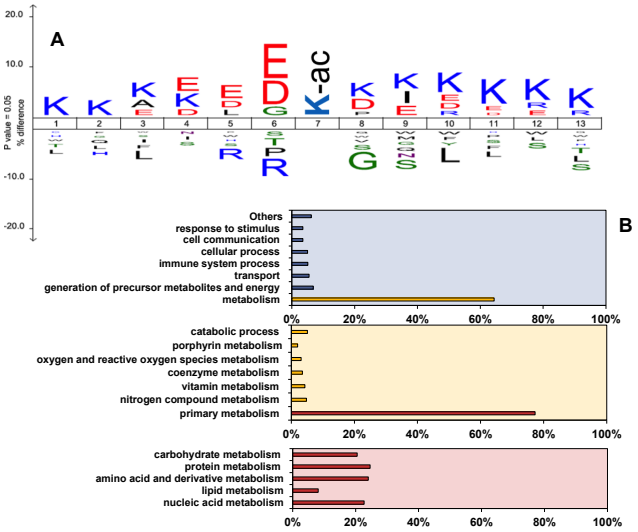


Fig. 2. Conserved sequence surrounding the acetylated lysine in the peptides identified in this study, obtained using IceLogo software with a $p<0.05$ **(A)**. More representative Biological processes found in the lysine acetylated proteins in this study, using PantherDB. **(B).**

Changes in the acetylation profiles do not provide sufficient evidence for true regulatory roles. Multiple proteins are affected by this PTM but, guided by the physiology, gene expression and protein acetylation profiles of the mutants, we focused on the pathways which were quantitatively most affected in attempt to identify physiologic roles of lysine acetylation. A clear shift in the acetate overflow led us to investigate the role of acetylation on the regulation of the two pathways that are essential for its assimilation: acetyl-CoA synthetase and the glyoxylate shunt. In addition, gene expression profiles underlined a clear effect on the motility and acid stress genes, which are both belonging to the RcsB regulon. In the following sections, major implications of protein lysine acetylation in these pathways will be dissected.

The relationship between protein acetylation and acetate metabolism in *E. coli*

In glucose limited chemostat cultures, acetate overflow is a function of the dilution rate due to catabolite repression of the acetyl-CoA synthetase encoding gene

(*acs*) (37, 55, 56). The Δ*cobB* strain produces acetate in low dilution rate glucose chemostat cultures; in fact, it has a phenotype similar to the Δ*acs* mutant (**Suppl. Fig. 1**), where yield is limited by its inability to scavenge overflown acetate (37). The importance of the de-acetylation of this enzyme for acetate metabolization has been demonstrated in a previous study, since the Δ*cobB* mutant cannot grow efficiently on acetate (31). However, the reduced growth rate and biomass yield under acetate and chemostat conditions cannot be explained by the modification of one single enzyme (**Table 1**) and, most likely, the phenotypes observed are the result of more profound effects exerted on other pathways essential for acetate assimilation.

To further demonstrate the effect of acetylation on acetate metabolism, the deacetylation of acetyl-CoA synthetase by CobB was demonstrated *in vitro*. This enzyme is more active in its deacetylated form: activity increased 40 times after incubation with CobB compared with the control without the deacetylase or with the CobB inhibitor nicotinamide (NAM). Deacetylation was confirmed by western blotting (**Fig. 3A**). In these *in vitro* assays, acetylation of K609 was detected by LC-MS/MS in the negative controls but not in the CobB deacetylation reaction. This conserved residue has been previously shown to be deacetylated in *Salmonella enterica* and other bacteria (16, 57)**.**

The over-acetylation of acetyl-CoA synthetase was reflected by *in vivo* activity levels. Acetyl-CoA synthetase activity in the Δ*cobB* mutant is almost half of that of the wild type in glucose-limited chemostat cultures and four times lower in acetate cultures (**Fig. 3B**). The peptide containing K609 was found in the wild type strain and the Δ*cobB* mutant but was not present in the *patZ* mutant (**Suppl. Fig. 3**). Eight additional acetylation sites were found in Acs in chemostat and acetate cultures, but none of their acetylation ratios showed significant changes in the Δ*cobB* mutant, suggesting that they cannot be deacetylated by CobB.

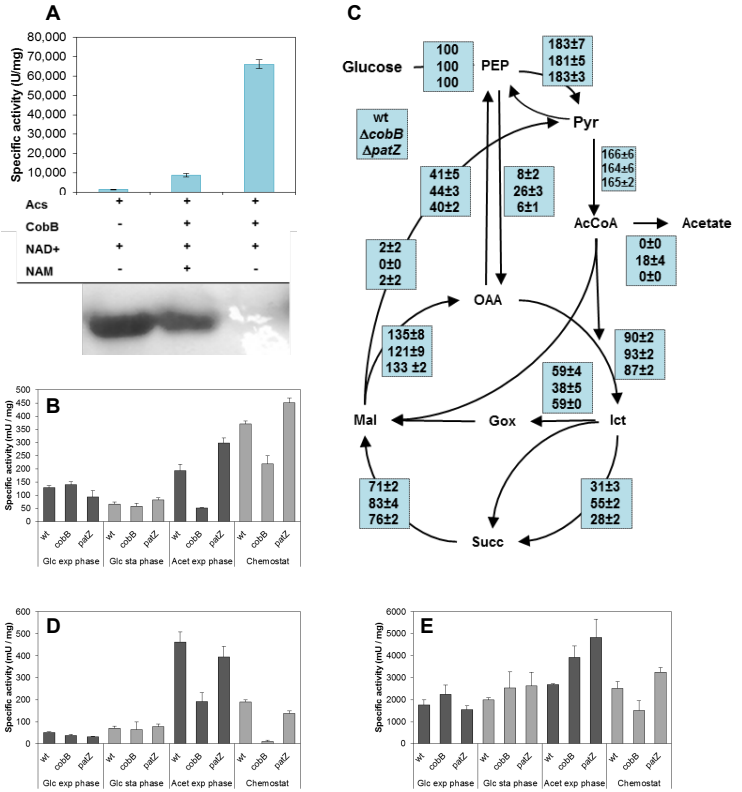


Fig. 3. *In vitro* deacetylation of Acs enzyme (**A**). Affinity purified Acs protein was deacetylated with purified CobB. Deacetylation controls were performed in the absence of CobB and in the presence of the CobB inhibitor nicotinamide (NAM). The effect of deacetylation was assessed by specific enzyme activity assays and western blotting using an anti-acetyl-lysine antibody. Cell physiology *of E. coli* BW25113 and its *cobB* and *patZ* mutants. Enzyme activities of (**B**) Acs, (**D**) AceA, (**E**) Icd in cell free extracts from batch glucose exponential and stationary phase cultures, acetate batch cultures and glucose limited cultures. (**C**) Metabolic fluxes in ^{13}C-labelled glucose limited chemostat cultures run at a D=0.2 h^{-1}.

The reduced growth rate and biomass yield of the Δ*cobB* mutant under acetate and chemostat conditions indicate that other acetate assimilation pathway could be affected. We hypothesized that probably the glyoxylate shunt was affected by an over-acetylation in the Δ*cobB* mutant. This pathway is essential for growth on acetate as the sole carbon source and for glucose catabolism in glucose limited conditions (48). To further explore the functional consequences of the acetylation of metabolic enzymes, fluxes were

determined **(Suppl. Table 4)**. Flux through the glyoxylate shunt decreased by 50% in the Δ*cobB* mutant in glucose chemostat cultures **(Fig 3)**. The isocitrate node is an important regulation point for anabolism and catabolism. Isocitrate lyase (glyoxylate shunt) and isocitrate dehydrogenase (TCA cycle) compete for their common substrate, and fluxes through the node are regulated by reversible phosphorylation of isocitrate dehydrogenase (58–60). It has been proposed in *S. enterica* that this metabolic node is controlled by the acetylation of the bifunctional isocitrate dehydrogenase phosphatase/kinase AceK, (14). However, acetylation of AceK was not detected in our proteomic study. Also, quantification of metabolic fluxes in the Δ*cobB*Δ*aceK*, Δ*patZ*Δ*aceK* mutants demonstrated that lysine acetylation was not affecting AceK function (See **Suppl. Mat.** for further information).

Proteomic evidences of the acetylation of isocitrate lyase have been found. Thirteen acetylation sites were found in isocitrate lyase, and acetylation levels of five of them (K13, K34, K308, K326 and K331) were increased in the Δ*cobB* mutant compared to the wild type strain in chemostat cultures, and one of them (K308) in acetate cultures. *In vitro* isocitrate lyase deacetylation assays were carried out but the enzyme activity was not altered after incubation with CobB, potentially because the enzyme was purified activated (*i.e.*, in the non-acetylated form) or acetylated at lysine residues that CobB is unable to remove. Relative quantification of the unmodified protein expression patterns in the different conditions revealed the big changes in protein levels in the Δ*cobB* mutant. Glyoxylate shunt proteins were less abundant in the Δ*cobB* mutant (approximately 50% of the levels observed in the wild type) (**Supplem. Fig. 4**). Finally, the enzyme activities of isocitrate lyase and isocitrate dehydrogenase were measured (**Fig. 3B and 3C**). In acetate cultures and glucose-limited cultures, the activity of isocitrate lyase was lower in the Δ*cobB* mutant compared with the wild type strain. This was especially true in chemostat cultures, where it decreased almost 20 times. This low activity, which is in agreement with the observed fluxes, cannot be explained by relative protein quantification.

All these evidences suggest that the regulation of the isocitrate node involves the cross-talk between phosphorylation and acetylation, both acting at different levels: a

gross regulation mechanism undertaken by AceK that blocks the flux through the TCA cycle, and a fine-tuning regulation of the glyoxylate shunt, which is partially inhibited by acetylation.

Protein lysine acetylation regulates cellular motility and acid stress response in *E. coli*.

A high number of acetylated transcriptional regulators have been found in our study. The post-translational modified transcriptional regulators identified in any condition (**Supplem. Table 5**) represent 7% of the total acetylation sites found. Many of the acetylated peptides identified showed increase in acetylation in the Δ*cobB* mutant. Since the acetylation of transcription factors may impair DNA-binding or protein-protein interaction affecting transcriptional regulation, changes in protein acetylation in the Δ*cobB* knockout mutant may indirectly tune the transcription of many genes. To test this, DNA microarray studies were performed in *E. coli* wild type and its knockout strain Δ*cobB* **(Suppl. Tables 7-10).** Genes differentially expressed in glucose exponential phase of batch cultures and steady state chemostat cultures in the *cobB* mutant were analysed using hierarchical clustering, showing that almost all significant changes were similar in both conditions (**Suppl. Fig. 5**). All the genes related to bacterial motility were the most up regulated in Δ*cobB*, while some genes involved in stress and pH response revealed down-regulation **(Fig. 4)**.

The genes, involved in bacterial motility, can be classified into three functional groups according to the hierarchical regulation of their transcription (**Fig. 4**) (61–63). Our data suggest that up-regulation of this pathway begins upstream of Class I genes (*flhDC* operon). This means that probably one of the transcription factors regulating this operon is responsible for this global de-regulation. None of the transcriptional regulators of this operon showed differential gene expression in the Δ*cobB* mutant, suggesting that increased acetylation of a transcriptional regulator in the Δ*cobB* mutant could be responsible for the differential expression of this regulon. The high alterations observed in the expression of motility and chemotaxis genes and the acid resistance system in the Δ*cobB* mutant led us to think that probably these effects were caused by a specific transcription factor (**Fig. 4**). All these evidences led us to

hypothesize that the differential gene expression observed in the Δ*cobB* mutant could be caused by the inactivation of the transcription factor RcsB.

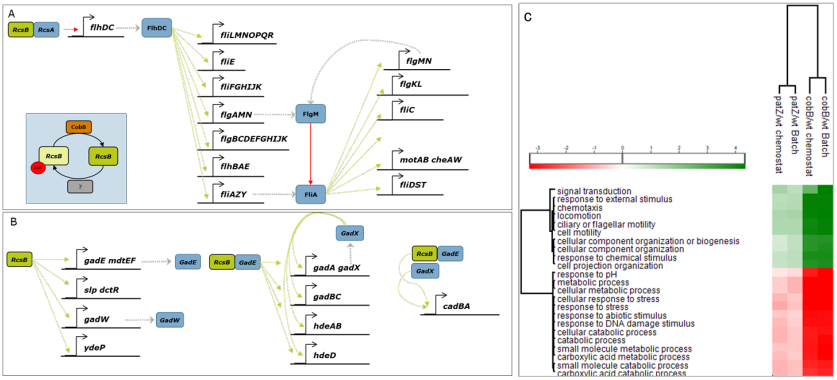


Fig. 4. (A) RcsB transcriptional regulation in the flagellar regulon and **(B)** acid stress response genes (64)**.(C)** Annotation matrix obtained from the gene expression microarray data where the main functions of the up and down regulated genes are grouped by their expression, for further information (65).

It has been previously described that *E. coli* RcsB is acetylated *in vitro* by a protein acetyltransferase from *S. enterica* at K180 (66). Also recently it has been observed that K154 of RcsB is chemically acetylated by acetyl-phosphate and this lysine is deacetylated by CobB (36). This lysine is located in the helix-turn-helix (HTH) LuxR-type domain that directly interacts with its consensus DNA binding sequence (67). The acetylation of this residue could modify the interaction of this protein with some of the promoters of the genes analysed. In our proteomic results, we observed a higher acetylation of lysine 154 of RcsB in the Δ*cobB* mutant. This fact, together with the differential gene expression of the flagella regulon and acid stress response genes in this mutant, indicates that the acetylation of this transcription factor impairs RcsB activity. In order to prove this hypothesis and observe the physiological consequences of the acetylation of this transcription factor, physiological tests were carried out.

The acetylation of lysine 154 in the Δ*cobB* mutant increased the expression of the flagella regulon, provoking an increase in motility and number of flagella compared

with the parent strain, showing a behaviour similar to the Δ*rcsB* null mutant (**Fig. 5 A1-2 and B1-2**). Loss of function of acetylated RcsB also triggered the down-regulation of the acid stress response genes. Survival in an acidic environment requires glutamate, lysine or arginine decarboxylase enzymes. These proteins catalyse the decarboxylation of these amino acids consuming a proton in the reaction, thus maintaining the pH homeostasis. The genes belonging to glutamate-dependent acid response system (AR2), the acid response chaperones *hdeA* and *hdeB*, and others belonging to this survival system (**Fig. 4**), all of them activated by RcsB, were down regulated in the Δ*cobB* mutant. Acid survival of *E. coli* strains was assessed after two hours incubation at low pH (2.5). The deletion of *rcsB* impaired acid stress survival. In contrast, survival of the Δ*cobB* mutant was slightly higher, which is consistent with low level expression of acid resistance related genes in this mutant.

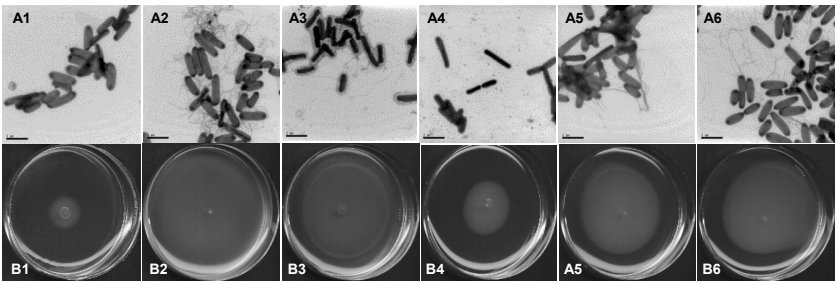


Fig. 5. Flagella **(A)** and mobility assays **(B)** of the *E. coli* wild type strain **(1)**, and mutants Δ*cobB* **(2)**, Δ*rcsB* **(3),** Δ*rcsB*+p*rcsB*-K154R **(4)** Δ*rcsB*+p*rcsB*-K154Q **(5)** and Δ*rcsB*+p*rcsB*-K154E **(6).**

To demonstrate that the acetylation of lysine 154 is responsible for the phenotypic and transcriptional changes, site directed mutagenesis was performed in order to mimic the electrostatic charge of a non-acetylated lysine (K154R) and an acetylated lysine (K154Q). These mutants were transformed into a Δ*rcsB* background. The non-acetylated K154R RcsB mutant has a similar phenotype compared with the native construction, meaning that this mutation does not affect its function. In contrast, the K154Q RcsB mutation, mimicking a permanently acetylated lysine showed the same

behaviour as the Δ*cobB* mutant (**Fig. 5**). Similarly, the mutations of K154 of RcsB also affected acid stress survival differently. Acid survival was higher in the non-acetylated lysine derivative (K154R) and almost negligible in the K154Q, as in the *rcsB* mutant. Accordingly, glutamate decarboxylase activity was high in the wild type, Δ*rcsB* complemented with *rcsB* and Δ*rcsB* containing the plasmid with the *rcsB* K154R mutant strains, while activity was low in Δ*cobB* and Δ*rcsB* mutants and *rcsB*-K154Q in Δ*rcsB* (**Fig. 6**).

Altogether, this demonstrates that acetylation of lysine 154 of RcsB transcription factor impairs its function, affecting flagella biosynthesis and bacterial motility and decreasing acid stress survival.

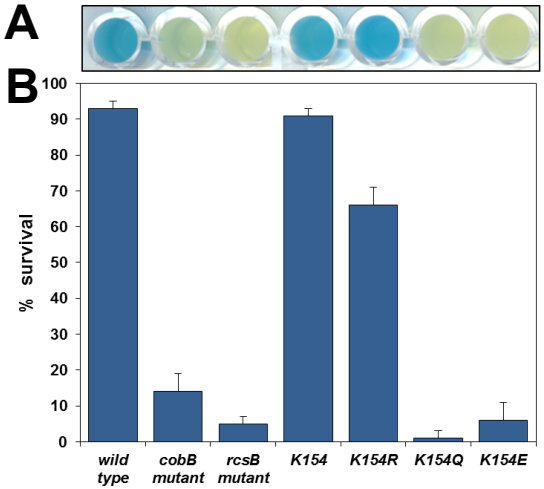


Fig.6. Acid stress response assays. **(A)** Colorimetric assay of glutamate descarboxilase enzyme activity. **(B)** Acid stress survival of the different *E. coli* mutants. The *rcsB* knockout mutant was complemented with the *rcsB* wild type gene (K154) and its different mutants mimicking different acetylation states of lysine 154 (K154R, K154Q and K154E).

Discussion

In the last years, several acetylome characterizations were reported in both prokaryotes and eukaryotes, but functional evidence for the roles of reversible protein acetylation is scarce. In this study we have addressed this problem taking a systems biology approach, merging proteomic, transcriptomic, metabolic and fluxomic data with molecular biology studies. This allowed us to demonstrate that the deacetylase activity of CobB is global, contributing to the deacetylation of a big number of substrates and, therefore, it has a major impact on bacterial physiology. Lysine acetylation affects protein functionality and cell phenotype directly, modulating the activity of metabolic enzymes, or indirectly, affecting transcriptional regulators. Regulation of particular physiological processes has been demonstrated *in vivo*: protein deacetylation by CobB activates acetate metabolism and regulates the expression of flagella biosynthesis, motility and acid stress survival.

The link between acetate metabolism and protein acetylation is well known (16, 22, 24). In addition to its well-demonstrated role in the regulation of acetyl-CoA synthetase, we have also shown that protein acetylation contributes to regulating the flux through the glyoxylate shunt. Wang and col. have recently described that protein acetylation affects the relative activity of glycolysis, gluconeogenesis and glyoxylate shunt in *S. enterica* by targeting the activity of the glyceraldehyde-phosphate dehydrogenase, isocitrate lyase and isocitrate dehydrogenase phosphatase/kinase (14). There is some controversy around these results; other authors have claimed that these results are not reproducible (29). In fact, we have not observed acetylation of AceK in *E. coli*, and our results suggest that its activity is not regulated by acetylation in this microorganism.

We have demonstrated that over-acetylation of RcsB exerts several physiological effects: it impairs flagella synthesis, motility and chemotaxis and compromises acid stress survival. Interestingly, the transcriptomic effects showed in the *cobB* mutant where similar to those observed in Δ*patZ*, probably due to increased chemical acetylation in this mutant (**Fig. 4**). Acetylation of RcsB by the

acetyltransferase from *S. enterica* has been previously reported, although modification occurred at a different lysine residue (66). More recently, acetylation of K154 of RcsB has been described, although these authors claimed that it did not affect motility, probably due to the use of a different strain for the motility tests. However, we also observed that over-acetylation of RcsB in K154 represses transcription of (**Suppl. Table 9**) gene as described in the same study (36).

The phenotype of the Δ*cobB* mutant was significantly affected, mirroring the peptide acetylation ratios of proteins, however, this was not the case of the *patZ* mutant which growth was not affected in acetate cultures despite the high peptide acetylation ratios assessed. An unaltered phenotype despite a severely affected acetylation pattern was also described before in an acetate kinase (*ackA*) mutant (30). This evidences that not all acetylation events have an effect on protein function, and an increase in acetylation of proteins may have no evident effect on cell physiology.

Besides the existence of several protein acetyltransferases which may differ in their specificity towards targeted proteins and in the environmental signals to which they respond, there are increasing evidences that many proteins are either chemically or autocatalytically acetylated (30, 68–71). The high protein acetylation ratios of the *patZ* mutant in acetate cultures are in agreement with this hypothesis and suggest that PatZ could modulate chemical acetylation by regulating the levels of acetylating metabolites. PatZ inhibits acetyl-CoA synthetase that could lead to increased intracellular concentration of acetyl-CoA. Acetyl-phosphate could also be responsible for this acetylation pattern; in fact, the activity of phosphotransacetylase (Pta) also increased in the Δ*patZ* mutant (**Suppl. Fig. 6**). It could be argued that, during evolution, organisms may have evolved two complementary strategies in order to fight chemical acetylation: acetyltransferases, regulating the synthesis of acetylating agents and deacetylases, removing acyl moieties from proteins.

The abundance of acid residues in the protein acetylation motif can shed light on the specificity of protein modification and explain why chemical acetylation is frequent even under mild physiological conditions. Acid residues are also over-represented in the acetylation motifs of mitochondrial and cytoplasmic proteins in rat

(8) and other microorganisms (54). Aspartate and glutamate residues in the vicinities of targeted lysines could enhance the nucleophilicity of lysil residues, which would attack acetylating agents. This mechanism would be in agreement with both enzymatic and chemical acetylation (30, 72, 73).

Despite the widespread acetylation of proteins, the number of known substrates of the sirtuin CobB is limited. The discovery of new substrates of CobB has been driven by *in vitro* techniques (16, 34, 36, 66) and also high-throughput MS-based proteomics. In a previous study (6), the number of CobB substrates (log_2 acetylation ratio >1 for Δ*cobB* mutant compared to wild type) was approximately 10% (≈366 peptides) while in our study 40% of all the acetylated peptides detected are over this cut-off value, in at least one condition (1025 peptides). Interestingly, both datasets reveal that there is no relevant deacetylation motif for CobB, which preferentially recognizes acetyl-lysine residues in disorganized regions of the proteins or in the protein termini (30, 74). The high chemical reactivity of the lysine side chain may indicate that neutralization of its positive charge by acetylation could alter function of proteins. A long list of targeted lysines has been built in this work, which should be validated before physiologic roles can be ascertained. We have only validated roles of the differential acetylation of three proteins, although other acetylation events can be understood based on bibliography. This is the case of K326 of the gluconeogenic fructose-1,6-bisphosphate aldolase class II (FbaA), which mutation leads to a loss of 94% of activity (75), and K119 of molybdopterin synthase (MoaE), which mutation inhibits its activity completely (76). Further work is needed to demonstrate other regulatory acetylations.

Current quantitative proteomics methods cannot determine the relative abundance of these modifications in the total population of a cellular protein, which is a key limitation in order to fully understand how will they impact cell physiology. The mechanisms and roles of lysine acetylation in bacteria are still unclear; the functionality of acetylated sites should be uncovered, and regulation of the synthesis of acetylating agents should be integrated in the context of metabolism. Important efforts are needed to complete the picture of the regulatory roles of post-translational acetylation of proteins in bacterial physiology.

Bibliography

1. Gerosa, L., Kochanowski, K., Heinemann, M., and Sauer, U. (2013) Dissecting specific and global transcriptional regulation of bacterial gene expression. *Mol. Syst. Biol.* 9, 658
2. Heinemann, M., and Sauer, U. (2010) Systems biology of microbial metabolism. *Curr. Opin. Microbiol.* 13, 337–43
3. Martin, C., and Zhang, Y. (2005) The diverse functions of histone lysine methylation. *Nat. Rev. Mol. Cell Biol.* 6, 838–49
4. Kai, Z., Chen, Y., Zhang, Z., and Zhao, Y. (2010) Identification and Verification of Lysine Propionylation and Butyrylation in Yeast Core Histones Using PTMap Software. *J Proteome Res* 8, 900–906
5. Hershko, A., and Ciechanover, A. (1998) The ubiquitin system. *Annu. Rev. Biochem.* 67, 425–79
6. Weinert, B. T., Schölz, C., Wagner, S. a, Iesmantavicius, V., Su, D., Daniel, J. a, Choudhary, C., and Scho, C. (2013) Lysine succinylation is a frequently occurring modification in prokaryotes and eukaryotes and extensively overlaps with acetylation. *Cell Rep.* 4, 842–51
7. Sterner, D. E., and Berger, S. L. (2000) Acetylation of Histones and Transcription-Related Factors. *Microbiol. Mol. Biol. Rev.* 64, 435–459
8. Lundby, A., Lage, K., Weinert, B. T., Bekker-Jensen, D. B., Secher, A., Skovgaard, T., Kelstrup, C. D., Dmytriyev, A., Choudhary, C., Lundby, C., and Olsen, J. V (2012) Proteomic analysis of lysine acetylation sites in rat tissues reveals organ specificity and subcellular patterns. *Cell Rep.* 2, 419–31
9. Henriksen, P., Wagner, S. a, Weinert, B. T., Sharma, S., Bacinskaja, G., Rehman, M., Juffer, A. H. A. H., Walther, T. C., Lisby, M., and Choudhary, C. (2012) Proteome-wide analysis of lysine acetylation suggests its broad regulatory scope in *Saccharomyces cerevisiae*. *Mol. Cell. Proteomics* 11, 1510–22
10. Van Noort, V., Seebacher, J., Bader, S., Mohammed, S., Vonkova, I., Betts, M. J., Kühner, S., Kumar, R., Maier, T., O'Flaherty, M., Rybin, V., Schmeisky, A., Yus, E., Stülke, J., Serrano, L., Russell, R. B., Heck, A. J. R., Bork, P., and Gavin, A.-C. (2012) Cross-talk between phosphorylation and lysine acetylation in a genome-reduced bacterium. *Mol. Syst. Biol.* 8, 571
11. Zhang, K., Zheng, S., Yang, J. S., Chen, Y., and Cheng, Z. (2012) Comprehensive Profiling of Protein Lysine Acetylation in *Escherichia coli*. *J. Proteome Res.* 12, 844–51
12. Jo, B., Kim, J. A., Moon, J. H., Ryu, S. E., Pan, J. G., Yu, B. J., and Yu, Byung Jo, Jung Ae Kim, Jeong Hee Moon, Seong Eon Ryu, A. J.-G. P. (2008) The Diversity of Lysine-Acetylated Proteins in *Escherichia coli*. *J. Microbiol. Biotechnol.* 18, 1529–1536
13. Zhang, J., Sprung, R., Pei, J., Tan, X., Kim, S., Zhu, H., Liu, C.-F., Grishin, N. V, and Zhao, Y. (2009) Lysine acetylation is a highly abundant and evolutionarily conserved modification in *Escherichia coli*. *Mol Cell Prot* 8, 215–225
14. Wang, Q., Zhang, Y., Yang, C., Xiong, H., Lin, Y., Yao, J., Li, H., Xie, L., Zhao, W., Yao, Y., Ning, Z.-B., Zeng, R., Xiong, Y., Guan, K.-L., Zhao, S., and Zhao, G.-P. (2010) Acetylation of metabolic enzymes coordinates carbon source utilization and metabolic flux. *Science* 327, 1004–7
15. Kim, D., Yu, B. J., Kim, J. A., Lee, Y.-J., Choi, S.-G., Kang, S., and Pan, J.-G. (2013) The Acetylproteome of Gram-positive Model Bacterium *Bacillus subtilis*. *Proteomics J.* 13, 1–28

16. Starai, V. J., Celic, I., Cole, R. N., Boeke, J. D., and Escalante-Semerena, J. C. (2002) Sir2-dependent activation of acetyl-CoA synthetase by deacetylation of active lysine. *Science (80-.).* 298, 2390–2392
17. Starai, V. J., and Escalante-Semerena, J. C. (2004) Identification of the protein acetyltransferase (Pat) enzyme that acetylates acetyl-CoA synthetase in *Salmonella enterica*. *J Mol Biol* 340, 1005–1012
18. Tucker, A. C., and Escalante-Semerena, J. C. (2010) Biologically Active Isoforms of CobB Sirtuin Deacetylase in Salmonella enterica and *Erwinia amylovora*. *J. Bacteriol.* 192, 6200–6208
19. Ho Jun, L., Lang, P. T., Fortune, S. M., Sassetti, C. M., and Alber, T. (2013) Cyclic-AMP regulation of protein lysine acetylation in *Mycobacterium tuberculosis*. *Nat. Struct. Biol.* 19, 811–818
20. Hayden, J. D., Brown, L. R., Gunawardena, H. P., Perkowski, E. F., Chen, X., and Braunstein, M. (2013) Reversible acetylation regulates acetate and propionate metabolism in *Mycobacterium smegmatis*. *Microbiology* 159, 1986–99
21. Nambi, S., Basu, N., and Visweswariah, S. (2010) cAMP-regulated Protein Lysine Acetylases in Mycobacteria. *J Biol Chem* 285, 24313–24323
22. Crosby, H. A., Heiniger, E. K., Harwood, C. S., and Escalante-Semerena, J. C. (2010) Reversible N-ε-lysine acetylation regulates the activity of acyl-CoA synthetases involved in anaerobic benzoate catabolism in *Rhodopseudomonas palustris*. *Mol Microbiol* 76, 874–888
23. Crosby, H. a, Rank, K. C., Rayment, I., and Escalante-Semerena, J. C. (2012) Structural insights into the substrate specificity of the *Rhodopseudomonas palustris* protein acetyltransferase RpPat: identification of a loop critical for recognition by RpPat. *J. Biol. Chem.* 287, 41392–404
24. Gardner, J. G., Grundy, F. J., Henkin, T. M., and Escalante-Semerena, J. C. (2006) Control of acetyl-coenzyme A synthetase (AcsA) activity by acetylation/deacetylation without NAD+ involvement in *Bacillus subtilis*. *J. Bacteriol.* 188, 5460–5468
25. Gardner, J. G., and Escalante-Semerena, J. C. (2009) In *Bacillus subtilis*, the sirtuin protein deacetylase, encoded by the *srtN* gene (formerly yhdZ), and functions encoded by the *acuABC* genes control the activity of acetyl coenzyme A synthetase. *J. Bacteriol.* 191, 1749–1755
26. Mikulik, K., Felsberg, J., Kudrnáčová, E., Bezoušková, S., Šetinová, D., Stodůlková, E., Zídková, J., Zídek, V., and Setinová, D. (2012) CobB1 deacetylase activity in *Streptomyces coelicolor*. *Biochem. Cell Biol.* 90, 179–87
27. Zhao, K., Chai, X., and Marmorstein, R. (2004) Structure and substrate binding properties of CobB, a Sir2 homolog protein deacetylase from *Escherichia coli*. *J Mol Biol* 337, 731–741
28. Tsang, a W., and Escalante-Semerena, J. C. (1998) CobB, a new member of the SIR2 family of eucaryotic regulatory proteins, is required to compensate for the lack of nicotinate mononucleotide:5,6-dimethylbenzimidazole phosphoribosyltransferase activity in cobT mutants during cobalamin biosynthesis. *J Biol Chem* 273, 31788–31794
29. Crosby, H. a, Pelletier, D. a, Hurst, G. B., and Escalante-Semerena, J. C. (2012) System-wide studies of N-lysine acetylation in *Rhodopseudomonas palustris* reveal substrate specificity of protein acetyltransferases. *J. Biol. Chem.* 287, 15590–601
30. Weinert, B. T. T., Iesmantavicius, V., Wagner, S. A. A., Schölz, C., Gummesson, B., Beli, P., Nyström, T., Choudhary, C., Scho, C., and Nystro, T. (2013) Acetyl-Phosphate Is a Critical Determinant of Lysine Acetylation in *E . coli*. *Mol. Cell* 51, 1–8
31. Castaño-Cerezo, S., Bernal, V., Blanco-Catalá, J., Iborra, J. L., and Cánovas, M. (2011) cAMP-CRP co-ordinates the expression of the protein acetylation pathway with central metabolism in *Escherichia coli*. *Mol. Microbiol.* 82, 1110–1128

32. Lima, B. P., Thanh Huyen, T. T., Bäsell, K., Becher, D., Antelmann, H., and Wolfe, A. J. (2012) Inhibition of acetyl phosphate-dependent transcription by an acetylatable lysine on RNA polymerase. *J. Biol. Chem.* 287, 32147–60
33. Li, R., Gu, J., Chen, Y.-Y., Xiao, C.-L., Wang, L.-W., Zhang, Z.-P., Bi, L.-J., Wei, H.-P., Wang, X.-D., Deng, J.-Y., and Zhang, X.-E. (2010) CobB regulates Escherichia coli chemotaxis by deacetylating the response regulator CheY. *Mol. Microbiol.* 76, 1162–1174
34. Zhang, Q., Gu, J., Gong, P., Wang, X., Tu, S., Bi, L., Yu, Z., Zhang, Z., Cui, Z., Wei, H., Tao, S., and Zhang, X. (2013) Reversibly acetylated lysine residues play important roles in the enzymatic activity of *Escherichia coli* N-hydroxyarylamine O-acetyltransferase. *FEBS J.* 280, 1966–79
35. Thao, S., Chen, C., Zhu, H., and Escalante-Semerena, J. C. (2010) Ne-Lysine Acetylation of a Bacterial Transcription Factor Inhibits Its DNA-Binding Activity. *PLoS One* 5, e15123
36. Hu, L. I., Chi, B. K., Kuhn, M. L., Filippova, E. V, Walker-Peddakotla, A. J., Bäsell, K., Becher, D., Anderson, W. F., Antelmann, H., and Wolfe, A. J. (2013) Acetylation of the Response Regulator RcsB Controls Transcription from a Small RNA Promoter. *J. Bacteriol.*,
37. Renilla, S., Bernal, V., Fuhrer, T., Castaño-Cerezo, S., Pastor, J. M., Iborra, J. L., Sauer, U., and Cánovas, M. (2012) Acetate scavenging activity in *Escherichia coli*: interplay of acetyl-CoA synthetase and the PEP-glyoxylate cycle in chemostat cultures. *Appl Microbiol Biotechnol* 95, 2109–2124
38. Nanchen, A., Schicker, A., and Sauer, U. (2006) Nonlinear Dependency of Intracellular Fluxes on Growth Rate in Miniaturized Continuous Cultures of *Escherichia coli* Nonlinear Dependency of Intracellular Fluxes on Growth Rate in Miniaturized Continuous Cultures of Escherichia coli. *Appl. Environ. Microbiol.* 72, 1164–1172
39. Boersema, P. J., Raijmakers, R., Lemeer, S., Mohammed, S., and Heck, A. J. R. (2009) Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics. *Nat. Protoc.* 4, 484–94
40. Choudhary, C., Kumar, C., Gnad, F., Nielsen, M. L., Rehman, M., Walther, T. C., Olsen, J. V, and Mann, M. (2009) Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science* 325, 834–40
41. Cox, J., and Mann, M. (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat. Biotechnol.* 26, 1367–72
42. Cox, J., Neuhauser, N., Michalski, A., Scheltema, R. A., Olsen, J. V, and Mann, M. (2011) Andromeda: a peptide search engine integrated into the MaxQuant environment. *J. Proteome Res.* 10, 1794–805
43. Castaño-Cerezo, S., Pastor, J. M., Renilla, S., Bernal, V., Iborra, J. L., and Cánovas, M. (2009) An insight into the role of phosphotransacetylase (*pta*) and the acetate/acetyl-CoA node in *Escherichia coli*. *Microb. Cell Fact.* 8, 54
44. Smyth, G. K. (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat. Appl. Genet. Mol. Biol.* 3, Article3
45. Medina, I., Carbonell, J., Pulido, L., Madeira, S. C., Goetz, S., Conesa, A., Tárraga, J., Pascual-Montano, A., Nogales-Cadenas, R., Santoyo, J., García, F., Marbà, M., Montaner, D., and Dopazo, J. (2010) Babelomics: an integrative platform for the analysis of transcriptomics, proteomics and genomic data with advanced functional profiling. *Nucleic Acids Res.* 38, W210–3
46. Kitagawa, M., Ara, T., Arifuzzaman, M., Ioka-Nakamichi, T., Inamoto, E., Toyonaga, H., and Mori, H. (2005) Complete set of ORF clones of Escherichia coli ASKA library (a complete set of *E. coli* K-12 ORF archive): unique resources for biological research. *DNA Res.* 12, 291–9

47. Zamboni, N., Fendt, S.-M., Ruhl, M., and Sauer, U. (2009) 13C-based metabolic flux analysis. *Nat Protoc* 4, 878–892
48. Fischer, E., and Sauer, U. (2003) A novel metabolic cycle catalyzes glucose oxidation and anaplerosis in hungry *Escherichia coli*. *J Biol Chem* 278, 46446–46451
49. Zamboni, N., Fischer, E., and Sauer, U. (2005) FiatFlux - a software for metabolic flux analysis from 13C-glucose experiments. *BMC Bioinformatics* 6, 209
50. Guzman, L. M., Belin, D., Carson, M. J., and Beckwith, J. (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J. Bacteriol.* 177, 4121–30
51. Krin, E., Danchin, A., and Soutourina, O. (2010) RcsB plays a central role in H-NS-dependent regulation of motility and acid stress resistance in *Escherichia coli*. *Res. Microbiol.* 161, 363–71
52. Deininger, K. N. W., Horikawa, A., Kitko, R. D., Tatsumi, R., Rosner, J. L., Wachi, M., and Slonczewski, J. L. (2011) A requirement of TolC and MDR efflux pumps for acid adaptation and GadAB induction in *Escherichia coli*. *PLoS One* 6, e18960
53. Weinert, B. T., Wagner, S. a, Horn, H., Henriksen, P., Wenshe, R., Olsen, J. V, Jensen, L. J., July, C. C., Liu, W. R., and Choudhary, C. (2011) Proteome-wide mapping of the *Drosophila* acetylome demonstrates a high degree of conservation of lysine acetylation. *Sci. Signal.* 4, ra48
54. Okanishi, H., Kim, K., Masui, R., and Kuramitsu, S. (2013) Acetylome with Structural Mapping Reveals the Significance of Lysine Acetylation in *Thermus thermophilus*. *J. Proteome Res.*,
55. Valgepea, K., Adamberg, K., Nahku, R., Lahtvee, P.-J., Arike, L., and Vilu, R. (2010) Systems biology approach reveals that overflow metabolism of acetate in *Escherichia coli* is triggered by carbon catabolite repression of acetyl-CoA synthetase. *BMC Syst Biol* 4, 166
56. Vemuri, G. N., Altman, E., Sangurdekar, D. P., Khodursky, A. B., and Eiteman, M. A. (2006) Overflow metabolism in *Escherichia coli* during steady-state growth: transcriptional regulation and effect of the redox ratio. *Appl. Environ. Microbiol.* 72, 3653–3661
57. Starai, V. J., Gardner, J. G., and Escalante-Semerena, J. C. (2005) Residue Leu-641 of Acetyl-CoA synthetase is critical for the acetylation of residue Lys-609 by the Protein acetyltransferase enzyme of *Salmonella enterica*. *J Biol Chem* 280, 26200–26205
58. Borthwick, A. C., Holms, W. H., and Nimmo, H. G. (1984) Isolation of active and inactive forms of isocitrate dehydrogenase from *Escherichia coli* ML 308. *Eur J Biochem* 141, 393–400
59. LaPorte, D. C., and Koshland, D. E. (1983) Phosphorylation of isocitrate dehydrogenase as a demonstration of enhanced sensitivity in covalent regulation. *Nature* 305, 286–290
60. LaPorte, D. C., Walsh, K., and Koshland, D. E. (1984) The branch point effect. Ultrasensitivity and subsensitivity to metabolic control. *J. Biol. Chem.* 259, 14068–75
61. Kalir, S., and Alon, U. (2004) Using a quantitative blueprint to reprogram the dynamics of the flagella gene network. *Cell* 117, 713–20
62. Claret, L., and Hughes, C. (2002) Interaction of the Atypical Prokaryotic Transcription Activator FlhD2C2 with Early Promoters of the Flagellar Gene Hierarchy. *J. Mol. Biol.* 321, 185–199
63. Liu, X., and Matsumura, P. (1995) An alternative sigma factor controls transcription of flagellar class-III operons in *Escherichia coli*: gene sequence, overproduction, purification and characterization. *Gene* 164, 81–4
64. Keseler, I. M., Mackie, A., Peralta-Gil, M., Santos-Zavaleta, A., Gama-Castro, S., Bonavides-Martínez, C., Fulcher, C., Huerta, A. M., Kothari, A., Krummenacker, M., Latendresse, M., Muñiz-Rascado, L., Ong, Q., Paley, S., Schröder, I., Shearer, A. G., Subhraveti, P., Travers, M., Weerasinghe, D., Weiss, V., Collado-Vides, J., Gunsalus, R. P., Paulsen, I., and Karp, P. D. (2013) EcoCyc: fusing model organism databases with systems biology. *Nucleic Acids Res.* 41, D605–12

65. Geiger, T., Velic, A., Macek, B., Lundberg, E., Kampf, C., Nagaraj, N., Uhlen, M., Cox, J., and Mann, M. (2013) Initial quantitative proteomic map of 28 mouse tissues using the SILAC mouse. *Mol. Cell. Proteomics* 12, 1709–22
66. Thao, S., Chen, C.-S., Zhu, H., and Escalante-Semerena, J. C. (2010) Ne-Lysine Acetylation of a Bacterial Transcription Factor Inhibits Its DNA-Binding Activity. *PLoS One* 5, e15123
67. Zhang, R., Pappas, K. M., Pappas, T., Brace, J. L., Miller, P. C., Oulmassov, T., Molyneaux, J. M., Anderson, J. C., Bashkin, J. K., Winans, S. C., and Joachimiak, A. (2002) Structure of a bacterial quorum-sensing transcription factor complexed with pheromone and DNA. *Nature* 417, 971–4
68. Barak, R., Prasad, K., Shainskaya, A., Wolfe, A. J., and Eisenbach, M. (2004) Acetylation of the chemotaxis response regulator CheY by acetyl-CoA synthetase purified from *Escherichia coli*. *J Mol Biol* 342, 383–401
69. Kuo, Y.-M., and Andrews, A. J. (2013) Quantitating the specificity and selectivity of Gcn5-mediated acetylation of histone H3. *PLoS One* 8, e54896
70. Schwer, B., Eckersdorff, M., Li, Y., Silva, J. C., Fermin, D., Kurtev, M. V, Giallourakis, C., Comb, M. J., Alt, F. W., and Lombard, D. B. (2009) Calorie restriction alters mitochondrial protein acetylation. *Aging Cell* 8, 604–6
71. Ramponi, G., Manao, G., and Camici, G. (1975) Nonenzymatic Acetylation of Histones with Acetyl Phosphate and Acetyl Adenylate. *Biochemistry* 14, 2681–2685
72. Smith, B. C., and Denu, J. M. (2009) Chemical mechanisms of histone lysine and arginine modifications. *Biochim. Biophys. Acta* 1789, 45–57
73. Yan, Y., Harper, S., Speicher, D. W., and Marmorstein, R. (2002) The catalytic mechanism of the ESA1 histone acetyltransferase involves a self-acetylated intermediate. *Nat. Struct. Biol.* 9, 862–9
74. Khan, A. N., and Lewis, P. N. (2005) Unstructured conformations are a substrate requirement for the Sir2 family of NAD-dependent protein deacetylases. *J. Biol. Chem.* 280, 36073–8
75. Zgiby, S. M., Thomson, G. J., Qamar, S., and Berry, A. (2000) Exploring substrate binding and discrimination in fructose1, 6-bisphosphate and tagatose 1,6-bisphosphate aldolases. *Eur. J. Biochem.* 267, 1858–68
76. Wuebbens, M. M., and Rajagopalan, K. V (2003) Mechanistic and mutational studies of *Escherichia coli* molybdopterin synthase clarify the final step of molybdopterin biosynthesis. *J. Biol. Chem.* 278, 14523–32
77. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) Mass Spectrometric Sequencing of Proteins from Silver-Stained Polyacrylamide Gels. *Anal. Chem.* 68, 850–858
78. Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K. A., Tomita, M., Wanner, B. L., and Mori, H. (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* 2, 2006.0008
79. Datsenko, K. A., and Wanner, B. L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* 97, 6640–6645

Appendix

Supplementary information.

The reduced growth rate and biomass yield under acetate and chemostat conditions of the Δ*cobB* mutant indicate that acetate assimilation pathways could be affected. The glyoxylate shunt is essential for growth on acetate as the sole carbon source. In fact, the isocitrate node is an important regulation point of anabolism and catabolism. Isocitrate is a substrate for two enzymes, isocitrate dehydrogenase (Icd) and isocitrate lyase (AceA). Isocitrate dehydrogenase is part of the TCA cycle and its function is mainly catabolic, while isocitrate lyase is part of the glyoxylate shunt, a primarily anabolic pathway that shortcuts the TCA cycle.

The complex regulation of the isocitrate node in bacteria complicates the straightforward identification of the molecular target determining this metabolic shift. The different affinity of isocitrate dehydrogenase and isocitrate lyase towards its common substrate is the driving force explaining flux distributions at this node. Isocitrate dehydrogenase outcompetes for substrate due to its higher affinity, and a net flux through the glyoxylate shunt is only observed upon isocitrate accumulation. The reversible phosphorylation of isocitrate dehydrogenase by the action of AceK (isocitrate dehydrogenase phosphatase/kinase decreases flux through the TCA cycle (57–59). It has been described that in *S. enterica* this metabolic node is also controlled by the acetylation of AceK (14).

In order to explore the functional consequences of metabolic enzymes acetylation contributing to acetate overflow and biomass yield, metabolic fluxes were determined in the *E. coli* mutants Δ*cobB and ΔpatZ*, on glucose batch cultures and low dilution rate glucose limited chemostat cultures **(Suppl. Table 1)**. Furthermore, in our proteomic study, acetylation of AceK was not detected. In order to demonstrate that the acetylation of AceK was not the causing the metabolic shift at the isocitrate node, metabolic fluxes the double (Δ*cobB*Δ*aceK*, Δ*patZ*Δ*aceK*) mutants were determined (**Suppl. Table 1**). In an *E. coli aceK* mutant, isocitrate dehydrogenase is permanently active, , thus decreasing the flux through the glyoxylate shunt (37). The deletion of *cobB*

decreased flux in the bypass by half compared to the parent strain, while deletion of *patZ* exerted almost no effect on fluxes. Interestingly, the Δ*cobB*Δ*aceK* and Δ*patZ*Δ*aceK* mutants were more affected than the simple knockout strain Δ*cobB* **(Suppl. Table 1)**. Although the shunt was less active in both double mutants, the overflow of acetate and lower biomass yield were only observed in the Δ*cobB*Δ*aceK* mutant, as in the Δ*cobB* mutant. Altogether, this shows that phosphorylation of isocitrate dehydrogenase by AceK is the main process regulating flux partitioning at the isocitrate node and that AceK is not affected by acetylation.

Supplementary Methods.

Western blotting.

Acs and AceA proteins were resolved in 12% acrylamide SDS page. Blotting was performed as into PVDF membranes. Membranes were proofed against rabbit anti acetyl lysine (InmuneChem, Burnaby, Canada) according to the manufacturer instructions. A goat anti rabbit antibody conjugated with HPR (Santa Cruz Biotechnology, Heidelberg, Germany) was used.

Mass spectrometry.

After electrophoresis and Blue Coomassie staining, samples were digested with the following standard procedure with some modifications (76). The separation and analysis of the tryptic digests of the samples were performed with a HPLC-MS/MS system consisting of an Agilent 1100 Series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with a μwellplate autosampler and a capillary pump, and connected to an Agilent Ion Trap XCT Plus mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) using an electrospray (ESI) interface.

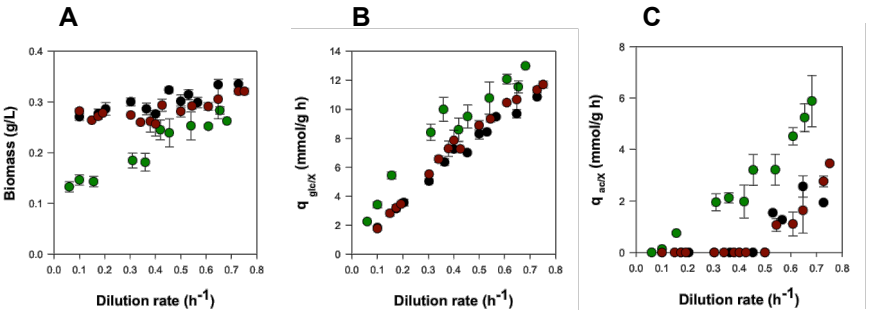
Dry samples from in-gel digestion were resuspended in 20 μl of buffer A, consisting in water/acetonitrile/formic acid (94.9:5:0.1). Sample was injected onto a Zorbax SB-C18 HPLC column (5 μm, 150 × 0.5 mm, Agilent Technologies, Santa Clara, CA, USA), thermostatted at 40 °C, at a flow rate of 10 μl/min. After the injection the column was washed with buffer A for 10 minutes and then the digested

peptides were eluted using a linear gradient 0-80% buffer B (buffer B: water/acetonitrile/formic acid, 10:89.9:0.1) for 180 min. The column was coupled online to an Agilent Ion Trap XCT Plus Mass Spectrometer using an electrospray interface. The mass spectrometer was operated in the positive mode with a capillary spray voltage of 3500 V, and a scan speed of 8100 (m/z)/sec from 50-2200 m/z, with a target mass of 1000 m/z, and 3 spectra averaging. The nebulizer gas pressure was set to 15 psi, whereas the drying gas was set to a flow of 5 l/min at a temperature of 350 °C. MS/MS data were collected in an automated data-dependent mode (AutoMS mode). The five most intense ions were sequentially fragmented using helium collision-induced dissociation (CID) with an isolation width of 2 m/z and a relative collision energy of 35%.

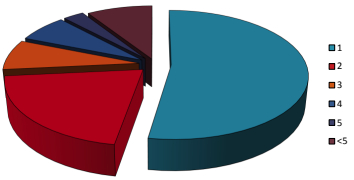
Data processing was performed with DataAnalysis program for LC/MSD Trap Version 3.3 (Bruker Daltonik, GmbH, Germany) and Spectrum Mill MS Proteomics Workbench (Rev A.03.02.060B, Agilent Technologies, Santa Clara, CA, USA) (references 2-3).

Briefly, raw mass spectra were extracted under default conditions as follows: unmodified or carbamidomethylated cysteines; sequence tag length >1; $[MH]^+$ 50–7000 m/z; maximum charge +7; minimum signal-to-noise (S/N) 25; finding ^{12}C signals. The extracted data were submitted to MS/MS search against the bacterial protein sequences of interest (Acs, AceA, IcdA), with the following criteria: identity search mode; tryptic digestion with 3 maximum missed cleavages; carbamidomethylated cysteines; peptide charge +1, +2, +3; monoisotopic masses; peptide precursor mass tolerance 2.5 Da; product ion mass tolerance 0.7 amu; ESI ion trap instrument; minimum matched peak intensity 50%; acetylated lysines, oxidized methionine, and N-terminal glutamine conversion to pyroglutamic acid as variable modifications. Finally, after automatic and manual validation of the results, a summary of the identified proteins with the sequence of the digested peptides was reported.

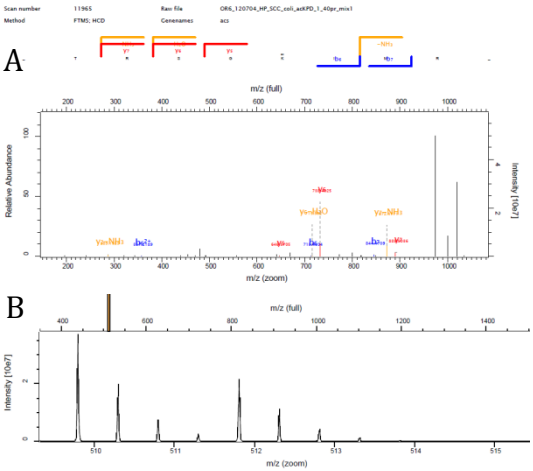
Supplementary figures



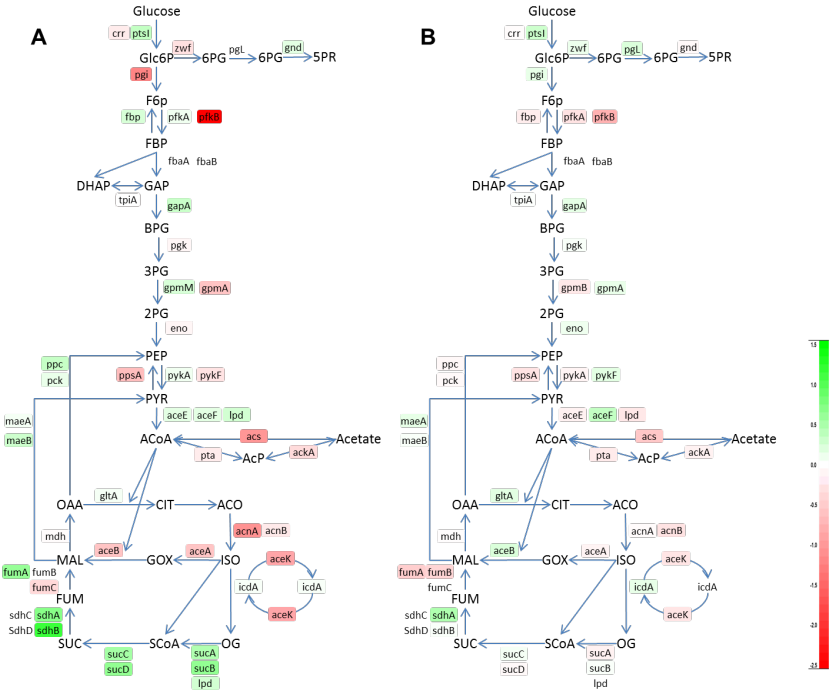
Supp. Fig 1. *Escherichia coli* BW25113 (black) and its knockout strains Δ*cobB* (green), Δ*patZ* (dark red) grown in glucose-limited cultures at different dilution rates. **A.** Cell Biomass, **B.** Glucose consumption rate and **C.** Acetate production rate variations in all strains at different dilution rates.



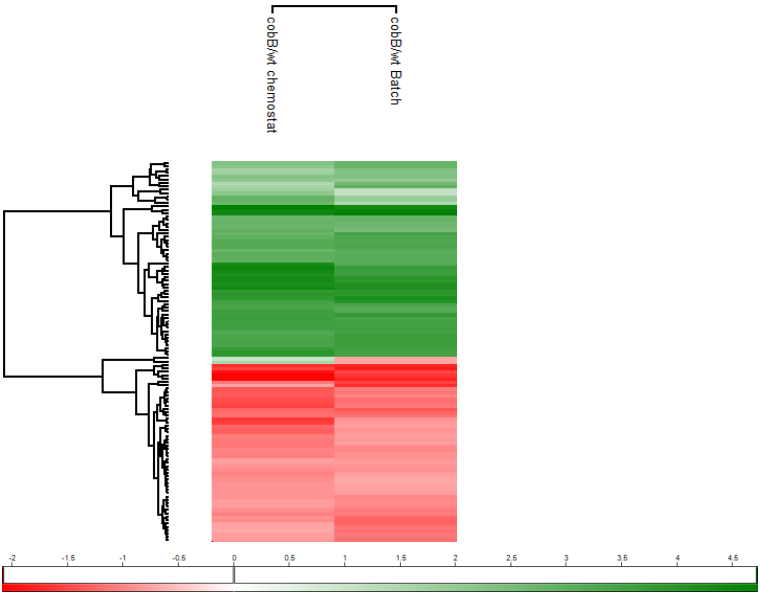
Suppl. Fig 2. Percentage of lysine acetylation sites per protein in all conditions assayed.



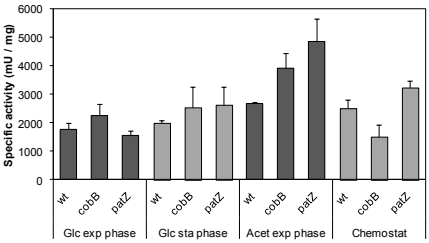
Suppl. Fig 3. The peptide containing the acetylated lysine 609 found in a different Maxquant analysis where lysine dimethylation label was changed to variable modification. **(A)** Fragmented peptide containing lysine 609 in chemostat cultures. **(B)** MS1 scan showing two of the three labelling states of the peptide shown in B. Only the light (wild type) and the medium (cobB) states are present in all the MS1 scan checked, which is consistent with lack of acetylation in the *patZ* mutant.



Supplementary Fig. 4. Central metabolism protein abundance ($\log_2$ ratio) **(A)** Δ*cobB*/wt and **(B)** Δ*patZ*/wt in chemostat cultures.



Supplementary Fig. 5. Hierarchical clustering of the significantly up- and down-regulated genes in the *cobB* mutant under two different condtions, glucose exponential phase and chemostat cultures (D=0.2 h^{-1}). Gene expression values expressed as $\log_2$ ratio Δ*cobB*/wt.



Supplementary Fig. 6. Phosphotransacetylase enzyme activity measured in cell crude extracts of *E. coli* BW25113 and its knockouts strains grown in glucose batch cultures, acetate batch cultures and glucose limited chemostat cultures.

Supplementary Tables

Suppl. Table 2. All strains and plasmids used in this study.

Strains	Relevant genotype	Source
E. coli BW25113	*lacI* q *rrnB*T14 D*lacZ*WJ16 *hsdR*514 Δ(*araBAD*)AH33 Δ(*rhaBAD*)LD78	Keio Collection(77)
BW25113 derivatives		
Δ*cobB*	[BW25113] *cobB:kan*	Keio Collection(77)
Δ*patZ*	[BW25113] *patZ:kan*	Keio Collection(77)
Δ*cobB*Δ*aceK*	[BW25113] *cobB:frt aceK:kan*	This study(78)
Δ*patZ* Δ*aceK*	[BW25113] *patZ:frt aceK:kan*	This study(78)
Δ*rcsB*	[BW25113] *rcsB:kan*	Keio Collection(77)
E. coli BL21 DE3 Δ*cobB*	F– ompT gal dcm lon hsdSB(rB- mB-) λ(DE3) *cobB*:kan	This study(78)
E. coli DH10B	F- *mcr*A D(*mrr-hsd*RMS-*mcr*BC) phi;80*lac*ZDM15 D*lac*X74 *recA*1 *endA*1 *araD*139 D(*ara, leu*) 7697 *gal*U *gal*K l- *rps*L *nup*G Host for cloning and plasmid propagation	Invitrogen
Plasmids		
pKD13	KanR/AmpR plasmid	Yale *E. coli* Genomic Resource Center
pKD46	AmpR plasmid that shows temperature-sensitive replication and thermal induction of FLP synthesis	Yale *E. coli* Genomic Resource Center
pCP20	AmpR/CmR plasmid that shows temperature-sensitive replication and thermal induction of FLP synthesis	Yale *E. coli* Genomic Resource Center
pBAD24	AmpR Arabinose promoter.	Invitrogen
pBAD24-*rcsB*	*rcsB* gene from *E. coli* BW25113	This study
pBAD24-*rcsB* K154R	*rcsB* substitution lysine 154 by arginine	This study
pBAD24-*rcsB* K154Q	*rcsB* substitution lysine 154 by glutamine	This study
pBAD24-*rcsB* K154E	*rcsB* substitution lysine 154 by glutamic	This study
pCA24N-*cobB*	GFP (-)	Aska library(45)
pCA24N-*acs*	GFP (-)	Aska library(45)
pCA24N-*aceA*	GFP (-)	Aska library(45)

Suppl. Table 2. Primers used in this study.

	Forward (5`→3`)	Reverse (5`→3`)
***cobB* KO**	GTGGTGCGGCCTTCCTACATCTAACCGATTAAACAACA GAGGTTGCTATGATTCCGGGGATCCGTCGACC	CCCCTTGCAGGCCTGATAAGCGTAGTGCATCAGGCA ATGCTTCCCGCTTTTGTAGGCTGGAGCTGCTTCG
***aceK* KO**	CGTTTACGCCGCATCCGGCAATTCTCTGCTCCTGATGA GGGCGCTAAATGATTCCGGGGATCCGTCGACC	TGCGGAGAAAAATTATATGGAAGCTTTACTCAAAA AAGCATCTCCCCATATGTAGGCTGGAGCTGCTTCG
***rcsB* cloning**	GGTGGTTCTAGAAATGAACAATATGAACGTAATTATTG	GGTGGTAAGCTTTTAGTCTTTATCTGCCGGACTT
***rcsB* K154R**	CAAGCGTCTCTCGCCACGTGAGAGTGAAGTTCTG	CAGAACTTCACTCTCACGTGGCGAGAGACGCTTG
***rcsB* K154Q**	CAAGCGTCTCTCGCCACAGGAGAGTGAAGTTCTG	CAGAACTTCACTCTCCTGTGGCGAGAGACGCTTG
***rcsB* K154E**	GCGTCTCTCGCCAGAAGAGAGTGAAG	CTTCACTCTCTTCTGGCGAGAGACGC

Suppl. Table 3. Number of lysine acetylated peptides and proteins in all replicates and conditions assayed.

acK peptides	**Replicate1**	**Replicate2**	**Replicate3**	**Replicate4**	**All**
Glucose batch exponential phase	294	725	1124	501	**1486**
Glucose batch stationary phase	840	939	778	958	**1674**
Acetate batch cultures	658	790	1090	1233	**1867**
Glucose chemostat cultures	243	104	619	731	**1001**
All conditions					**2502**

acK proteins	**Replicate1**	**Replicate2**	**Replicate3**	**Replicate4**	**All**
Glucose batch exponential phase	177	370	366	179	**444**
Glucose batch stationary phase	392	451	288	336	**508**
Acetate batch cultures	331	401	366	408	**565**
Glucose chemostat cultures	147	75	229	270	**330**
All conditions					**809**

Supl. Table 4. Metabolic fluxes of *Escherichia coli* and its knockout mutants (Δ*cobB*, Δ*patZ*, Δ*cobB*Δ*aceK* and Δ*patZ*Δ*aceK*) grown in glucose batch and glucose limited chemostat cultures.

	Glucose batch cultures					**Glucose chemostat cultures (D=0.2h^{-1})**				
	wt	**Δ*cobB***	**Δ*patZ***	**Δ*cobB*Δ*aceK***	**Δ*patZ*Δ*aceK***	**wt**	**Δ*cobB***	**Δ*patZ***	**Δ*cobB*Δ*aceK***	**Δ*patZ*Δ*aceK***
glucose in	100 (8.66±0.21)	100 (9.08±0.06)	100 (8.17±0.01)	100 (8.4±0.26)	100 (7.95±0.02)	100 (2.73±0.03)	100 (3.55±0.09)	100 (2.7±0.07)	100 (2.55±0.04)	100 (2.86±0.08)
zwf	23.73±0.79	23.03±0.05	22.49±1.78	*24.59±1.45*	*22.68±1*	33.74±1.9	26.5±0.86	34.61±2.54	25.16±1.16	28.58±0.61
gnd	23.1±0.37	23.03±0.05	22.39±1.67	21.43±2.57	22.24±0.27	26.91±0.81	21.1±0.35	28.35±1.76	18.1±0.33	25.77±1.78
pgi	75.8±0.79	76.56±0.06	77.05±1.78	74.99±1.44	76.86±1	65.82±1.88	73.2±0.87	64.94±2.51	74.44±1.16	70.98±0.6
edd	0.63±0.75	0±0	0.1±0.17	3.16±3.73	0.44±0.77	6.83±1.63	5.41±0.91	6.26±4.28	7.06±1.45	2.8±2.29
pfk-fba	86.19±0.78	87.54±0.13	87.04±0.66	84.85±2.81	86.85±0.83	79.35±1.44	84.21±0.93	79.34±3.43	82.44±1.33	83.62±1.53
tktA	7.27±0.11	7.33±0.02	7.07±0.56	6.82±0.85	7.04±0.09	8.89±0.27	6.98±0.12	9.37±0.59	5.97±0.11	8.5±0.6
tktB	3.75±0.03	4.19±0.08	3.54±0.57	3.6±0.82	3.56±0.1	5.23±0.35	4.43±0.09	5.63±0.39	2.56±0.09	4.74±0.43
tal	7.27±0.11	7.33±0.02	7.07±0.56	6.82±0.85	7.04±0.09	8.89±0.27	6.98±0.12	9.37±0.59	5.97±0.11	8.5±0.6
gap-pgk	175.77±0.91	178.39±0.36	176.73±0.64	175.59±2.62	176.74±0.8	169.88±0.96	177.67±1.03	169.69±2.92	173.71±1.27	173.9±1.23
PGA > PEP	162.75±1.13	166.85±0.66	163.77±0.61	163.92±2.4	164.01±0.77	157.7±0.47	168.86±1.15	157.29±2.4	162.25±1.08	161.12±1.08
Pyk	128.61±1.87	135.65±2.32	129.54±0.5	132.97±2.12	130.28±0.68	182.98±6.53	180.78±5.01	182.97±3.1	145.42±8.09	141.34±6.24
PYR-AcCoA	100.78±2.42	110.67±2.14	101.05±0.27	110.31±3.38	102.59±0.16	165.97±5.89	163.94±5.91	164.77±2.08	127.97±5.74	114.41±4.01
TCA1	29.17±4.2	26.6±4.16	24.13±2.97	19.79±6.49	26.03±0.36	89.39±2.28	93.04±3.45	87.36±2.24	69.1±5.31	78.08±2.8
TCA2	29.17±4.2	26.6±4.16	24.13±2.97	19.79±6.49	26.03±0.36	30.97±3.45	54.57±1.86	28.4±2.18	52.46±8	61.79±7.73
TCA3	16.68±4.52	15.35±4.46	11.58±3.02	8.45±6.91	13.67±0.39	20.13±3.47	44.37±1.27	17.42±1.95	40.91±8.72	48.51±7.63
TCA4	16.68±4.52	15.35±4.46	11.58±3.02	8.45±6.91	13.67±0.39	78.54±2.13	82.84±3.77	76.38±1.95	57.55±6.24	64.79±2.65
mdh	16.68±4.52	14.75±5.26	11.58±3.02	8.45±6.91	13.67±0.39	134.81±8.25	121.31±8.62	133.49±2.24	72.23±10.44	80.83±4.06
mae	0±0	0.61±1.05	0±0	0±0	0±0	2.14±2.26	0±0	1.85±1.55	1.96±3.39	0.25±0.32
pck	6.75±1.16	7.64±3.25	8.65±1.7	9.56±2.85	6.07±1.32	41.06±4.72	43.6±2.48	39.78±1.72	24.14±5.69	30.84±1.27
pcc	33.4±0.52	32.15±1.74	35.38±1.64	33.68±2.1	32.38±1.28	8.17±2.07	26.2±3.19	6.37±0.48	33.8±2.66	42.68±6.16
acetate	51.08±1.26	65.85±1.55	56.47±3.17	72.14±2.49	56.5±0.15	0±0	18.11±3.73	0±0	24.46±1.18	0±0
gox	0±0	0±0	0±0	0±0	0±0	58.41±4.56	38.47±4.87	58.96±0.59	16.63±4.96	16.29±5.44
TH	64.43±6.97	51.87±7.27	70.99±6.69	61.35±10.94	67.2±1.95	50.43±9.15	0±0	53.28±11.16	34.56±8.76	27.2±13.87
Respiration	140.65±11.49	150.01±11.36	130.22±7.41	133.47±16.36	135.85±1.21	269.37±9.24	301.01±8.27	264.18±7.56	227.95±13.51	236.7±9.14
growth	8.67±0.22	7.61±0.19	8.58±0.02	7.74±0.25	8.43±0.02	8.16±0.47	5.49±0.17	8.31±0.48	7.45±0.01	8.24±0.47

Suppl. Table 5. Lysine acetylated proteins related transcription in *E. coli* (GO:0006351 and GO:0006355). Transcription factors marked with an asterisk are transcriptional regulators of flagella biosynthesis and chemotaxis and the ones marked with a cross are transcriptional regulators of acid stress response.

Gene name	Uniprot ID	Number acetylation sites	Acetylated lysine position in the protein
agaR	P0ACK2	1	93
appY	P05052	2	47,55
arcA	P0A9Q1	3	187,195,160
arcB	P0AEC3	1	93
argR	P0A6D0	3	15,19,6
cheY	P0AE67	1	126
cpxR	P0AE88	1	219
crl	P24251	2	15,88
crp*†	P0ACJ8	3	101,153,167
csgD	P52106	1	201
cspC	P0A9Y6	3	9,15,59
cspE	P0A972	1	9
cysB	P0A9F3	1	320
dosP	P76129	1	95
ebgR	P06846	1	319
evgA	P0ACZ4	1	159
fis	P0A6R3	2	25,32
frlR	P45544	1	104
fur*	P0A9A9	1	117
glnB	P0A9Z1	2	85,90
greA	P0A6W5	2	63,116
gyrA	P0AES4	10	239,253,276,282,308,465,473516,573,671
gyrB	P0AES6	4	231,299,339,592
Hns*†	P0ACF8	5	6,57,120,128,136
hupA	P0ACF0	6	3,13,18,67,83,86
hupB	P0ACF4	5	3,9,18,67,86
ihfA*	P0A6X7	3	20,86,97
ihfB*	P0A6Y1	3	3,69,81
kdgR	P76268	1	170
lrp	P0ACJ0	4	10,25,36,129
malY	P23256	1	261
mprA	P0ACR9	1	97
nadR	P27278	1	47
nagC	P0AF20	1	63
narL	P0AF28	1	77
nusA	P0AFF6	2	117,243
nusB	P0A780	3	67,94,121
nusG	P0AFG0	2	106,121
ompR*	P0AA16	1	184
pcnB	P0ABF1	1	396
pepA	P68767	3	6,90,216
rcsB*†	P69407	2	125,154
relE	P0C077	1	13
rho	P0AG30	4	100,105,123,367
rplD	P60723	5	106,123,132,137,166
rpoA	P0A7Z4	5	95,246,291,297,304
rpoB	P0A8V2	19	115,191,236,279,639,844,890900,909,914,954,988,991,1027,1065,1133,1140,1200,1242
rpoC	P0A8T7	15	13,39,50,66,74,87,570603,649,850,959,983,996,1072,1132
rpoE	P0AGB6	1	16
rpoZ	P0A800	1	35
rpsD	P0A7V8	7	8,31,33,77,156,177,183
rsd	P0AFX4	2	17,79
rtcR	P38035	1	206
seqA	P0AFY8	1	60
stpA	P0ACG1	2	66,98
wrbA	P0A8G6	2	37,50
ydjF	P77721	2	80,158
yebC	P0A8A0	1	215
yeeN	P0A8A2	2	66,116
ygbI	P52598	1	188
yhaJ	P67660	1	62
yhaV	P64594	1	87
yiaU	P37682	1	7

Suppl. Table 6. Up regulated genes (log_2) in the *cobB* mutant compared with the wild type in glucose exponential phase cultures (FDR<0.05).

Genes	Fold change	Description
Policistronic operons		
fliLMNOPQR		
fliL	3.275	flagellar basal body-associated protein FliL
fliM	3.090	flagellar motor switch protein FliM
fliN	3.150	flagellar motor switch protein FliN
fliO	2.798	flagellar biosynthesis protein FliO
fliP	2.597	flagellar biosynthesis protein FliP
fliQ	2.294	flagellar biosynthesis protein FliQ
fliR	2.264	flagellar biosynthesis protein FliR
fliFGIJK		
fliF	3.081	flagellar MS-ring protein
fliG	3.200	flagellar motor switch protein G
fliI	2.849	flagellum-specific ATP synthase
fliJ	3.080	flagellar biosynthesis chaperone
fliK	2.797	flagellar hook-length control protein
FlgBCDEFGHIJKL		
flgB	3.617	flagellar basal body rod protein FlgB
flgC	3.484	flagellar basal body rod protein FlgC
flgD	3.354	flagellar basal body rod modification protein
flgE	3.477	flagellar hook protein
flgF	3.477	flagellar component of cell-proximal portion of basal-body rod
flgG	3.325	flagellar basal body rod protein FlgG
flgH	3.274	flagellar basal body L-ring protein
flgI	3.665	flagellar basal body P-ring protein
flgJ	3.293	flagellar rod assembly protein
flgK	3.853	flagellar hook-associated protein FlgK
flgL	3.526	flagellar hook-associated protein FlgL
FlhBAE		
flhB	2.341	flagellar biosynthesis protein FlhB
flhA	2.058	flagellar biosynthesis protein FlhA
flhE	2.322	flagellar protein
FlgAMN		
flgA	3.158	flagellar basal body P-ring biosynthesis protein FlgA
flgM	3.115	anti-sigma28 factor FlgM
flgN	3.447	flagella synthesis protein FlgN
FliAZ		
fliA	3.693	flagellar biosynthesis sigma factor
fliZ	3.572	flagella biosynthesis protein FliZ
tar-tap-cheRYZ		
tar	4.561	methyl-accepting chemotaxis protein II
tap	4.123	methyl-accepting protein IV
cheR	3.533	chemotaxis methyltransferase CheR
cheB	3.583	chemotaxis-specific methylesterase
cheY	3.618	chemotaxis regulatory protein CheY
cheZ	3.505	chemotaxis regulator CheZ
fliDST		
fliD	4.380	flagellar capping protein
fliS	3.981	flagellar protein FliS
fliT	3.054	flagellar biosynthesis protein FliT
motAB-CheAW		
motA	3.916	flagellar motor protein MotA
motB	4.004	flagellar motor protein MotB
cheA	4.175	chemotaxis protein CheA
cheW	4.149	purine-binding chemotaxis protein
flhCD		
flhC	1.925	transcriptional activator FlhC
flhD	1.921	transcriptional activator FlhD
Monocistronic operons		
fliC	4.808	flagellar filament structural protein (flagellin)
fliE	2.907	flagellar hook-basal body protein FliE
tsr	3.883	methyl-accepting chemotaxis protein I
ycgR	3.717	hypothetical protein
yhjH	3.613	EAL domain-containing protein
flxA	3.519	Qin prophage; predicted protein
yecR	2.813	hypothetical protein
ymdA	2.688	hypothetical protein

yjcZ	2.630	hypothetical protein
ynjH	2.477	hypothetical protein
aer	1.467	aerotaxis receptor

Suppl. Table 7. Down regulated genes (log_2) in the *cobB* mutant compared with the wild type in glucose exponential phase cultures (FDR<0.05).

Genes	fold change	Description
policistronic operons		
gadBC		
gadB	-1.870	glutamate decarboxylase isozyme
gadC	-1.259	acid sensitivity protein
hdeAB-yhiD		
hdeA	-1.819	acid-resistance protein
hdeB	-1.627	acid-resistance protein
entCEBAH		
entC	-1.531	isochorismate synthase
entE	-1.804	2,3-dihydroxybenzoate-AMP ligase component of enterobactin synthase multienzyme complex
entB	-1.191	2,3-dihydro-2,3-dihydroxybenzoate synthetase
gadAXW		
gadA	-1.156	glutamate decarboxylase isozyme
gadX	-1.718	DNA-binding transcriptional regulator GadX
ybaST		
ybaS	-1.548	glutaminase
puuRDCBE		
puuR	-1.263	DNA-binding transcriptional repressor PuuR
puuD	-1.288	gamma-glutamyl-gamma-aminobutyrate hydrolase
puuC	-1.465	gamma-glutamyl-gamma-aminobutyraldehyde dehydrogenase
puuB	-1.420	gamma-Glu-putrescine oxidase, FAD
talA-tktB		
tktB	-1.268	transketolase
yeaGH		
yeaG	-1.180	hypothetical protein
acs-yjcH-actP		
acs	-1.153	acetyl-CoA synthetase
gadE-mdtEF		
gadE	-1.149	hypothetical protein
fadBA		
fadB	-1.126	multifunctional fatty acid oxidation complex subunit alpha
monocistronic operons		
mscS	-1.160	mechanosensitive channel MscS
katE	-1.114	hydroperoxidase II
yciE	-1.114	hypothetical protein
entF	-1.112	enterobactin synthase multienzyme complex component, ATP-dependent
hchA	-1.103	chaperone protein HchA
pfkB	-1.760	6-phosphofructokinase 2
fiu	-1.477	catecholate siderophore receptor Fiu
hdeD	-1.376	acid-resistance membrane protein
cirA	-1.241	colicin I receptor
ydiZ	-1.153	hypothetical protein
ydhS	-1.131	hypothetical protein
ygiW	-1.487	hypothetical protein

Suppl. Table 8. Up regulated genes (log_2) in the *cobB* mutant compared with the wild type in glucose chemostat cultures (FDR<0.05).

Genes	Fold change	Description
policistronic operons		
tar-tap-cheRBYZ		
tar	4.448	methyl-accepting chemotaxis protein II
tap	4.204	methyl-accepting protein IV
cheR	3.524	chemotaxis methyltransferase CheR
cheB	3.344	chemotaxis-specific methylesterase
cheY	4.200	chemotaxis regulatory protein CheY
cheZ	3.538	chemotaxis regulator CheZ
fliDST		
fliD	4.944	flagellar capping protein
fliS	4.306	flagellar protein FliS
fliT	2.994	flagellar biosynthesis protein FliT
motAB-cheAW		
motA	3.933	flagellar motor protein MotA
motB	3.500	flagellar motor protein MotB
cheA	3.834	chemotaxis protein CheA
cheW	4.397	purine-binding chemotaxis protein
flgKL		
flgK	4.355	flagellar hook-associated protein FlgK
flgL	3.764	flagellar hook-associated protein FlgL
fliAZY		
fliA	4.497	flagellar biosynthesis sigma factor
fliZ	4.380	flagella biosynthesis protein FliZ
fliY		
flgBCDEFGHIJ		
flgB	3.415	flagellar basal body rod protein FlgB
flgC	3.185	flagellar basal body rod protein FlgC
flgD	3.626	flagellar basal body rod modification protein
flgE	2.778	flagellar hook protein
flgF	3.880	flagellar component of cell-proximal portion of basal-body rod
flgG	3.394	flagellar basal body rod protein FlgG
flgH	3.134	flagellar basal body L-ring protein
flgI	3.279	flagellar basal body P-ring protein
flgJ	2.981	flagellar rod assembly protein
flgAMN		
flgA	3.357	flagellar basal body P-ring biosynthesis protein FlgA
flgM	4.290	anti-sigma28 factor FlgM
flgN	3.864	flagella synthesis protein FlgN
fliLMNOPQ		
fliL	3.113	flagellar basal body-associated protein FliL
fliM	2.961	flagellar motor switch protein FliM
fliN	3.555	flagellar motor switch protein FliN
fliO	2.285	flagellar biosynthesis protein FliO
fliP	1.437	flagellar biosynthesis protein FliP
fliQ	1.676	flagellar biosynthesis protein FliQ
fliFGHIJK		
fliF	2.918	flagellar MS-ring protein
fliG	3.159	flagellar motor switch protein G
fliH		
fliI	2.785	flagellum-specific ATP synthase
fliJ	2.764	flagellar biosynthesis chaperone
fliK	2.704	flagellar hook-length control protein
yjfAZ		
yjcZ	2.754	hypothetical protein
flhBA		
flhB	2.248	flagellar biosynthesis protein FlhB
flhA	2.072	flagellar biosynthesis protein FlhA
flhCD		
flhC	2.873	transcriptional activator FlhC
flhD	2.844	transcriptional activator FlhD
fimAICDFGH		
fimA	2.177	major type 1 subunit fimbrin (pilin)
codAB		
codA	1.524	cytosine deaminase
codB		
modABC		

modC	1.466	molybdate transporter ATP-binding protein
upp-uraA		
upp	1.454	uracil phosphoribosyltransferase
uraA		
purMN		
purM	1.414	phosphoribosylaminoimidazole synthetase
ykfBFXGHWI		
ykfB	2.088	CP4-6 prophage; predicted protein
nuoMN		
nuoM	1.560	NADH dehydrogenase subunit M
monocistronic operons		
fliC	4.418	flagellar filament structural protein (flagellin)
tsr	3.771	methyl-accepting chemotaxis protein I
fliE	1.591	flagellar hook-basal body protein FliE
ndk	1.621	nucleoside diphosphate kinase
aer	2.673	aerotaxis receptor
ycfQ	1.795	hypothetical protein
ycgR	3.577	hypothetical protein
yecR	2.139	hypothetical protein
ymdA	2.819	hypothetical protein
ynjH	2.970	hypothetical protein
flxA	3.543	Qin prophage; predicted protein
yhjH	3.395	EAL domain-containing protein

Suppl. Table 9. Down regulated genes (log_2) in the *cobB* mutant compared with the wild type in glucose chemostat cultures (FDR<0.05).

Genes	Fold change	Description
policistronic operons		
prpBCDE		
prpB	-2.866	2-methylisocitrate lyase
prpC	-2.869	methylcitrate synthase
prpD	-3.099	2-methylcitrate dehydratase
prpE	-2.729	propionate--CoA ligase
lsrACDHFG-tam		
lsrA	-2.340	putative ATP-binding component of a transport system
hdeAB-yhiD		
hdeA	-2.139	acid-resistance protein
hdeB	-2.298	acid-resistance protein
ydcSTUV		
ydcS	-1.640	putative transport protein
yddLKJ		
yddK	-1.540	predicted protein
gadBC		
gadB	-1.530	glutamate decarboxylase isozyme
gadE-mdtEF		
gadE	-1.465	hypothetical protein
yeaGH		
yeaG	-1.458	hypothetical protein
yaiXO		
yaiX	-1.368	insertion element IS2 transposase InsD
ostBA		
otsB	-1.346	trehalose-6-phosphate phosphatase
yjbLM		
yjbL	-1.240	predicted protein
lsrRK		
lsrR	-1.230	putative SORC-type transcriptional regulator
yjdKO		
yjdK	-1.220	hypothetical protein
yhcADE		
yhcE	-1.203	pseudogene
gadAXW		
gadW	-1.192	putative ARAC-type regulatory protein
yciGFE		
yciG	-1.151	hypothetical protein
elfDCG-ycbUVF		
elfD	-1.119	putative chaperone
monocistronic operons		
ryjA	-2.368	ncRNA
ryhB	-1.415	ncRNA
rprA	-1.351	ncRNA
micF	-1.311	ncRNA
rpsV	-1.113	30S ribosomal subunit S22
pfkB	-1.715	6-phosphofructokinase 2
aldB	-1.598	aldehyde dehydrogenase B (lactaldehyde dehydrogenase)
ecnB	-1.624	entericidin B membrane lipoprotein
csiE	-1.570	stationary phase inducible protein CsiE
katE	-1.349	hydroperoxidase II
ycgB	-1.534	SpoVR family protein
yafF	-1.461	H repeat-containing protein
insB-1	-1.301	IS1 transposase B
ompC	-1.289	outer membrane porin protein C
hdeD	-1.238	acid-resistance membrane protein
ldrA	-1.162	toxic polypeptide, small
ldrB	-1.121	toxic polypeptide, small
ymeL	-1.111	hypothetical protein
yodD	-1.101	hypothetical protein
yjfN	-2.204	hypothetical protein
ygiW	-1.934	hypothetical protein
ydcH	-1.869	hypothetical protein
yebV	-1.371	hypothetical protein
yiaG	-1.281	putative transcriptional regulator
yqgD	-1.275	hypothetical protein
yjiC	-1.261	predicted protein

yohC	-1.252	hypothetical protein
phnB	-1.184	hypothetical protein
ZO115	-1.157	hypothetical protein
ydiZ	-1.150	hypothetical protein
ytfK	-1.128	hypothetical protein

Chapter 7

Final discussion, outlook and conclusions

"Nothing in life is to be feared, it is only to be understood"
Marie Curie

Discussion

The main objective of this dissertation was investigating the involvement of acetate metabolism in central carbon metabolism and physiology, and its regulation in *Escherichia coli*. Most of the results presented are focused in the regulation of acetate metabolism, although other pathways have also been studied.

In the first chapter, the link between acetate metabolism and central carbon metabolism was studied, showing the high importance of the main acetate-producing pathway, and revealing its importance in *E. coli* metabolic homeostasis. Some of the results observed in this chapter induced us to investigate the regulation of the acetate metabolism by post-translational modifications. In the second chapter, the role of lysine acetylation in the regulation of the acetate metabolism in *E. coli* was studied. We described how acetyl-CoA synthetase, and therefore acetate metabolism, is regulated *in vivo* by the only known deacetylase, the sirtuin CobB, and the best-known protein acetyltransferase, PatZ. Furthermore, it was described for the first time how lysine acetylation is transcriptionally regulated in bacteria, this being controlled by the metabolic state of the cell. The regulation of acetate metabolism by lysine acetylation showed in this chapter and the well-known differences in acetate production in two different *E. coli* strains, K-12 and BL21, made us hypothesise that protein lysine acetylation might be different in these two strains. In this chapter we described that acetate metabolism in the B strain is even more tightly controlled by the sirtuin CobB and PatZ than in the K12. Finally, we aimed at discerning if this post-translational modification was also involved in the regulation of other physiological functions in *E. coli*. In the final chapter of this PhD thesis, we have identified other major physiological functions that are controlled by CobB, such as the isocitrate node and the activity of the transcription factor RcsB, modifying cellular process such us the biosynthesis of flagella, cellular motility and acid stress survival.

The main results of this dissertation have been summarised above, but for a better integration, a final discussion of all the results obtained is presented in the following.

The characterization of the mutants lacking the acetate production/consumption pathways in *E. coli* has been presented in the first chapter. Important effects were observed in the Δ*pta* mutant. As it was expected, much lesser acetate was produced in glucose cultures in this mutant. But this was not the only effect observed in this mutant. Other enzymes, genes and metabolites reporting the cellular energetic levels were unbalanced as a result of *pta* deletion. This showed the direct link between the central carbon and the acetate metabolism and its importance for global metabolic fitness. The results suggested that the deletion of *pta* increased the acetyl-CoA concentration inhibiting pyruvate dehydrogenase and leading to increased levels of pyruvate. Its accumulation affected the expression of several genes of central carbon metabolism, and cellular energetic levels. Also, it was proposed that an accumulation of acetyl-phosphate could take place in Δ*pta* in acetate cultures. A careful observation of some of the changes in gene expression and enzyme activity revealed a low correlation between them. This puzzling result led us to think that probably an unidentified regulation mechanism was operating under those conditions, which was causing these effects in the Δ*pta* genomic background.

When this study was carried out, back in 2009, the regulation of the metabolism of bacteria by post-translational modifications was still in its infancy. The effect of the inactivation of isocitrate dehydrogenase in *E. coli* by phosphorylation was known since the early 80's, and other post-translational modification mechanisms had not been reported yet (1–4). Little was known about protein lysine acetylation, especially in *E. coli*. The regulation of acetate metabolism in *S. enterica* and *B. subtilis* by protein lysine acetylation had been well described, and many evidences pointed that they might also occur in *E. coli* (5–8). At that point we thought that it was worth dissecting the roles and the regulation of protein acetylation in the best-characterized model bacterium. We demonstrated that lysine acetylation inactivates the enzyme acetyl-CoA synthetase *in vivo* by the protein acetyltransferase PatZ and this inactivation is reverted by the sirtuin CobB.

Apart from this, we showed for the first time how the proteins involved in lysine acetylation are transcriptionally regulated. We demonstrated that the protein

acetyltransferase (*patZ*) gene expression is controlled by one of the main transcription factors in *E. coli*, CRP, and therefore lysine acetylation is directly linked to the metabolic state of the cell. This means that expression of the genes encoding the protein acetyltransferase *patZ* and the acetyl-CoA synthetase *acs* occurs simultaneously in *E. coli*. This finding was interesting but puzzling at the same time. Why does *E. coli* transcribe an enzyme needed for acetate consumption and, at the same time, the enzyme that inactivates it? In previous studies, Escalante-Semerena and collaborators hypothesised that the inactivation of the acetyl-CoA synthetase protein could contribute to preserve cellular homeostasis. They argued that a pool of too active acetyl-CoA synthetase would result in the accumulation of AMP (7, 9). But, could exist another reason for this simultaneous transcriptional activation?

In the last chapter of this PhD Thesis, the relative quantification of lysine acetylation between the mutants *cobB* and *patZ* and their parent strain under different environmental conditions was shown. In these experiments expected results were a higher acetylation pattern in the *cobB* mutant (deacetylase deficient) and a lower acetylation pattern in the *patZ* mutant (protein acetyltransferase deficient). As expected, the Δ*cobB* mutant showed a higher acetylation pattern in all conditions assayed. The *patZ* deficient showed almost no changes in acetylation in some of the conditions, as previously described by Choudhary and collaborators (10). But under those conditions where the expression of *patZ* is maximal, a much higher acetylation pattern was observed, especially in acetate cultures where more than 75% of the sites were more acetylated in the *patZ* deletion mutant. This higher acetylation observed in the Δ*patZ* mutant could be explained by taking into consideration two facts: the modulation of the activity of acetyl-CoA synthetase activity by PatZ, and the fact that acetyl-CoA can act as an acetyl donor itself.

We have previously described that PatZ inhibits acetyl-CoA synthetase activity and, therefore, higher activity was shown in the Δ*patZ* mutant, especially in acetate cultures. This increase in the activity would lead to a higher rate of acetyl-CoA production. If this metabolite was not consumed at the same rate as it is produced, it would accumulate and could act as a chemical acetyl donor, therefore increasing

protein acetylation in this mutant. This indicates that the most likely function of PatZ is the regulation of chemical acetylation by acetyl-CoA, which would explain that *acs* and *patZ* present the same transcription activation pattern.

The role of lysine acetylation on the differences shown in acetate metabolism between the K and B strains was studied. The main difference observed between both strains was the higher transcription of *acs* and *patZ* at the early exponential phase of glucose cultures. Simultaneous expression of *acs* and *patZ* genes was also observed in the B strain, showing that acetate metabolism in the low-acetate producer strain was regulated similarly to the K strain. In fact, we observed that protein acetylation increased both in the BL21 and K12 Δ*patZ* mutants in acetate cultures, confirming that probably the main function of PatZ is the regulation of acetyl-CoA synthetase and, therefore, chemical acetylation. This also highlights that, even though the acetyl-CoA synthetase activity is higher in the BL21 strain, this enzyme should remain partially inactive in order to maintain the levels of acetyl-CoA and avoid an increase of protein acetylation. Despite this observation, protein acetylation in acetate cultures of BL21 Δ*patZ* was lower than in the same mutant of K12. This effect was probably due to lower acetyl-CoA pools in the BL21 mutant caused by a higher rate of the acetyl-derivatives consuming pathways.

Chemical lysine acetylation carried out by acetyl- derivatives has been known since the 70s (11). Until few years ago, not much attention had been paid to it. Different acetylating agents like acetyl-CoA, acetyl-AMP or acetyl-phosphate can perform lysine chemical acetylation (11, 12). In the first chapter of this Thesis, we observed that some enzyme activities and gene expression profiles did not correlate well, especially in the *pta* mutant. We suspected that a post-translational modification could be altered in the Δ*pta* mutant producing those uncorrelated results. The deletion of the *pta* gene might alter the levels of acetyl-phosphate in all the conditions where this pathway should be active.

But back in 2009, only chemical protein phosphorylation by acetyl-phosphate had been demonstrated in some *E. coli* proteins (13–16). Later on, our original suspicion was confirmed, when Choudhary and collaborators showed that the deletion of phosphotransacetylase (*pta*) or acetate kinase (*ackA*) genes alters protein lysine acetylation

(10). This effect was explained by the accumulation of acetyl-phosphate, the intermediate metabolite of the Pta-AckA pathway, this being one of the main acetylating agents in *E. coli*. These authors explained that, in glucose cultures, lysine acetylation was increased in the Δ*ackA* mutant while this post-translational modification was increased in the Δ*pta* mutant in acetate cultures (10). They also stated that the over-acetylation observed in the Δ*ackA* did not alter its phenotype, showing that lysine acetylation deregulation does not always involve changes in bacterial physiology. However in our results, the knockout mutant Δ*pta,* that might have an increase (in acetate cultures) or a decrease (in glucose cultures) of acetyl-phosphate and therefore in protein lysine acetylation, had a severely altered phenotype. Although it should not be forgotten the role of acetyl phosphate in chemical phosphorylation (17). Probably an alteration of the phosphoproteome in the Δ*pta* mutant caused by the increase/decrease of acetyl-phosphate could also contribute to altering other physiological features. More efforts should be devoted to understand the role of this high-energy metabolite in the physiology in *E. coli.*

Regarding the functionality of this post-translational modification several examples of how acetylated lysines can affect protein function have been shown in the introduction. However, over 2000 lysines can be acetylated in *E. coli*, which makes us wonder if all of them alter protein function. In this Thesis we have demonstrated how lysine acetylation affects *in vivo* acetyl-CoA synthetase and isocitrate lyase enzyme activities and the DNA binding activity of the transcription factor RcsB, which indirectly alters motility and stress survival. Quite probably, we have only demonstrated few of the most prevalent functions of lysine acetylation and the acetylation of other lysines might only play a role in the fine-tuning regulation of other physiological functions. Moreover, acetylation of proteins can also alter their stability (*e.g.* RNase) (18). Other mechanisms of modification of protein activity by acetylation, such as the alteration of protein-protein interaction, have not been studied widely. In our proteomic study, we have identified that RNA polymerase and some transcription factors are extensively acetylated and also other transcription factors. It is well known that protein-protein interaction is sometimes necessary for proper transcriptional regulation (19, 20).

The neutralization of the positive charge of lysine side chain by acetylation could imply changes in protein-protein interaction and, consequently in the transcriptional regulation.

It is known that lysine acetylation can modify itself protein function, but State of the Art proteomic technologies, do not allow the quantification of the abundance of a post-translational modification in the cell and, therefore, the real implications that this modification could have in cell physiology are difficult to predict. Despite this, it has been proposed that lysine acetylation is not so important in *E. coli* physiology arguing the low abundance of this post-translational modification in the global proteome (10). Moreover, this was proposed because of the unaltered physiology observed in the highly acetylated Δ*ackA* mutant (10). The physiological data presented in this study does not support this idea, and suggest that lysine acetylation plays indeed a regulatory function in *E. coli*. In fact, in our characterization of the Δ*pta* mutant in acetate cultures, where its acetylation is much higher, we observed that its phenotype was severely affected. Not only growth rate was affected, but also gene expression, enzyme activities and metabolic state. This indicates that also chemical acetylation alters protein functionality. In fact, the Δ*patZ* mutant showed almost no impairment in its growth in acetate cultures but other features like gene expression and probably metabolite pools were importantly altered. These evidences suggest that the study of post-translational modifications using high-resolution proteomics should be carried out in parallel with a deep physiological characterization. Otherwise, the insights of these regulatory mechanisms would be lost and the true significance of this mechanism could not be judged.

Although this dissertation has shown new knowledge about how acetate metabolism can be linked to central carbon metabolism and the different roles of the post-translational modifications, several questions remain open for future studies. It is more than evident that the side chain of lysines is chemically promiscuous, given the many different modifications that they can suffer (21–25). Actually, a recent study has described that the glycolytic intermediate 1,3-bisphosphoglycerate reacts with a lysine of the glyceraldehyde-3-phosphate dehydrogenase enzyme forming 3-phosphoglyceryl-lysine (26). As described in the introduction, there is increasing evidence that other

protein lysine acylations, like succinylation, propionylation and butirylation are present in all domains of life (21, 23, 27). This different acylations occur enzymatically and also chemically (23, 28–30). Actually, it is well documented that acetyl-CoA synthetase can catalyse the activation of propionate to propionyl-CoA (31). Also, in *Rhodopseudomonas palustris* it has been described that the substrates for its protein acetyltransferase (*Rp*Pat) are all of them acyl-CoA ligases (32). Having in mind the results presented in this PhD Thesis, and especially the high acetylation level observed in the Δ*patZ* mutant, we can hypothesize that acetyltransferases control protein chemical acylation. Probably, this role could be general, not being restricted only to *E. coli*. During evolution, organisms may have evolved two complementary strategies in order to fight chemical acetylation: acetyltransferases, regulating the synthesis of acetylating agents and deacetylases, removing acyl moieties from proteins.

In this PhD Thesis we have demonstrated that lysine acetylation decreases acid stress survival. Probably other mechanisms of bacterial survival are affected by this post-translational modification. Many other transcription factors in our study are differentially regulated and most of them are involved in stress responses. From the evolutionary point of view, the role of lysine acetylation in the capability of organisms to survive or adapt to different stresses or changing environmental conditions could have the clue. In a comparative genomics study between a mesophile fungi and a thermophile fungi it was observed that the most prevalent substitutions between both genomes were arginines to lysines. The authors proposed that these protein changes could contribute to protein thermostability (33). In these two very related fungi, lysine acylation studies have not been carried out but, probably, the presence of permanent deacetylated state of the arginine in the thermophile organism would help for the maintenance of cellular stability.

Another unclear point about protein acylation is the differential functionality of these modifications in the protein. Could the size of the modification alter differentially protein function? Furthermore, it has been observed that protein succinylation and acetylation overlap in *E. coli* (23). Probably these post-translational modifications can co-occur in the same protein. In fact, in the reduced genome

microorganism, *Mycoplasma pneumoniae*, it has been described the crosstalk between lysine acetylation and phosphorylation (34). In this Thesis, an example of functional crosstalk between lysine acetylation and phosphorylation in *E. coli* has been described. Phosphorylation acts as a gross regulator and lysine acetylation as a fine-tuning regulator of the isocitrate node. It is interesting to highlight the bi-functional post-translational function of acetyl-phosphate in *E. coli*, and probably in other organisms. The regulation of the pool of this metabolite and the differential reactivity towards phosphorylation and acetylation makes the crosstalk provoked by this metabolite very interesting for future studies.

Summarising, in this PhD Thesis we have not only demonstrated that acetate metabolism is regulated by lysine acetylation but, furthermore, we have shown that this metabolism is part of this post-translational modification. High-throughput proteomic techniques have increased the knowledge in the number of acetylation sites, but the functionality and physiological implications of lysine acetylation should be extensively considered and be the main aim in future studies. Additionally, deciphering the role of the protein acetyltransferase PatZ in the regulation of chemical acylation would increase the importance of this regulation and also the evolutionary paths of regulation in living organisms. Finally, the co-occurrence of different post-translational modifications in the same protein and the implications of this crosstalk in physiology will give us a more realistic picture on how biological functions operate and are regulated in living beings.

Conclusions

The main aim of this PhD thesis was to increase our understanding of acetate metabolism and its link with the central carbon metabolism of *E. coli* and to determine the function of lysine acetylation in the regulation of metabolism and other cellular processes. All the results obtained in this thesis have been extensively presented in the previous experimental chapters but, for a clearest comprehension, the main conclusions have been summarised.

1. The deletion of the main acetate-producing pathway (*pta*) alters *E. coli* physiology, decreasing acetate production 14-fold and increasing lactate production 10-fold in glucose cultures. This altered phenotype was the consequence of the incapacity of acetate production in the *pta* mutant and, therefore, the accumulation of acetyl-CoA and pyruvate, this leading to alter the gene expression pattern and several enzyme activities. This indicates the importance of this pathway for continued glycolytic activity and in the maintenance of metabolite pools and redox homeostasis.
2. The phosphotransacetylase enzyme is not essential for growth under anaerobic conditions. Although its deletion drastically decreases growth efficiency under certain conditions, this pathway is highly relevant for ensuring the adaptability of this bacterium to environmental changes.
3. The enzyme acetyl-CoA synthetase is regulated by the acetylation of lysine 609 in *E. coli*. In low concentration acetate cultures, the growth of the Δ*cobB* mutant was almost negligible because of the inactivation of the acetyl-CoA synthetase, the deletion of the *patZ* gene recovered the Δ*cobB* phenotype. In fact, the CobB activation of the acetyl-CoA synthetase by deacetylation increased its activity more than 50 times. Moreover, the acetylation of lysine 609 is responsible, *in vitro* and *in vivo*, for the low activity of acetyl-CoA synthetase in the Δ*cobB* mutant in acetate and chemostat cultures.
4. The gene *cobB* is transcribed constitutively from the region upstream *nagK*. However, the expression of the *patZ* gene occurs from its own upstream

region, and is transcriptionally activated by CRP consequently, it is controlled by the metabolic state of the cell. Two CRP binding sites were identified in the region upstream *patZ*, localized at -41.5 and -81.5 bp from the RNA polymerase binding site.

5. Protein acetylation contributes to the differences in the acetate metabolism in *E. coli* BL21 and K12. Protein acetylation is lower in the BL21 strain, especially in acetate and stationary phase glucose batch cultures. Acetate overflow was completely supressed in the BL21 Δ*patZ* mutant, and growth in acetate minimal medium was higher in this mutant. Acetate overflow and acetate consumption are tightly regulated by protein acetylation in *E. coli* BL21.
6. A high number of lysine-acetylated residues were observed in all the conditions assayed. More than 2000 acetylated peptides belonging to 809 proteins were identified and quantified in *E. coli* K12. 65% of those acetylated proteins were related to metabolism, especially to primary metabolism. 10% of the acetylated proteins were DNA binding proteins.
7. Lysines and acid amino acids are very characteristic of the consensus acetylation motif observed in our data set. Aspartate and glutamate residues could be part of the chemo-catalytic mechanism of lysine acetylation, since they can enhance the nucleophilicity of lysil residues, which would attack acetylating agents.
8. The sirtuin CobB has a major role as protein deacetylase. The deletion of this sirtuin increased the acetylation in more than 1000 lysines in *E. coli.* This increase in protein acetylation mirrored the altered phenotype of the Δ*cobB* mutant, showing the importance and functionality of lysine acetylation.
9. The deletion of the protein acetyltransferase did not severely alter the phenotype and protein acetylation patterns in glucose cultures. In acetate cultures, where *patZ* expression is maximal, protein acetylation increased: 75% of the peptides were much more acetylated. PatZ could regulate chemical acetylation in *E. coli*, showing an evolutionary strategy to control the levels of acetyl- donors in the bacterial cytoplasm.

10. Lysine acetylation controls the activity of isocitrate lyase, decreasing its activity more than 20 times and limiting flux through the glyoxylate shunt. Further, isocitrate lyase is acetylated on 13 different sites, but higher acetylation of lysine 308 was observed in the Δ*cobB* mutant. This post-translational modification acts as a fine-tuning regulation of the isocitrate node while phosphorylation would act as a gross regulator.
11. The up regulation of the flagella and motility genes and down regulation of the acid stress response genes was caused by the higher acetylation of lysine 154 of the transcription factor RcsB. The acetylation of lysine 154 impairs RcsB binding to DNA due to the loss of its positive charge. The changes in gene expression mirrored the *E. coli* K12 phenotype, having higher number of flagella and motility, but lower survival under acid stress response.

Bibliography.

1. LaPorte, D. C., and Koshland, D. E. (1983) Phosphorylation of isocitrate dehydrogenase as a demonstration of enhanced sensitivity in covalent regulation. *Nature* 305, 286–290
2. Borthwick, A. C., Holms, W. H., and Nimmo, H. G. (1984) Isolation of active and inactive forms of isocitrate dehydrogenase from *Escherichia coli* ML 308. *Eur J Biochem* 141, 393–400
3. Hurley, J. H., Thorsness, P. E., Ramalingam, V., Helmers, N. H., Koshland, D. E., and Stroud, R. M. (1989) Structure of a bacterial enzyme regulated by phosphorylation, isocitrate dehydrogenase. *Proc Natl Acad Sci U S A* 86, 8635–8639
4. Hurley, J. H., Dean, A. M., Sohl, J. L., Koshland, D. E., and Stroud, R. M. (1990) Regulation of an enzyme by phosphorylation at the active site. *Science (80-.).* 249, 1012–1016
5. Starai, V. J., Celic, I., Cole, R. N., Boeke, J. D., and Escalante-Semerena, J. C. (2002) Sir2-dependent activation of acetyl-CoA synthetase by deacetylation of active lysine. *Science (80-.).* 298, 2390–2392
6. Starai, V. J., and Escalante-Semerena, J. C. (2004) Identification of the protein acetyltransferase (Pat) enzyme that acetylates acetyl-CoA synthetase in *Salmonella enterica*. *J Mol Biol* 340, 1005–1012
7. Gardner, J. G., Grundy, F. J., Henkin, T. M., and Escalante-Semerena, J. C. (2006) Control of acetyl-coenzyme A synthetase (AcsA) activity by acetylation/deacetylation without NAD+ involvement in *Bacillus subtilis*. *J. Bacteriol.* 188, 5460–5468
8. Gardner, J. G., and Escalante-Semerena, J. C. (2009) In *Bacillus subtilis*, the sirtuin protein deacetylase, encoded by the *srtN* gene (formerly yhdZ), and functions encoded by the *acuABC* genes control the activity of acetyl coenzyme A synthetase. *J. Bacteriol.* 191, 1749–1755
9. Chan, C. H., Garrity, J., Crosby, H. A., and Escalante-Semerena, J. C. (2011) In *Salmonella enterica*, the sirtuin-dependent protein acylation/deacylation system (SDPADS) maintains energy homeostasis during growth on low concentrations of acetate. *Mol Microbiol* 80, 168–183
10. Weinert, B. T. T., Iesmantavicius, V., Wagner, S. A. A., Schölz, C., Gummesson, B., Beli, P., Nyström, T., Choudhary, C., Scho, C., and Nystro, T. (2013) Acetyl-Phosphate Is a Critical Determinant of Lysine Acetylation in *E . coli*. *Mol. Cell* 51, 1–8
11. Ramponi, G., Manao, G., and Camici, G. (1975) Nonenzymatic Acetylation of Histones with Acetyl Phosphate and Acetyl Adenylate. *Biochemistry* 14, 2681–2685
12. Kuo, Y.-M., and Andrews, A. J. (2013) Quantitating the specificity and selectivity of Gcn5-mediated acetylation of histone H3. *PLoS One* 8, e54896
13. McCleary, W. R., Stock, J. B., and Ninfa, A. J. (1993) Is acetyl phosphate a global signal in *Escherichia coli*? *J Bacteriol* 175, 2793–2798
14. Mika, F., and Hengge, R. (2005) A two-component phosphotransfer network involving ArcB, ArcA , and RssB coordinates synthesis and proteolysis of S (RpoS) in *E . coli*. *Genes Dev.*, 2770–2781
15. Pruteanu, M., and Hengge-aronis, R. (2002) The cellular level of the recognition factor RssB is rate-limiting for s S proteolysis : implications for RssB regulation and signal transduction in s S turnover in *Escherichia coli*. *Mol. Microbiol.* 45, 1701–1713
16. Klauck, E., and Hengge-aronis, R. (1998) Regulation of RssB-dependent proteolysis in *Escherichia coli*: a role for acetyl phosphate in a response regulator-controlled process. *Mol. Microbiol.* 27, 787–795
17. McCleary, W. R., and Stock, J. B. (1994) Acetyl phosphate and the activation of 2-component response regulators. *J Biol Chem* 269, 31567–31572

18. Liang, W., and Deutscher, M. P. (2012) Transfer-messenger RNA-SmpB protein regulates ribonuclease R turnover by promoting binding of HslUV and Lon proteases. *J. Biol. Chem.* 287, 33472–9
19. Busby, S., and Ebright, R. H. (1997) Transcription activation at class II CAP-dependent promoters. *Mol. Microbiol.* 23, 853–9
20. Niu, W., Kim, Y., Tau, G., Heyduk, T., and Ebright, R. H. (1996) Transcription activation at class II CAP-dependent promoters: two interactions between CAP and RNA polymerase. *Cell* 87, 1123–34
21. Kai, Z., Chen, Y., Zhang, Z., and Zhao, Y. (2010) Identification and Verification of Lysine Propionylation and Butyrylation in Yeast Core Histones Using PTMap Software. *J Proteome Res* 8, 900–906
22. Wagner, G. R., and Payne, R. M. (2013) Metabolism : Widespread and enzyme-independent N ε-acetylation and N ε-succinylation in the chemical conditions of the mitochondrial matrix. *J. Biol. Chem.* 288, 29036–45
23. Weinert, B. T., Schölz, C., Wagner, S. a, Iesmantavicius, V., Su, D., Daniel, J. a, Choudhary, C., and Scho, C. (2013) Lysine succinylation is a frequently occurring modification in prokaryotes and eukaryotes and extensively overlaps with acetylation. *Cell Rep.* 4, 842–51
24. Wagner, S. a, Beli, P., Weinert, B. T., Nielsen, M. L., Cox, J., Mann, M., and Choudhary, C. (2011) A proteome-wide, quantitative survey of in vivo ubiquitylation sites reveals widespread regulatory roles. *Mol. Cell. Proteomics* 10, M111.013284
25. Smith, B. C., and Denu, J. M. (2009) Chemical mechanisms of histone lysine and arginine modifications. *Biochim. Biophys. Acta* 1789, 45–57
26. Moellering, R. E., and Cravatt, B. F. (2014) Functional Lysine Modification by an Intrinsically Reactive Primary Glycolytic Metabolite. *Science (80-.).* 549, 549–
27. Zhilhoung, Z., Ninji, T., Zhongyu, X., Lunzhi, D., Yue, C., Tingming, Z., Zhihong, Z., and Minjia, T. (2011) Identification of lysine succinylation as a new post-translational modification. *Nat. Chem. Biol.* 7, 58–63
28. Colak, G., Xie, Z., Zhu, A. Y., Dai, L., Lu, Z., Zhang, Y., Wan, X., Chen, Y., Cha, Y. H., Lin, H., Zhao, Y., and Tan, M. (2013) Identification of Lysine Succinylation Substrates and the Succinylation Regulatory Enzyme CobB in *Escherichia coli*. *Mol. Cell. Proteomics* 12, 3509–20
29. Hayden, J. D., Brown, L. R., Gunawardena, H. P., Perkowski, E. F., Chen, X., and Braunstein, M. (2013) Reversible acetylation regulates acetate and propionate metabolism in *Mycobacterium smegmatis*. *Microbiology* 159, 1986–99
30. Garrity, J., Gardner, J. G., Hawse, W., Wolberger, C., and Escalante-Semerena, J. C. (2007) Nε-lysine propionylation controls the activity of propionyl-CoA synthetase. *J Biol Chem* 282, 30239–30245
31. Barak, R., Yan, J., Shainskaya, A., and Eisenbach, M. (2006) The chemotaxis response regulator CheY can catalyze its own acetylation. *J. Mol. Biol.* 359, 251–65
32. Crosby, H. a, Pelletier, D. a, Hurst, G. B., and Escalante-Semerena, J. C. (2012) System-wide studies of N-lysine acetylation in *Rhodopseudomonas palustris* reveal substrate specificity of protein acetyltransferases. *J. Biol. Chem.* 287, 15590–601
33. Van Noort, V., Bradatsch, B., Arumugam, M., Amlacher, S., Bange, G., Creevey, C., Falk, S., Mende, D. R., Sinning, I., Hurt, E., and Bork, P. (2013) Consistent mutational paths predict eukaryotic thermostability. *BMC Evol. Biol.* 13, 7
34. Beltrao, P., Bork, P., Krogan, N. J., and van Noort, V. (2013) Evolution and functional cross-talk of protein post-translational modifications. *Mol. Syst. Biol.* 9, 714