UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

POSGRADO EN CIENCIAS BIOLÓGICAS

FACULTAD DE CIENCIAS

EL CICLO DEL GLIOXILATO EN *Rhizobium*, VIDA LIBRE Y EN LA INTERACCIÓN CON PLANTAS LEGUMINOSAS

T E S I S

QUE PARA OBTENER EL GRADO ACADÉMICO DE

DOCTOR EN CIENCIAS

P R E S E N T A

JOSÉ AUGUSTO RAMÍREZ TRUJILLO

TUTOR PRINCIPAL DE TESIS: DR. ISMAEL HERNÁNDEZ LUCAS

COMITÉ TUTOR: DR. JUAN MIRANDA RÍOS
DR. MICHAEL F. DUNN

MÉXICO, D.F. **JUNIO, 2010**

VNIVERSIDAD NACIONAL AVTÓNOMA DE MEXICO

POSGRADO EN CIENCIAS BIOLÓGICAS
FACULTAD DE CIENCIAS
DIVISIÓN DE ESTUDIOS DE POSGRADO

OFICIO FCIE/DEP/158/10

ASUNTO: Oficio de Jurado

Dr. Isidro Ávila Martínez
Director General de Administración Escolar, UNAM
P r e s e n t e

Me permito informar a usted que en la reunión ordinaria del Comité Académico del Posgrado en Ciencias Biológicas, celebrada el día 8 de febrero de **2010**, se aprobó el siguiente jurado para el examen de grado de **DOCTOR EN CIENCIAS** del (la) alumno (a) **RAMIREZ TRUJILLO JOSE AUGUSTO** con número de cuenta **501049065** con la tesis titulada: **"El ciclo del glioxilato en *Rhizobium*, vida libre y en la interacción con plantas leguminosas"**, realizada bajo la dirección del (la) **DR. ISMAEL HERNANDEZ LUCAS:**

Presidente:	DRA. GLORIA SOBERON CHAVEZ
Vocal:	DR. JUAN MIRANDA RIOS
Vocal:	DR. DANIEL GENARO SEGURA GONZALEZ
Vocal:	DR. ALFREDO MARTINEZ JIMENEZ
Secretario:	DR. ISMAEL HERNANDEZ LUCAS
Suplente:	DR. MARIO SOBERON CHAVEZ
Suplente	DR. ALEJANDRO GARCIA DE LOS SANTOS

Sin otro particular, me es grato enviarle un cordial saludo.

A t e n t a m e n t e
"POR MI RAZA HABLARA EL ESPIRITU"
Cd. Universitaria, D.F. a 26 de marzo de 2010

Dr. Juan Núñez Farfán
Coordinador del Programa

FACULTAD DE CIENCIAS
DIVISION DE ESTUDIOS DE POSGRADO

JNF/DCRV/ASR/mnm

AGRADECIMIENTOS

Al Posgrado en Ciencias Biológicas, UNAM.

Al CONACYT por la beca otorgada para realizar los estudios de doctorado (No. de becario 167227).

A la DGAPA/PAPIIT por los apoyos otorgados para la realización de este trabajo (proyectos IN206802 y IN206705).

A los miembros del comité tutor, el Dr. Ismael Hernández Lucas, el Dr. Juan Miranda Ríos y el Dr. Michael F. Dunn, por su valiosa participación critica durante el desarrollo de este trabajo.

AGRADECIMIENTOS PERSONALES

A mis Padres Aurelio y Carmen, por el apoyo recibido durante mi formación profesional.

A mis hijos Oscar y Oswaldo, porque su presencia ha sido el motivo más grande que me ha impulsado para lograr esta meta.

A mi esposa Xochitl, por su comprensión y tolerancia.

Al Dr. Ismael Hernández Lucas por haberme permitido trabajar en su grupo y por transmitirme su conocimiento.

Al Dr. Jesús Caballero por brindarme su apoyo estos últimos años.

Al Dr. Alejandro García de los Santos por su enorme disponibilidad y orientación proporcionada en diversos aspectos relacionados con mi proyecto.

A los miembros del jurado por la revisión de esta tesis: Dres. Juan Miranda Ríos, Alejandro García de los Santos, Gloria Soberón Chávez, Daniel Genaro Segura González, Alfredo Martínez Jiménez y Mario Soberón Chávez.

A mis compañeros del laboratorio de Ecología Genómica del Centro de Ciencias Genómicas y del laboratorio del doctor Edmundo calva/José Luís Puente del Instituto de Biotecnología.

ÍNDICE

ABREVIATURAS

PHB	poli-β-hidroxibutirato.
Factor Nod	factor de nodulación.
ORF	del ingles open reading frame; marco de lectura abierto
ATP	trifosfato de adenosina
GTP	trifosfato de guanosina
Acetil-CoA	acetil coenzima A
ACN	aconitaza
IDH	isocitrato deshidrogenasa
α-CDH	α-cetoglutarato deshidrogenasa
SCS	succinil coenzima A sintetasa
SDH	Succinato deshidrogenasa
FUM	fumarato deshidrogenasa
MDH	malato deshidrogenasa
DME	enzima málica
TME	enzima málica
NAD	nicotinamida adenina dinucleótido
NADP	nicotinamida adenina dinucleótido fosfato
PDH	piruvato deshidrogenasa
GOGAT	glutamato-α-cetoglutarato deshidrogenasa
GS	glutamino sintetasa
ICL	isocitrato liasa
MS	malato sintasa

RESUMEN

El ciclo del glioxilato es una ruta anaplerótica del ciclo de Krebs que permite a los microorganismos crecer en compuestos de dos carbonos. Las enzimas de esta ruta son la isocitrato liasa y la malato sintasa, codificadas por los genes *aceA* y *glcB* (o *aceB*), respectivamente. La isocitrato liasa transforma el isocitrato en glioxilato y succinato, mientras que la malato sintasa condensa una molécula de acetil-CoA con glioxilato para formar malato. Los productos finales del ciclo del glioxilato son utilizados en gluconeogénesis u otros procesos biosintéticos. En el presente trabajo se evaluó el papel de los genes *aceA, glcB* y SMc00767 en la vida libre y simbiótica de *S. meliloti*. Los resultados mostraron que en *S. meliloti,* así como en la mayoría de las bacterias, el gen *aceA* es necesario para el metabolismo del acetato. Contrario a lo reportado la mutante en malato sintasa no se ve afectada en el crecimiento en acetato, sugiriendo una ruta alterna para la utilización del glioxilato generado por la isocitrato liasa. El gen *aceA* esta formando un operón con el ORF SMc00767 que se encuentra corriente abajo. La mutante en el gen SMc00767 muestra crecimiento pobre en acetato, sugiriendo un papel en el metabolismo del acetato. Por medio de fusiones transcripcionales y midiendo la actividad de isocitrato liasa en la cepa silvestre y mutante observamos que SMc00767 regula negativamente la expresión de *aceA.* El análisis del transcriptoma de *S. meliloti* silvestre y mutante mostró que SMc00767 además de regular a *aceA* también regula negativamente la expresión de SMa2071 (proteína hipotética), SMb21456 (proteína hipotética), SMb21473 (proteína hipotética), SMc00561 (probable *dnaB*), SMc00769 (proteína hipotética) y SMc00772 (probable *potH*). Los genes *aceA* y *glcB* no son necesarios en la simbiosis *Rhizobium*-Leguminosa. Resultados adicionales revelan que el ciclo del glioxilato se encuentra involucrado en la utilización de poli-β-hidroxibutirato, lo cual sugiere que el ciclo podría ser necesario para la sobrevivencia de la bacteria en condiciones limitantes de carbono. Adicionalmente, el ciclo del glioxilato podría estar operando con fuentes de carbono provistas por la raíz, ya que se conoce que el frijol secreta grandes cantidades de acetato. El conocimiento de las condiciones naturales en las cuales el ciclo del glioxilato opera es fundamental para entender el funcionamiento de las vías metabólicas en el ciclo vital de bacterias del género *Rhizobium*.

ABSTRACT

The glyoxylate cycle is an anaplerotic route to the tricarboxylic acid cycle that enables growth in two-carbon compounds, avoiding the decarboxylation steps of the Krebs cycle. The main enzymes of this pathway are isocitrate lyase and malate synthase, encoded by the *aceA* and *glcB* (or *aceB*) genes respectively. The isocitrate lyase breaks the isocitrate into glyoxylate and succinate, while malate synthase transforms the glyoxylate and one molecule of acetyl-CoA to malate. The final products of the glyoxylate cycle are used in gluconeogenesis or other biosynthetic processes. In the present work the role of *aceA* and *glcB* genes in free-living and symbiotic state of *Sinorhizobium meliloti* was evaluated. The results showed that in *S. meliloti*, as in most bacteria and other microorganisms, the gene *aceA* is necessary for the acetate metabolism. Contrary to what has been reported, the malate synthase mutant is not affected in growth on acetate, suggesting an alternate route for the use of generated glyoxylate by isocitrate lyase. The gene *aceA* is forming an operon with the ORF SMc00767 found downstream. The SMc00767 mutant shows poor growth on acetate, suggesting a role in the acetate metabolism. Gene fusions and enzimatic activities showed that SMc00767 negatively regulates the *aceA* expression. The transcriptome analysis of *S. meliloti* wild type and mutant showed that SMc00767 not only regulates *aceA* but will also negatively regulates the expression of SMa2071 (hypothetical protein), SMb21456 (hypothetical protein), SMb21473 (hypothetical protein), SMc00561 (probable *dnaB*), SMc00769 (hypothetical protein) and SMc00772 (probable *potH*). This work also shows that genes *aceA* and *glcB* are not esential during the *Rhizobium*-Legume interaction. Unpublished results of this investigation show that the glyoxylate cycle is involved in the use of storage carbon sources such as poly-β-hidroxybutyrate, which suggests that the cycle is required under carbon starvation. Additionally, the glyoxylate cycle may be operating with carbon sources provided by the root, since it is known that bean secret large amounts of acetate. No doubt thet trying to know the natural conditions in which the glyoxylate cycle operates in symbionts is a fascinating task that undoubtedly generates relevant answers about the functioning of this metabolic pathway in the life cycle of *Rhizobium.*

INTRODUCCIÓN

En esta sección se presenta una revisión, que abarca los aspectos bioquímicos, genéticos y de regulación del ciclo del glioxilato, así como de su importancia para la virulencia de microorganismos patógenos de plantas y animales.

Dunn, M. F., **Ramírez-Trujillo, J. A**. y Hernández-Lucas I. (2009). Major roles of isocitrate lyase and malate synthase in bacterial and fungal pathogenesis. *Microbiology* **155:**3166-3175.

Microbiology (2009), 155, 3166–3175 DOI 10.1099/mic.0.030858-0

Review

Major roles of isocitrate lyase and malate synthase in bacterial and fungal pathogenesis

M. F. Dunn,[1]† J. A. Ramírez-Trujillo[1]† and I. Hernández-Lucas[2]

Correspondence
I. Hernández-Lucas
ismaelh@ibt.unam.mx

[1]Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Cuernavaca, Morelos 62210, Mexico

[2]Departamento de Microbiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos 62210, Mexico

The glyoxylate cycle is an anaplerotic pathway of the tricarboxylic acid (TCA) cycle that allows growth on C_2 compounds by bypassing the CO_2-generating steps of the TCA cycle. The unique enzymes of this route are isocitrate lyase (ICL) and malate synthase (MS). ICL cleaves isocitrate to glyoxylate and succinate, and MS converts glyoxylate and acetyl-CoA to malate. The end products of the bypass can be used for gluconeogenesis and other biosynthetic processes. The glyoxylate cycle occurs in Eukarya, Bacteria and Archaea. Recent studies of ICL- and MS-deficient strains as well as proteomic and transcriptional analyses show that these enzymes are often important in human, animal and plant pathogenesis. These studies have extended our understanding of the metabolic pathways essential for the survival of pathogens inside the host and provide a more complete picture of the physiology of pathogenic micro-organisms. Hopefully, the recent knowledge generated about the role of the glyoxylate cycle in virulence can be used for the development of new vaccines, or specific inhibitors to combat bacterial and fungal diseases.

Introduction

More than half a century ago, Smith & Gunsalus (1954) reported the existence of the enzyme isocitrate lyase (ICL), which cleaves isocitrate to glyoxylate and succinate, in extracts prepared from *Pseudomonas aeruginosa*. Shortly thereafter Ajl (1956) showed that malate synthase (MS) is able to convert acetyl-CoA and glyoxylate to malate in *Escherichia coli*. In 1957 Kornberg and co-workers demonstrated that the synthesis of C_4 dicarboxylic acids from acetate occurs by a modified tricarboxylic acid (TCA) cycle that was termed the glyoxylate cycle or glyoxylate bypass (Kornberg & Krebs, 1957; Kornberg & Madsen, 1957; Fig. 1). The pathway consists of the two initial steps of the TCA cycle (catalysed by citrate synthase and aconitase) followed by ICL, MS and malate dehydrogenase (Fig. 1). The glyoxylate cycle serves to bypass the CO_2-generating steps of the TCA cycle and allow the net assimilation of carbon from C_2 compounds, allowing micro-organisms to replenish the pool of TCA cycle intermediates necessary for gluconeogenesis and other biosynthetic processes. The net result of the glyoxylate cycle is the production of malate and succinate from two molecules of acetyl-CoA derived from acetate or from the degradation of ethanol, fatty acids or poly-β-hydroxybutyrate (Fig. 1). During growth on these compounds, ICL competes with isocitrate dehydrogenase (IDH) for their common substrate, isocitrate. IDH has a much higher affinity for isocitrate and, in bacteria, it is inactivated by phosphorylation by a bifunctional IDH kinase-phosphatase, thus directing isocitrate towards the biosynthetic reactions of the glyoxylate cycle. IDH dephosphorylation (activation) occurs when glycolytic and TCA cycle intermediates are present in the medium, causing isocitrate to be directed towards the energy-yielding TCA cycle (Cozzone, 1998).

The glyoxylate cycle is widespread and well documented in archaea, bacteria, protists, plants, fungi and nematodes [the latter contain an ICL–MS gene fusion (Kondrashov *et al.*, 2006)]. The presence of this metabolic pathway in animals is controversial. ICL and MS activities have been reported in birds and amphibians (Davis *et al.*, 1986, 1990) and a recent comparative genomic study showed the presence of an ICL gene in nematodes and cnidaria and an MS gene in nematodes, cnidaria, echinoderms, amphibians, fish, and insects. Interestingly, in placental mammals the MS gene is a pseudogene and the ICL gene is absent (Kondrashov *et al.*, 2006). In addition to allowing the growth of bacteria on C_2 compounds, the glyoxylate cycle is important for the growth of higher plant seedlings under most environmental conditions, since it participates in the conversion of stored lipids to carbohydrates that serve as a primary nutrient source prior to the commencement of photosynthesis (Eastmond *et al.*, 2000; Kornberg & Beevers, 1957a, b). In plants, the glyoxylate cycle enzymes are usually localized in peroxisomes, but recent studies with protein-targeting mutants have shown that they can also function effectively

†These authors contributed equally to this work.

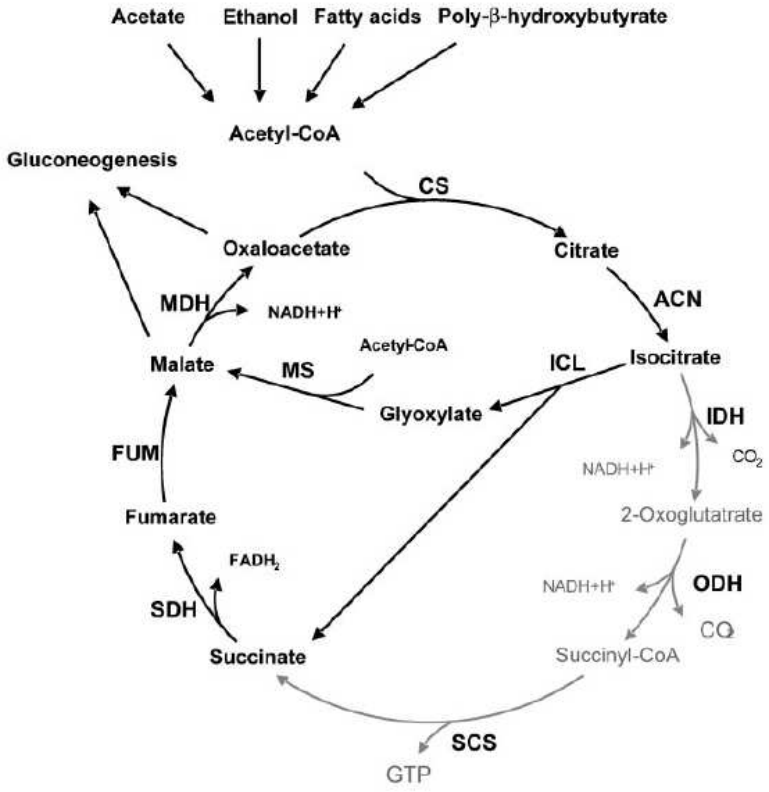

Fig. 1. Enzymic reactions of the glyoxylate and TCA cycles. A variety of metabolic processes can generate acetyl-CoA, the carbon from which can be preserved by metabolism via the glyoxylate cycle, which bypasses the CO_2-generating steps of the TCA cycle. Abbreviations: CS, citrate synthase; ACN, aconitase; IDH, isocitrate dehydrogenase, ODH, 2-oxoglutarate dehydrogenase; SCS, succinyl-CoA synthetase; SDH, succinate dehydrogenase; FUM, fumarase; MDH, malate dehydrogenase; MS, malate synthase; ICL, isocitrate lyase.

in the cytosol (Kunze *et al.*, 2002; McCammon *et al.*, 1990; Piekarska *et al.*, 2008). There is evidence supporting the expression of the glyoxylate cycle during embryogenesis of the nematode *Caenorhabditis elegans* (Kahn & McFadden, 1980; Liu *et al.*, 1997) and it was suggested that the glyoxylate cycle may allow the growth of halophytic archaea in hypersaline lakes (Oren & Gurevich, 1994; Serrano & Bonete, 2001).

The genetic regulation of the glyoxylate cycle during bacterial growth on acetate has been reviewed (Cozzone, 1998) and in the last several years it has become evident that the pathway is important in fungal and bacterial pathogenesis (Lorenz & Fink, 2002; Vereecke *et al.*, 2002a). This review focuses on the latter aspect and summarizes the functional role of the glyoxylate cycle in human, animals and plant pathogens as well as in symbionts.

Biochemical characteristics of ICL and MS

As described above, the glyoxylate cycle is a specialized pathway that has been extensively studied in connection with bacterial growth on C_2 compounds, and ICL and MS are the signature enzymes of the pathway (Fig. 1). ICL is a homotetramer requiring Mg^{2+} or Mn^{2+} and a thiol for activity. During catalysis, isocitrate is deprotonated, forming succinate and glyoxylate. In the ICL of *E. coli*, Lys-193, Cys-195, His-197 and His-356 are catalytic, active-site residues, while His-184 is involved in the assembly of the tetrameric enzyme (Diehl & McFadden, 1993, 1994; Rehman & Mcfadden, 1996, 1997). The recent structural determination of the ICLs from *E. coli* (Britton *et al.*, 2001), *Mycobacterium tuberculosis* (Sharma *et al.*, 2000) and *Aspergillus nidulans* (Britton *et al.*, 2000) has revealed key aspects of their functionality. For instance, a remarkable difference between prokaryotic and eukaryotic ICLs is the presence of an additional approximately 100 amino acids located near the centre of the eukaryotic enzyme that have been proposed to function in the localization of ICL in peroxisomes. This also explains the subunit molecular mass difference between prokaryotic (48 kDa) and eukaryotic (67 kDa) enzymes.

The second enzyme of the glyoxylate cycle is MS, which condenses glyoxylate with an acetyl group from acetyl-CoA to produce malate. MS requires Mg^{2+} for activity and is competitively inhibited by oxalate, a glyoxylate analogue (Dixon *et al.*, 1960). Two MSs are present in *E. coli*: MSG and MSA. MSG, with a molecular mass of around 80 kDa, is a monomeric enzyme (encoded by *glcB*) that functions during growth on glycolate as sole carbon source, and has been found only in bacteria. The active site is composed of the catalytic Asp-631, the residues that bind Mg^{2+} (Glu-427, Asp-455), Arg-338, which hydrogen bonds with glyoxylate, and the residues interacting with acetyl-CoA (Tyr-126, Pro-538, Val-18 and Pro-536) (Howard *et al.*, 2000; Tugarinov *et al.*, 2005). Genetic data support these functional assignments, since a D631N mutant had no

detectable activity and an R338K mutant showed only 6% of wild-type activity (Anstrom *et al.*, 2003).

MSA is a multimeric enzyme with a molecular mass of about 65 kDa (per subunit) that is indispensable for growth on acetate and is found in plants, fungi and bacteria. Lohman *et al.* (2008) showed recently that the structures of MSA and MSG are very similar and that the difference in size is due to the presence of an additional α/β domain in the G isoform. The active site is formed by Glu-250 and Asp-278, which bind Mg^{2+}, while the adenine ring or ribose of acetyl-CoA binds to residues Pro-369, Met-102, Thr-95 Ala-367, Asn-105, Lys-101, Tyr-154 and His-368. MSA contains a highly conserved Cys-438 in the active site, which corresponds to Cys-617 in MSG.

Importance of ICL and MS in fungal pathogens

Plant-pathogenic fungi

Several reports indicate an important role for ICL in fungal virulence on plants. Studies with *Leptosphaeria maculans*, causal agent of blackleg of crucifers (e.g. broccoli, canola and cauliflower) have shown that the *icl1* gene is expressed during its infection of *Brassica napus* cotyledons and inactivation of this locus causes low germination rates of pycnidiospores, reducing the pathogenicity of the fungus on cotyledons as well as limiting its hyphal growth on canola. It was suggested that the reduced pathogenicity of the mutant is due to its inability to utilize carbon sources provided by the plant (Idnurm & Howlett, 2002). Another role for ICL in fungal phytopathogenesis has been reported in *Magnaporthe grisea*, the rice blast pathogen, which can also infect a number of other agriculturally important cereals including wheat, rye and barley. *M. grisea* reproduces both sexually and asexually to produce specialized infectious structures known as appressoria. The appressoria attach to the cuticle of the host and from them hyphae emerge and penetrate the plant, the inside of which is rapidly colonized by the fungus, with disease symptoms being observable in a few days. During infection by *M. grisea*, significant ICL gene expression was found in conidia, appressoria, mycelia and hyphae. Deletion of the *M. grisea ICL1* gene caused a reduction in appressorium formation, conidiogenesis and cuticle penetration, and an overall decrease in damage to leaves of rice and barley. Thus ICL is essential for full virulence in this organism (Rauyaree *et al.*, 2001; Wang *et al.*, 2003).

Stagonospora nodorum is a necrotropic fungal pathogen producing leaf and glume blotch disease on wheat and other cereals of economic importance. Infection begins by germination of pycnidiospores on the leaf followed by hyphal penetration of the host and sporulation at the end of the infection cycle. In the initial steps of infection the pathogen depends on internal, stored carbon sources and it is thought that lipids are metabolized via the glyoxylate pathway to produce glucose and support fungal development. Expression analysis of the MS gene (*mls1*) in this pathogen during its interaction with wheat showed an increased expression in ungerminated spores followed by a dramatic decrease in transcription after germination. Paradoxically, the opposite pattern was seen using MS activity measurements, where activity was undetectable in ungerminated spores but increased to a significant level after germination. Spores of an *mls1* null mutant inoculated onto wheat seedlings and leaves were unable to induce necrotic lesions on either tissue, indicating that this gene is essential for virulence on wheat. The biological reason for this phenotype is that the *mls1*-deficient strain has dramatically decreased spore germination and a reduction in the length of hyphae (Solomon *et al.*, 2004).

Asakura *et al.* (2006) demonstrated the functional role of ICL in *Colletotrichum lagenarium*, which causes anthracnose on a considerable number of plants of agricultural interest such as cucumber, watermelon, muskmelon, cantaloupe, winter squash and bittermelon. An *icl1* mutant of *C. lagenarium* failed to grow on acetate or fatty acids, similar to other ICL mutants of fungi and prokaryotes. For the cellular localization of ICL, the encoding gene was fused to that of the green fluorescent protein (GFP) and *icl1–gfp* expression was detected in peroxisomes, conidia, appressoria and hyphae of the fungus. The *icl1* mutant was able to germinate and develop appressoria and was capable of degrading lipid bodies as well as the wild-type strain. However, conidia from the *icl1*-deficient mutant inoculated onto cucumber leaves and cotyledons formed a reduced number of lesions on leaves, and especially on cotyledons, but nevertheless remained pathogenic. In invasive experiments such as the inoculation of conidia into wound sites, no defect was observed in the *icl1* mutant, while in penetration assays on cucumber cotyledons the mutant was unable to develop penetrating hyphae, indicating a requirement for ICL at this early stage of *C. lagenarium* infection.

Candida albicans

Support for the direct involvement of ICL in virulence has also come from studies of *Candida albicans*, a commensal of the mammalian microbiota inhabiting the skin, mouth, gastrointestinal tract, gut and vagina. In immunocompromised patients, this diploid fungus is responsible for mucosal surface infections as well as life-threatening systemic infections. *C. albicans* is able to survive and grow inside macrophages. Transcriptional profiles of phagocytosed populations of *C. albicans* showed that all the steps of the glyoxylate cycle are induced (Lorenz *et al.*, 2004). Northern blot and differential display experiments with *C. albicans* in the presence of macrophages revealed that both ICL and MS are induced (Lorenz & Fink, 2001; Prigneau *et al.*, 2003). In addition, both enzymes are induced in *C. albicans* exposed to human neutrophils (Fradin *et al.*, 2005). High enzymic activities of ICL and MS were detected in *C. albicans* strains isolated from diabetic patients suffering from vulvovaginal candidiasis (Lattif, *et al.*, 2006). Furthermore, evaluation of ICL mutants in a

mouse model demonstrated that activity of this enzyme is essential for fungal virulence (Lorenz & Fink, 2001). In a model that mimics *C. albicans* bloodstream infection, ICL and MS were downregulated in the initial stages of infection (10 min), upregulated beginning about 20 min after infection and reached a 20-fold increase after 60 min, suggesting blood-specific expression and an important biological role for the glyoxylate cycle genes in bloodstream infections (Fradin *et al.*, 2003). In an interesting study Barelle *et al.* (2006) showed a specific activation of the *C. albicans ICL1* when the pathogen was exposed to neutrophils or macrophages, but because *icl* was not expressed in infected kidney cells it was concluded that *ICL1* contributes to virulence but is not essential for systemic infection.

A clear role for ICL in the pathogenesis of *C. albicans* emerges from the data summarized above. However, the function of this enzyme in *C. albicans* is peculiar because it is probably involved in processes other than lipid utilization or gluconeogenesis, since an ICL-deficient mutant is unable to utilize acetate, ethanol, citrate, glycerol, oleate, lactate, pyruvate, peptone, glutamate or alanine for growth, unlike the parental strain (Ramírez & Lorenz, 2007; Piekarska *et al.*, 2006, 2008; Brock, 2009). ICL expression is also detected during growth on Casamino acids, glutamate or peptone, and under starvation conditions (Barelle *et al.*, 2006; Brock, 2009). Interestingly a *fox2* mutant lacking the second enzyme of the β-oxidation pathway is also unable to utilize acetate, ethanol, lactate and oleic acid and is significantly attenuated in virulence. Therefore both the *fox2* mutant and the ICL-deficient mutant are unable to utilize nonfermentable carbon sources and have reduced virulence in mice, indicating a role for the β-oxidation pathway in virulence. This contention is not supported by the finding that a mutant in peroxisome biogenesis (*pex5*), which is strongly reduced in β-oxidation activity and is unable to utilize oleic acid, is able to use acetate, ethanol or lactate and is not affected in virulence (Piekarska *et al.*, 2006). This shows that acetyl-CoA derived from β-oxidation is not the carbon source inducing the glyoxylate cycle in the *pex5* mutant. Thus, *ICL1* expression in virulence may result not from lipid metabolism (Piekarska *et al.*, 2006; Ramírez & Lorenz, 2007; Brock, 2009) but from the conversion of carbon sources such as lactate into C_2 units for metabolism by the glyoxylate cycle (Piekarska *et al.*, 2006). ICL in *C. albicans*, unlike other organisms, is necessary for the utilization of a large variety of carbon sources. The fact that ICL is probably interconnected with multiple metabolic networks important in virulence encourages the development of specific inhibitors against this enzyme.

Other fungal pathogens

Upregulation of the glyoxylate cycle genes has also been detected in other intracellular fungal pathogens such as *Paracoccidioides brasiliensis*, which causes paracoccidioidomycosis in humans. RT-PCR analysis showed that transcript levels of the ICL and MS genes in this fungus increased following phagocytosis by murine macrophages (Derengowski *et al.*, 2008). *Penicillium marneffei* is a dimorphic fungus that can cause systemic mycosis in humans. The incidence of this fungal infection has increased substantially during the past few years, occurring most often in patients infected with HIV. To evaluate if the glyoxylate cycle was involved in the virulence of *P. marneffei*, Northern blot experiments were performed; these showed that after macrophage internalization of conidia the ICL-encoding gene (*acuD*) was highly expressed, suggesting a potential role for the cycle in the pathogen's adaptation inside macrophages (Thirach *et al.*, 2008). Together, these data directly or indirectly support the relevance of the glyoxylate pathway in fungal virulence in plants, animals and humans.

While a functional role for the glyoxylate cycle in fungal virulence is widespread, it is not universal. In the animal pathogen *Aspergillus fumigatus*, ICL expression was detected in hyphae and in conidia (Ebel *et al.*, 2006) but tissues of patients infected with the fungus were negative for the enzyme after immunostaining, and a mutant deleted of the ICL gene (*acuD*) was fully virulent in a murine model (Schöbel *et al.*, 2007). Similar results were obtained with the human-pathogenic fungus *Cryptococcus neoformans*, where ICL in a rabbit meningitis model was upregulated after 7 days in the subarachnoid space but an *icl1* mutant showed the same number of subarachnoidal yeast cells as the wild-type after 10 days in immunosuppressed rabbits. In addition, in an inhalation model of murine cryptococcosis, no differences in survival were observed between an *icl1* mutant and the wild-type, and similar growth was observed for both *C. neoformans* strains inside macrophages (Rude *et al.*, 2002). These findings show a lack of correlation between ICL gene expression and biological function in these systems.

Functional role of ICL and MS in phagocytosed bacteria

Mycobacterium tuberculosis

The actinobacterium *Mycobacterium tuberculosis* kills 3 000 000 people worldwide every year and is assumed to utilize fatty acid degradation products when growing in the host (Segal & Bloch, 1956; Srivastava *et al.*, 2008). The genome sequence of this bacterium (Cole *et al.*, 1998) contains more than 250 genes encoding proteins annotated as being involved in fatty acid metabolism, providing indirect support for the role of fatty acid degradation and C_2 metabolism in the pathogenesis of *M. tuberculosis*. ICL activity increases in pellicles in synthetic media as a consequence of fatty acid degradation (Murthy *et al.*, 1973) as well as under microaerophilic growth conditions (Wayne & Lin, 1982). Increased *aceA* (*icl*) mRNA expression in response to human macrophages is also documented (Dubnau *et al.*, 2002; Graham & Clark-

Curtiss, 1999). *M. tuberculosis* expresses a 50 kDa protein during intracellular infection (Sturgill-Koszycki *et al.*, 1997) and this was shown to be encoded by a second copy of an ICL gene that is present in several *Mycobacterium* species. Expression analysis and biochemical characterization of ICL activity clearly show that *M. tuberculosis* and *Mycobacterium avium* have two functional ICLs, ICL and AceA (Höner Zu Bentrup *et al.*, 1999). ICL seems to be the principal enzyme in the processing of isocitrate and McKinney *et al.* (2000) reported that single ICL mutations had no dramatic effect on the growth of *M. tuberculosis* in mouse lung during the first 2 weeks of infection. However, lungs infected with the mutant showed few changes between 2 and 16 weeks, suggesting that the ICL mutant had a reduced ability to sustain the infection. In contrast, at 16 weeks the lungs of mice infected with the virulent Erdman strain showed inflammatory lesions, enlargement and multiple expanding and coalescing tubercles. *icl* mRNA levels markedly increase in lungs of mice (Timm *et al.*, 2003) and in human lung granulomas, as well as in the lymphocyte region of necrotic granulomas. In contrast, *icl* expression was not detected in the transition zone and in the central region of necrotic granulomas, supporting the notion that *icl* has a pivotal role in bacterial persistence in the host (Fenhalls *et al.*, 2002). Muñoz-Elías & McKinney (2005) reported that single mutations in *icl* or *aceA* had no dramatic effect on bacterial growth on fatty acids, while an *icl aceA* double mutant was unable to grow on this carbon source. The double mutant inoculated into mice was eliminated from lungs and spleen and was unable to induce splenomegaly or alterations in lungs. ICL activity is thus essential for *M. tuberculosis* survival in the host. Additionally, ICL and to a lesser extent AceA were required for the growth of *M. tuberculosis* on propionate and on odd-chain fatty acids as a carbon source (Muñoz-Elías & McKinney, 2005; Muñoz-Elías *et al.*, 2006). The propionate or propionyl-CoA derived from β-oxidation of odd-chain fatty acids can be catabolized by the methylcitrate cycle, consisting of the enzymes 2-methylisocitrate lyase (MICL), methylcitrate synthase and methylcitrate dehydratase, encoded by the *prpB*, *prpC* and *prpD* genes, respectively. The *M. tuberculosis* genome contains homologues of *prpC* and *prpD*, but not *prpB*. However, structural and biochemical studies have demonstrated that unlike other ICLs and MICLs, the *M. tuberculosis* ICL possesses dual ICL/MICL activity and can support growth on acetate and propionate (Gould *et al.*, 2006). The *prpC* and *prpD* genes are upregulated during infection of macrophages (Schnappinger *et al.*, 2003), suggesting that the methylcitrate cycle could be important in *M. tuberculosis* pathogenesis. However, studies with a *prpC prpD* double mutant show that the methylcitrate cycle is required for *M. tuberculosis* replication in non-activated murine bone-marrow-derived macrophages, but that in IFN-γ-activated macrophages or in the lungs and spleen of inoculated mice the double mutant shows no alteration of *in vivo* growth, persistence or virulence. Thus, the functional role of ICL in *M. tuberculosis* virulence is in the glyoxylate cycle rather than the methylcitrate cycle (Muñoz-Elías *et al.*, 2006). Recent reports show that *M. tuberculosis phoP* mutants are attenuated but persist in macrophages and mouse organs apparently because the mutant expresses higher levels of *icl*. The PhoP-deficient strain would be a good candidate for vaccine production, since prolonged exposure of the immune system to the persistent, attenuated strain could result in long-term immunogenicity (Gonzalo-Asensio *et al.*, 2008).

The single MS present in *M. tuberculosis* may also contribute to pathogenicity. Studies *in vitro* show that MS is secreted and enhances the adherence of the pathogen to lung epithelial cells, supporting the notion that it may function as an adhesin as well as an enzyme (Kinhikar *et al.*, 2006). MS can also be used as a biomarker because it is recognized in the humoral response of tuberculosis patients, making possible its use in serodiagnostic assays for identification of this pulmonary disease (Achkar *et al.*, 2006; Melo Cardoso Almeida *et al.*, 2008; Samanich *et al.*, 2001; Wanchu *et al.*, 2008).

Other bacterial pathogens

The importance of a functional glyoxylate cycle in some intracellular human pathogens such as *Salmonella* has been evaluated, and the results obtained with an ICL-deficient strain of *Salmonella enterica* serovar Typhimurium show that ICL is required for persistence during chronic infection, but not for acute lethal infection in mice (Fang *et al.*, 2005; Kim *et al.*, 2006; Tchawa Yimga *et al.*, 2006). In *Brucella suis*, the glyoxylate cycle has been shown to be unnecessary for virulence (Kohler *et al.*, 2002). The pathogens *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* induce ICL and MS during growth on acetate but not on xylose, while *Yersinia pestis* synthesizes both enzymes on both carbon sources (Hillier & Charnetzky, 1981b). A natural mutation in *iclR*, encoding a repressor protein, explains why *Y. pestis* constitutively produces ICL (Sebbane *et al.*, 2004), and this constitutive enzymic activity has been used to identify *Y. pestis* in humans, animals, water, soil and food (Hillier & Charnetzky, 1981a; Quan *et al.*, 1982). Sebbane *et al.* (2004) showed that mutations in the sole ICL gene (*aceA*) of *Y. pestis* prevented growth on acetate but did not affect pathogenesis in a mouse model.

Rhodococcus equi is a Gram-positive intracellular bacillus causing pneumonia and enteritis in foals and is also able to infect cats, dogs and pigs, producing submandibular adenitis. *R. equi* causes infection in immunocompromised humans and is easily isolated from soil contaminated with faeces. The fact that *R. equi* uses acetate (Kelly *et al.*, 2002) and probably lipids as carbon sources in soil and within macrophages suggests that it may utilize the glyoxylate cycle in its interaction with foals. To validate this hypothesis an ICL-deficient (*aceA* mutant) strain of *R. equi* was evaluated in macrophages (Wall *et al.*, 2005). The population of the ICL-deficient strain increased in macro-

phages after 12 h but then declined significantly, indicating that ICL is essential for long-term survival and proliferation in macrophages, consistent with the finding that the *aceA* mutant was partially attenuated in a mouse model. In foals, the wild-type *R. equi* strain induced severe lesions of suppurative to pyogranulomatous bronchopneumonia in lung, while the *aceA* mutant was unable to produce any alteration, probably because the mutant population was six orders of magnitude lower than that of the wild-type strain (Wall *et al.*, 2005).

Another pathogen infecting lungs is *Pseudomonas aeruginosa*, which produces lung dysfunction and mortality in humans with cystic fibrosis. *P. aeruginosa* upregulates genes *in vivo* that are needed for replication in the lung environment, including those encoding enzymes for fatty acid and lipid metabolism (alcohol dehydrogenase), choline metabolism (betaine aldehyde dehydrogenase), amino acid degradation (arginine deiminase), nitrogen metabolism (respiratory nitrate reductase) and the glyoxylate cycle (ICL). These metabolic genes almost certainly contribute to carbon and nitrogen nutrition, allowing the replication and persistence of *P. aeruginosas* inside the host (Son *et al.*, 2007). Support for this hypothesis was obtained by a study in which several metabolic mutants of *P. aeruginosa* were isolated and evaluated on alfalfa seedlings and in a mouse model (Lindsey *et al.*, 2008). An ICL (*aceA*) mutant derived from strain PAO1 displayed reduced virulence on alfalfa seedlings and a reduction in histopathology in rat lungs. Thus the glyoxylate cycle has a pivotal role in the interaction of *P. aeruginosa* with both plant and mammalian hosts.

Inhibitors of ICL and MS

The development of specific inhibitors against ICL and MS is an attractive prospect, since in a variety of human-pathogenic bacteria and fungi the expression of the glyoxylate cycle genes is detected in specific stages of the interaction (Table 1). Several inhibitors of ICL have been identified, including itaconate, itaconic anhydride, bromopyruvate, nitropropionate, oxalate and malate (Höner Zu Bentrup *et al.*, 1999; McFadden & Purohit, 1977). However, these are not pharmacologically suitable for use *in vivo* since they are toxic and non-specific. For instance, nitropropionate inhibits ICL but also inhibits succinate dehydrogenase, a pivotal enzyme of the TCA cycle (Alston *et al.*, 1977; Fig. 1). Efforts to isolate natural ICL inhibitors from plants revealed that extracts of *Illicium verum* and *Zingiber officinale* inhibit the ICL of *Mycobacterium tuberculosis* (Bai *et al.*, 2007).

Because the glyoxylate cycle is important in many types of fungal pathogenesis, natural inhibitors of fungal ICLs have been sought. Those isolated from the tropical sponge *Hippospongia* sp. are halisulfates that are able to inhibit ICL activity, appressorium formation and C_2 utilization in the rice blast fungus *Magnaporthe grisea* (Lee *et al.*, 2007). Natural glyoxylate cycle inhibitors such as 5-hydroxyindole-type alkaloids are potent inhibitors of the *Candida albicans* ICL (Lee *et al.*, 2009).

It was recently proposed that instead of ICL or MS, other enzymes could make better targets for bringing about inhibition of the glyoxylate cycle. For instance, inactivation of the kinase-phosphatase that phosphorylates and inactivates isocitrate dehydrogenase would be a good candidate, since its absence would promote carbon flow through the full TCA cycle and avoid its assimilation by ICL (Singh & Ghosh, 2006). Other proposed targets for indirect inhibition of the glyoxylate cycle are the enzymes of the PHB cycle, since this metabolic route can provide acetyl-CoA in a manner that circumvents the link between glycolysis and the TCA cycle (Purohit *et al.*, 2007).

Roles of ICL and MS in other plant–bacteria interactions

The functionality of the glyoxylate cycle was evaluated in the plant pathogen *Rhodococcus fascians*, which causes leafy gall disease on a variety of monocots and dicots, including *Nicotiana tabacum* (tobacco). A malate synthase mutant of *R. fascians* gave a diminished number of bacteria inside symptomatic tobacco tissues in comparison to tissues infected by the wild-type (Vereecke *et al.*, 2002a, b). In the plant pathogen *Xanthomonas campestris,* MS was induced during infection of tomato plants and a MS-deficient strain induced fewer and smaller lesions on 75 % of inoculated leaves as compared to the wild-type strain (Tamir-Ariel *et al.*, 2007). Thus, in these two pathogens the glyoxylate cycle has an important role in the plant–microbe interaction.

Carbon metabolism has long been studied in *Rhizobium* spp., bacteria that form a nitrogen-fixing symbiosis with leguminous plants. Large quantities of acetate and fatty acids were reported in soybean nodules formed by *Bradyrhizobium japonicum* (Johnson *et al.*, 1966), and radiorespirometric studies of *B. japonicum* bacteroids indicated that up to 50 % of the acetyl-CoA entering the TCA cycle was metabolized via MS (Stovall & Cole, 1978). It was also shown that acetate can be used by isolated *B. japonicum* bacteroids to support *ex planta* nitrogen fixation (Peterson & LaRue, 1981, 1982). The existence of a complete glyoxylate cycle in nitrogen-fixing bacteroids is in doubt because ICL activity is not detected in the microsymbionts isolated from soybean, pea, alfalfa and clover nodules (Green *et al.*, 1998; Johnson *et al.*, 1966). However, ICL activity has been detected in bacteroids from senesced nodules formed by *B. japonicum* (Wong & Evans, 1971). In contrast, MS activity was found in bacteroids isolated from pea, alfalfa and clover nodules, and substantially higher activities were detected in bacteroids isolated from bean, cowpea and soybean nodules (Green *et al.*, 1998; Johnson *et al.*, 1966). Based on these data we decided to genetically evaluate the role of the glyoxylate cycle in the *Rhizobium*–Leguminosae symbiosis. We showed that neither MS nor or ICL is required for symbiosis, since MS (*glcB*) mutants of *Rhizobium legumi-*

Table 1. ICL and MS gene expression and mutant virulence phenotypes during interactions with host organisms

Organism	Host	Gene*	Expression†	Mutant phenotype virulence†	Reference
Aspergillus fumigatus	Human	*id*	Hyphae and conidia	Virulent	Schöbel *et al.* (2007)
Brucella suis	Human	*id*	ND	Virulent	Kohler *et al.* (2002)
Candida albicans	Human	*id*	Macrophages	Less virulent	Lorenz & Fink (2001)
		ms	Macrophages	ND	
Cryptococcus neoformans	Human	*id*	Rabbit subarachnoid space	Virulent	Rude *et al.* (2002)
Mycobacterium tuberculosis	Human	*id1*	Lung of mice and human	Double mutant (*icl1 icl2*)	McKinney *et al.* (2000)
		id2		Avirulent	Muñoz-Elías & McKinney (2005)
		ms	Lung	ND	Kinhikar *et al.* (2006)
Paracoccidioides brasiliensis	Human	*id*	Macrophages	ND	Derengowski *et al.* (2008)
		ms	Macrophages	ND	
Penicllium marneffei	Human	*id*	Macrophages	ND	Thirach *et al.* (2008)
Pseudomonas aeruginosa	Human	*id*	ND	Less virulent	Lindsey *et al.* (2008)
Salmonella enterica serovar Typhimurium	Human	*id*	ND	Virulent	Fang *et al.* (2005); Kim *et al.* (2006)
Yersinia pestis	Human	*id*	Constitutive	Virulent	Sebbane *et al.* (2004)
		ms	Constitutive	ND	
Rhodococcus equi	Foals	*id*	ND	Avirulent	Wall *et al.* (2005)
Colletotrichum lagenarium	Cucumber	*id*	Appressorium, conidia, hyphae	Less virulent	Asakura *et al.* (2006)
Leptosphaeria maculans	Canola	*id*	Cotyledons	Less virulent	Idnurm & Howlett (2002)
Magnaporthe grisea	Rice	*id*	Appressorium, conidia, mycelia, hyphae	Less virulent	Wang *et al.* (2003)
Rhodococcus fascians	Tobacco	*ms*	ND	Less virulent	Vereecke *et al.* (2002)
Stagonospora nodorum	Wheat	*ms*	Ungerminated spores	Avirulent	Solomon *et al.* (2004)
Xanthomonas campestris	Tomato	*ms*	ND	Less virulent	Tamir-Ariel *et al.* (2007)
Rhizobium leguminosarum	Pea	*ms*	ND	No effect on symbiosis	García-de los Santos *et al.* (2002)
Rhizobium tropici	Bean	*id*	ND	No effect on symbiosis	Ramírez-Trujillo *et al.* (2007)
Sinorhizobium meliloti	Alfalfa	*id*	ND	No effect on symbiosis	Ramírez-Trujillo *et al.* (2007)
		ms	ND	No effect on symbiosis	Ramírez-Trujillo *et al.* (2007)

*Various names have been used in the literature for the genes encoding ICL and MS in different organisms; for clarity, the designations *icl* and *ms* are used here.
†ND, Not determined.

nosarum evaluated during their interaction with *Pisum sativum* have the same levels of nitrogen fixation and nodulation as the wild-type strain (García-de los Santos *et al.*, 2002). Similarly, a *glcB* mutant of *Sinorhizobium meliloti* is able to normally nodulate and fix nitrogen in symbiosis with *Medicago sativa*. To evaluate the role of ICL, null mutants were constructed in *S. meliloti* and *Rhizobium tropici* and their symbiotic performance was evaluated on *M. sativa* and *Phaseolus vulgaris*, respectively. The results demonstrated that in both symbiotic models ICL is not involved in nodulation or nitrogen fixation (Ramírez-Trujillo *et al.*, 2007).

Conclusions

The study of the metabolic pathways involved in the pathogenesis of bacterial and fungal infections is critical for public health, crop productivity and animal welfare. The glyoxylate cycle is an important metabolic pathway in this regard, since substantial evidence supports its importance in many host–pathogen systems. The knowledge generated about the role of this pathway in pathogenesis is important, since it provides the opportunity to develop specific inhibitors of ICL and MS that could be used to combat fungal and bacterial diseases.

Acknowledgements

We thank F. J. Santana, A. Vazquez, M. Fernández-Mora, A. L. Gallego-Hernández, L. M. Aparicio, J. E. Rebollar-Flores, S. Ainsworth and E. Calva for useful scientific comments to improve the manuscript. This research was supported by grants to I. H.-L. from DGAPA/UNAM (IN206802, IN206705 IN214808) and Conacyt (89337) J. A. R.-T. was a PhD student in the Programa de Doctorado en Ciencias Biológicas-UNAM and acknowledges the fellowships from the Consejo Nacional de Ciencia y Tecnología (México).

References

Achkar, J. M., Dong, Y., Holzman, R. S., Belisle, J., Kourbeti, I. S., Sherpa, T., Condos, R., Rom, W. N. & Laal, S. (2006). *Mycobacterium tuberculosis* malate synthase- and MPT51-based serodiagnostic assay as an adjunct to rapid identification of pulmonary tuberculosis. *Clin Vaccine Immunol* **13**, 1291–1293.

Ajl, S. J. (1956). Conversion of acetate and glyoxylate to malate. *J Am Chem Soc* **78**, 3230–3231.

Alston, T. A., Mela, L. & Bright, H. J. (1977). 3-Nitropropionate, the toxic substance of *Indigofera*, is a suicide inactivator of succinate dehydrogenase. *Proc Natl Acad Sci U S A* **74**, 3767–3771.

Anstrom, D. M., Kallio, K. & Remington, S. J. (2003). Structure of the *Escherichia coli* malate synthase G : pyruvate : acetyl-coenzyme A abortive ternary complex at 1.95 Å resolution. *Protein Sci* **12**, 1822–1832.

Asakura, M., Okuno, T. & Takano, Y. (2006). Multiple contributions of peroxisomal metabolic function to fungal pathogenicity in *Colletotrichum lagenarium*. *Appl Environ Microbiol* **72**, 6345–6354.

Bai, B., Xie, J.-P., Yan, J.-F., Wang, H.-H. & Hu, C.-H. (2007). A high throughput screening approach to identify isocitrate lyase inhibitors from traditional Chinese medicine sources. *Drug Dev Res* **67**, 818–823.

Barelle, C. J., Priest, C. L., Maccallum, D. M., Gow, N. A., Odds, F. C. & Brown, A. J. (2006). Niche-specific regulation of central metabolic pathways in a fungal pathogen. *Cell Microbiol* **8**, 961–971.

Britton, K. L., Langridge, S., Baker, P. J., Weeradechapon, K., Sedelnikova, S. E., De Lucas, J. R., Rice, D. W. & Turner, G. (2000). The crystal structure and active site location of isocitrate lyase from the fungus *Aspergillus nidulans*. *Structure* **8**, 349–362.

Britton, K. L., Abeysinghe, I. S. B., Baker, P. J., Barynin, V., Diehl, P., Langridge, S. J., McFadden, B. A., Sedelnikova, S. E., Stillman, T. J. & other authors (2001). The structure and domain organization of *Escherichia coli* isocitrate lyase. *Acta Crystallogr D Biol Crystallogr* **57**, 1209–1218.

Brock, M. (2009). Fungal metabolism in host niches. *Curr Opin Microbiol* **12**, 371–376.

Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, K., Gas, S. & other authors (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**, 537–544.

Cozzone, A. J. (1998). Regulation of acetate metabolism by protein phosphorylation in enteric bacteria. *Annu Rev Microbiol* **52**, 127–164.

Davis, W. L., Jones, R. G. & Goodman, D. B. (1986). Cytochemical localization of malate synthase in amphibian fat body adipocytes: possible glyoxylate cycle in a vertebrate. *J Histochem Cytochem* **34**, 689–692.

Davis, W. L., Jones, R. G., Farmer, G. R., Dickerson, T., Cortinas, E., Cooper, O. J., Crawford, L. & Goodman, D. B. (1990). Identification of glyoxylate cycle enzymes in chick liver – the effect of vitamin D3: cytochemistry and biochemistry. *Anat Rec* **227**, 271–284.

Derengowski, L. S., Tavares, A. H., Silva, S., Procópio, L. S., Felipe, M. S. & Silva-Pereira, I. (2008). Upregulation of glyoxylate cycle genes upon *Paracoccidioides brasiliensis* internalization by murine macrophages and in vitro nutritional stress condition. *Med Mycol* **46**, 125–134.

Diehl, P. & McFadden, B. A. (1993). Site-directed mutagenesis of lysine 193 in *Escherichia coli* isocitrate lyase by use of unique restriction enzyme site elimination. *J Bacteriol* **175**, 2263–2270.

Diehl, P. & McFadden, B. A. (1994). The importance of four histidine residues in isocitrate lyase from *Escherichia coli*. *J Bacteriol* **176**, 927–931.

Dixon, G. H., Kornberg, H. L. & Lund, P. (1960). Purification and properties of malate synthetase. *Biochim Biophys Acta* **41**, 217–233.

Dubnau, E., Fontán, P., Manganelli, R., Soares-Appel, S. & Smith, I. (2002). *Mycobacterium tuberculosis* genes induced during infection of human macrophages. *Infect Immun* **70**, 2787–2795.

Eastmond, P. J., Germain, V., Lange, P. R., Bryce, J. H., Smith, S. M. & Graham, I. A. (2000). Postgerminative growth and lipid catabolism in oilseeds lacking the glyoxylate cycle. *Proc Natl Acad Sci U S A* **97**, 5669–5674.

Ebel, F., Schwienbacher, M., Beyer, J., Heesemann, J., Brakhage, A. A. & Brock, M. (2006). Analysis of the regulation, expression, and localisation of the isocitrate lyase from *Aspergillus fumigatus*, a potential target for antifungal drug development. *Fungal Genet Biol* **43**, 476–489.

Fang, F. C., Libby, S. J., Castor, M. E. & Fung, A. M. (2005). Isocitrate lyase (AceA) is required for *Salmonella* persistence but not for acute lethal infection in mice. *Infect Immun* **73**, 2547–2549.

Fenhalls, G., Stevens, L., Moses, L., Bezuidenhout, J., Betts, J. C., van Helden, P., Lukey, P. T. & Duncan, K. (2002). In situ detection of *Mycobacterium tuberculosis* transcripts in human lung granulomas reveals differential gene expression in necrotic lesions. *Infect Immun* **70**, 6330–6338.

Fradin, C., Kretschmar, M., Nichterlein, T., Gaillardin, C., d'Enfert, C. & Hube, B. (2003). Stage-specific gene expression of *Candida albicans* in human blood. *Mol Microbiol* **47**, 1523–1543.

Fradin, C., De Groot, P., MacCallum, D., Schaller, M., Klis, F., Odds, F. C. & Hube, B. (2005). Granulocytes govern the transcriptional response, morphology and proliferation of *Candida albicans* in human blood. *Mol Microbiol* **56**, 397–415.

García-de los Santos, A., Morales, A., Baldomá, L., Clark, S. R., Brom, S., Yost, C. K., Hernández-Lucas, I., Aguilar, J. & Hynes, M. F. (2002). The *glcB* locus of *Rhizobium leguminosarum* VF39 encodes an arabinose-inducible malate synthase. *Can J Microbiol* **48**, 922–932.

Gonzalo-Asensio, J., Mostowy, S., Harders-Westerveen, J., Huygen, K., Hernández-Pando, R., Thole, J., Behr, M., Gicquel, B. & Martín, C. (2008). PhoP: a missing piece in the intricate puzzle of *Mycobacterium tuberculosis* virulence. *PLoS One* **3**, e3496.

Gould, T. A., van de Langemheen, H., Muñoz-Elías, E. J., McKinney, J. D. & Sacchettini, J. C. (2006). Dual role of isocitrate lyase 1 in the glyoxylate and methylcitrate cycles in *Mycobacterium tuberculosis*. *Mol Microbiol* **61**, 940–947.

Graham, J. E. & Clark-Curtiss, J. E. (1999). Identification of *Mycobacterium tuberculosis* RNAs synthesized in response to phagocytosis by human macrophages by selective capture of transcribed sequences (SCOTS). *Proc Natl Acad Sci U S A* **96**, 11554–11559.

Green, L. S., Karr, D. B. & Emerich, D. W. (1998). Isocitrate dehydrogenase and glyoxylate cycle enzyme activities in *Bradyrhizobium japonicum* under various growth conditions. *Arch Microbiol* **169**, 445–451.

Hillier, S. L. & Charnetzky, W. T. (1981). Rapid diagnostic test that uses isocitrate lyase activity for identification of *Yersinia pestis*. *J Clin Microbiol* **13**, 661–665.

Hillier, S. & Charnetzky, W. T. (1981a). Glyoxylate bypass enzymes in *Yersinia* species and multiple forms of isocitrate lyase in *Yersinia pestis*. *J Bacteriol* **145**, 452–458.

Höner Zu Bentrup, K., Miczak, A., Swenson, D. L. & Russell, D. G. (1999). Characterization of activity and expression of isocitrate lyase in *Mycobacterium avium* and *Mycobacterium tuberculosis*. *J Bacteriol* **181**, 7161–7167.

Howard, B. R., Endrizzi, J. A. & Remington, S. J. (2000). Crystal structure of *Escherichia coli* malate synthase G complexed with

magnesium and glyoxylate at 2.0 Å resolution: mechanistic implications. *Biochemistry* **39**, 3156–3168.

Idnurm, A. & Howlett, B. J. (2002). Isocitrate lyase is essential for pathogenicity of the fungus *Leptosphaeria maculans* to canola (*Brassica napus*). *Eukaryot Cell* **1**, 719–724.

Johnson, G. V., Evans, H. J. & Ching, T. (1966). Enzymes of glyoxylate cycle in rhizobia and nodules of legumes. *Plant Physiol* **41**, 1330–1336.

Kahn, F. R. & McFadden, B. A. (1980). Embryogenesis and the glyoxylate cycle. *FEBS Lett* **115**, 312–314.

Kelly, B. G., Wall, D. M., Boland, C. A. & Meijer, W. G. (2002). Isocitrate lyase of the facultative intracellular pathogen *Rhodococcus equi*. *Microbiology* **148**, 793–798.

Kim, Y. R., Brinsmade, S. R., Yang, Z., Escalante-Semerena, J. & Fierer, J. (2006). Mutation of phosphotransacetylase but not isocitrate lyase reduces the virulence of *Salmonella enterica* serovar Typhimurium in mice. *Infect Immun* **74**, 2498–2502.

Kinhikar, A. G., Vargas, D., Li, H., Mahaffey, S. B., Hinds, L., Belisle, J. T. & Laal, S. (2006). *Mycobacterium tuberculosis* malate synthase is a laminin-binding adhesin. *Mol Microbiol* **60**, 999–1013.

Kohler, S., Foulongne, V., Ouahrani-Bettache, S., Bourg, G., Teyssier, J., Ramuz, M. & Liautard, J. P. (2002). The analysis of the intramacrophagic virulome of *Brucella suis* deciphers the environment encountered by the pathogen inside the macrophage host cell. *Proc Natl Acad Sci U S A* **99**, 15711–15716.

Kondrashov, F. A., Koonin, E. V., Morgunov, I. G., Finogenova, T. V. & Kondrashova, M. N. (2006). Evolution of glyoxylate cycle enzymes in Metazoa: evidence of multiple horizontal transfer events and pseudogene formation. *Biol Direct* **1**, 31.

Kornberg, H. L. & Beevers, H. (1957a). The glyoxylate cycle as a stage in the conversion of fat to carbohydrate in castor beans. *Biochim Biophys Acta* **26**, 531–537.

Kornberg, H. L. & Beevers, H. (1957b). A mechanism of conversion of fat to carbohydrate in castor beans. *Nature* **180**, 35–36.

Kornberg, H. L. & Krebs, H. A. (1957). Synthesis of cell constituents from C_2-units by a modified tricarboxylic acid cycle. *Nature* **179**, 988–991.

Kornberg, H. L. & Madsen, N. B. (1957). Synthesis of C_4-dicarboxylic acids from acetate by a glyoxylate bypass of the tricarboxylic acid cycle. *Biochim Biophys Acta* **24**, 651–653.

Kunze, M., Kragler, F., Binder, M., Hartig, A. & Gurvitz, A. (2002). Targeting of malate synthase 1 to the peroxisomes of *Saccharomyces cerevisiae* cells depends on growth on oleic acid medium. *Eur J Biochem* **269**, 915–922.

Lattif, A. A., Prasad, R., Banerjee, U., Gupta, N., Mohammad, S. & Baquer, N. Z. (2006). The glyoxylate cycle enzyme activities in the pathogenic isolates of *Candida albicans* obtained from HIV/AIDS, diabetic and burn patients. *Mycoses* **49**, 85–90.

Lee, H. S., Lee, T. H., Yang, S. H., Shin, H. J., Shin, J. & Oh, K. B. (2007). Sesterterpene sulfates as isocitrate lyase inhibitors from tropical sponge *Hippospongia* sp. *Bioorg Med Chem Lett* **17**, 2483–2486.

Lee, H. S., Yoon, K. M., Han, Y. R., Lee, K. J., Chung, S. C., Kim, T. I., Lee, S. H., Shin, J. & Oh, K. B. (2009). 5-Hydroxyindole-type alkaloids, as *Candida albicans* isocitrate lyase inhibitors, from the tropical sponge *Hyrtios* sp. *Bioorg Med Chem Lett* **19**, 1051–1053.

Lindsey, T. L., Hagins, J. M., Sokol, P. A. & Silo-Suh, L. A. (2008). Virulence determinants from a cystic fibrosis isolate of *Pseudomonas aeruginosa* include isocitrate lyase. *Microbiology* **154**, 1616–1627.

Liu, F., Thatcher, J. D. & Epstein, H. F. (1997). Induction of glyoxylate cycle expression in *Caenorhabditis elegans*: a fasting response throughout larval development. *Biochemistry* **36**, 255–260.

Lohman, J. R., Olson, A. C. & Remington, S. J. (2008). Atomic resolution structures of *Escherichia coli* and *Bacillus anthracis* malate synthase A: comparison with isoform G and implications for structure-based drug discovery. *Protein Sci* **17**, 1935–1945.

Lorenz, M. C. & Fink, G. R. (2001). The glyoxylate cycle is required for fungal virulence. *Nature* **412**, 83–86.

Lorenz, M. C. & Fink, G. R. (2002). Life and death in a macrophage: role of the glyoxylate cycle in virulence. *Eukaryot Cell* **1**, 657–662.

Lorenz, M. C., Bender, J. A. & Fink, G. R. (2004). Transcriptional response of *Candida albicans* upon internalization by macrophages. *Eukaryot Cell* **3**, 1076–1087.

McCammon, M. T., Veenhuis, M., Trapp, S. B. & Goodman, J. M. (1990). Association of glyoxylate and beta-oxidation enzymes with peroxisomes of *Saccharomyces cerevisiae*. *J Bacteriol* **172**, 5816–5827.

McFadden, B. A. & Purohit, S. (1977). Itaconate, an isocitrate lyase-directed inhibitor in *Pseudomonas indigofera*. *J Bacteriol* **131**, 136–144.

McKinney, J. D., Höner zu Bentrup, K., Muñoz-Elías, E. J., Miczak, A., Chen, B., Chan, W. T., Swenson, D., Sacchettini, J. C., Jacobs, W. R., Jr & Russell, D. G. (2000). Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature* **406**, 735–738.

Melo Cardoso Almeida, C., Vasconcelos, A. C., Jr, Kipnis, A., Andrade, A. L. & Junqueira-Kipnis, A. P. (2008). Humoral immune responses of tuberculosis patients in Brazil indicate recognition of *Mycobacterium tuberculosis* MPT-51 and GlcB. *Clin Vaccine Immunol* **15**, 579–581.

Muñoz-Elías, E. J. & McKinney, J. D. (2005). *Mycobacterium tuberculosis* isocitrate lyases 1 and 2 are jointly required for in vivo growth and virulence. *Nat Med* **11**, 638–644.

Muñoz-Elías, E. J., Upton, A. M., Cherian, J. & McKinney, J. D. (2006). Role of the methylcitrate cycle in *Mycobacterium tuberculosis* metabolism, intracellular growth, and virulence. *Mol Microbiol* **60**, 1109–1122.

Murthy, P. S., Sirsi, M. & Ramakrishnan, T. (1973). Effect of age on the enzymes of tricarboxylic acid and related cycles in *Mycobacterium tuberculosis* H37Rv. *Am Rev Respir Dis* **108**, 689–690.

Oren, A. & Gurevich, P. (1994). Production of D-lactate, acetate, and pyruvate from glycerol in communities of halophilic archaea in the Dead sea and in saltern crystallizer ponds. *FEMS Microbiol Ecol* **14**, 147–156.

Peterson, J. B. & LaRue, T. A. (1981). Utilization of aldehydes and alcohols in soybean bacteroids. *Plant Physiol* **68**, 489–493.

Peterson, J. B. & LaRue, T. A. (1982). Soluble aldehyde dehydrogenase and metabolism of aldehydes by soybean bacteroids. *J Bacteriol* **151**, 1473–1484.

Piekarska, K., Mol, E., van den Berg, M., Ardi, G., van den Burg, J., van Roermund, C., MacCallum, D., Odds, F. & Distel, B. (2006). Peroxisomal fatty acid beta-oxidation is not essential for virulence of *Candida albicans*. *Eukaryot Cell* **5**, 1847–1856.

Piekarska, K., Hardy, G., Mol, E., van den Burg, J., Strijbis, K., van Roermund, C., van den Berg, M. & Distel, B. (2008). The activity of the glyoxylate cycle in peroxisomes of *Candida albicans* depends on a functional beta-oxidation pathway: evidence for reduced metabolite transport across the peroxisomal membrane. *Microbiology* **154**, 3061–3072.

Prigneau, O., Porta, A., Poudrier, J. A., Colonna-Romano, S., Noël, T. & Maresca, B. (2003). Genes involved in beta-oxidation, energy metabolism and glyoxylate cycle are induced by *Candida albicans* during macrophage infection. *Yeast* **20**, 723–730.

Purohit, H. J., Cheema, S., Lal, S., Raut, C. P. & Kalia, V. C. (2007). In search of drug targets for *Mycobacterium tuberculosis*. *Infect Disord Drug Targets* **7**, 245–250.

Quan, T. J., Vanderlinden, J. J. & Tsuchiya, K. R. (1982). Evaluation of a qualitative isocitrate lyase assay for rapid presumptive identification of *Yersinia pestis* cultures. *J Clin Microbiol* **15**, 1178–1179.

Ramírez, M. A. & Lorenz, M. C. (2007). Mutations in alternative carbon utilization pathways in *Candida albicans* attenuate virulence and confer pleiotropic phenotypes. *Eukaryot Cell* **6**, 280–290.

Ramírez-Trujillo, J. A., Encarnación, S., Salazar, E., de los Santos, A. G., Dunn, M. F., Emerich, D. W., Calva, E. & Hernández-Lucas, I. (2007). Functional characterization of the *Sinorhizobium meliloti* acetate metabolism genes *aceA*, SMc00767, and *glcB*. *J Bacteriol* **189**, 5875–5884.

Rauyaree, P., Choi, W., Fang, E., Blackmon, B. & Dean, R. A. (2001). Genes expressed during early stages of rice infection with the rice blast fungus *Magnaporthe grisea*. *Mol Plant Pathol* **2**, 347–354.

Rehman, A. & Mcfadden, B. A. (1996). The consequences of replacing histidine 356 in isocitrate lyase from *Escherichia coli*. *Arch Biochem Biophys* **336**, 309–315.

Rehman, A. & Mcfadden, B. A. (1997). Lysine 194 is functional in isocitrate lyase from *Escherichia coli*. *Curr Microbiol* **35**, 14–17.

Rude, T. H., Toffaletti, D. L., Cox, G. M. & Perfect, J. R. (2002). Relationship of the glyoxylate pathway to the pathogenesis of *Cryptococcus neoformans*. *Infect Immun* **70**, 5684–5694.

Samanich, K., Belisle, J. T. & Laal, S. (2001). Homogeneity of antibody responses in tuberculosis patients. *Infect Immun* **69**, 4600–4609.

Schnappinger, D., Ehrt, S., Voskuil, M. I., Liu, Y., Mangan, J. A., Monahan, I. M., Dolganov, G., Efron, B., Butcher, P. D. & other authors (2003). Transcriptional adaptation of *Mycobacterium tuberculosis* within macrophages: insights into the phagosomal environment. *J Exp Med* **198**, 693–704.

Schöbel, F., Ibrahim-Granet, O., Avé, P., Latgé, J. P., Brakhage, A. A. & Brock, M. (2007). *Aspergillus fumigatus* does not require fatty acid metabolism via isocitrate lyase for development of invasive aspergillosis. *Infect Immun* **75**, 1237–1244.

Sebbane, F., Jarrett, C. O., Linkenhoker, J. R. & Hinnebusch, B. J. (2004). Evaluation of the role of constitutive isocitrate lyase activity in *Yersinia pestis* infection of the flea vector and mammalian host. *Infect Immun* **72**, 7334–7337.

Segal, W. & Bloch, H. (1956). Biochemical differentiation of *Mycobacterium tuberculosis* grown in vivo and in vitro. *J Bacteriol* **72**, 132–141.

Serrano, J. A. & Bonete, M. J. (2001). Sequencing, phylogenetic and transcriptional analysis of the glyoxylate bypass operon (*ace*) in the halophilic archaeon *Haloferax volcanii*. *Biochim Biophys Acta* **1520**, 154–162.

Sharma, V., Sharma, S., Höner zu Bentrup, K., McKinney, J. D., Russell, D. G., Jacobs, W. R., Jr & Sacchettini, J. C. (2000). Structure of isocitrate lyase, a persistence factor of *Mycobacterium tuberculosis*. *Nat Struct Biol* **7**, 663–668.

Singh, V. K. & Ghosh, I. (2006). Kinetic modeling of tricarboxylic acid cycle and glyoxylate bypass in *Mycobacterium tuberculosis*, and its application to assessment of drug targets. *Theor Biol Med Model* **3**, 27.

Smith, R. A. & Gunsalus, I. C. (1954). Isocitritase: a new tricarboxylic acid cleavage system. *J Am Chem Soc* **76**, 5002–5003.

Solomon, P. S., Lee, R. C., Wilson, T. J. & Oliver, R. P. (2004). Pathogenicity of *Stagonospora nodorum* requires malate synthase. *Mol Microbiol* **53**, 1065–1073.

Son, M. S., Matthews, W. J., Jr, Kang, Y., Nguyen, D. T. & Hoang, T. T. (2007). In vivo evidence of *Pseudomonas aeruginosa* nutrient acquisition and pathogenesis in the lungs of cystic fibrosis patients. *Infect Immun* **75**, 5313–5324.

Srivastava, V., Jain, A., Srivastava, B. S. & Srivastava, R. (2008). Selection of genes of *Mycobacterium tuberculosis* upregulated during residence in lungs of infected mice. *Tuberculosis (Edinb)* **88**, 171–177.

Stovall, I. & Cole, M. (1978). Organic acid metabolism by isolated *Rhizobium japonicum* bacteroids. *Plant Physiol* **61**, 787–790.

Sturgill-Koszycki, S., Haddix, P. L. & Russell, D. G. (1997). The interaction between *Mycobacterium* and the macrophage analyzed by two-dimensional polyacrylamide gel electrophoresis. *Electrophoresis* **18**, 2558–2565.

Tamir-Ariel, D., Navon, N. & Burdman, S. (2007). Identification of genes in *Xanthomonas campestris* pv. vesicatoria induced during its interaction with tomato. *J Bacteriol* **189**, 6359–6371.

Tchawa Yimga, M., Leatham, M. P., Allen, J. H., Laux, D. C., Conway, T. & Cohen, P. S. (2006). Role of gluconeogenesis and the tricarboxylic acid cycle in the virulence of *Salmonella enterica* serovar Typhimurium in BALB/c mice. *Infect Immun* **74**, 1130–1140.

Thirach, S., Cooper, C. R., Jr & Vanittanakom, N. (2008). Molecular analysis of the *Penicillium marneffei* glyceraldehyde-3-phosphate dehydrogenase-encoding gene (*gpdA*) and differential expression of *gpdA* and the isocitrate lyase-encoding gene (*acuD*) upon internalization by murine macrophages. *J Med Microbiol* **57**, 1322–1328.

Timm, J., Post, F. A., Bekker, L. G., Walther, G. B., Wainwright, H. C., Manganelli, R., Chan, W. T., Tsenova, L., Gold, B. & other authors (2003). Differential expression of iron-, carbon-, and oxygen-responsive mycobacterial genes in the lungs of chronically infected mice and tuberculosis patients. *Proc Natl Acad Sci U S A* **100**, 14321–14326.

Tugarinov, V., Choy, W. Y., Orekhov, V. Y. & Kay, L. E. (2005). Solution NMR-derived global fold of a monomeric 82-kDa enzyme. *Proc Natl Acad Sci U S A* **102**, 622–627.

Vereecke, D., Cornelis, K., Temmerman, W., Jaziri, M., Van Montagu, M., Holsters, M. & Goethals, K. (2002a). Chromosomal locus that affects pathogenicity of *Rhodococcus fascians*. *J Bacteriol* **184**, 1112–1120.

Vereecke, D., Cornelis, K., Temmerman, W., Holsters, M. & Goethals, K. (2002b). Versatile persistence pathways for pathogens of animals and plants. *Trends Microbiol* **10**, 485–488.

Wall, D. M., Duffy, P. S., Dupont, C., Prescott, J. F. & Meijer, W. G. (2005). Isocitrate lyase activity is required for virulence of the intracellular pathogen *Rhodococcus equi*. *Infect Immun* **73**, 6736–6741.

Wanchu, A., Dong, Y., Sethi, S., Myneedu, V. P., Nadas, A., Liu, Z., Belisle, J. & Laal, S. (2008). Biomarkers for clinical and incipient tuberculosis: performance in a TB-endemic country. *PLoS One* **3**, e2071.

Wang, Z. Y., Thornton, C. R., Kershaw, M. J., Debao, L. & Talbot, N. J. (2003). The glyoxylate cycle is required for temporal regulation of virulence by the plant pathogenic fungus *Magnaporthe grisea*. *Mol Microbiol* **47**, 1601–1612.

Wayne, L. G. & Lin, K. Y. (1982). Glyoxylate metabolism and adaptation of *Mycobacterium tuberculosis* to survival under anaerobic conditions. *Infect Immun* **37**, 1042–1049.

Wong, P. P. & Evans, H. J. (1971). Poly-β-hydroxybutyrate utilization by soybean (*Glycine max* Merr) nodules and assessment of its role in maintenance of nitrogenase activity. *Plant Physiol* **47**, 750–755.

Fijación de Nitrógeno

A pesar de que el 80% de la atmósfera terrestre es nitrógeno, este compuesto es inerte y no puede ser aprovechado directamente por la mayoría de los seres vivos. El nitrógeno es incorporado a la biósfera por medio de la fijación de nitrógeno. Este proceso puede ocurrir de forma abiótica como consecuencia de tormentas eléctricas y procesos de combustión. Además, la transformación de nitrógeno en amonio es realizada de forma biológica por bacterias que poseen la enzima nitrogenasa. Algunas bacterias como *Klebsiella*, *Anabaena*, *Azotobacter* y *Azospirillum* lo fijan en vida libre en condiciones de microaerobiosis para satisfacer sus requerimientos nitrogenados (Brewin *et al.*, 1991, Mylona *et al.*, 1995). Las bacterias del género *Rhizobium* fijan el nitrógeno en asociación simbiótica con plantas leguminosas. Actualmente se conoce que 10 géneros pertenecientes a las α-proteobacterias (*Azorhizobium*, *Bradirhizobium*, *Devosia*, *Mezorhizobium*, *Methylobacterium*, *Ocrhobactrum*, *Phylobacterium*, *Rhizobium*, *Shinella* y *Sinorhizobium*) y 3 géneros pertenecientes a las β-proteobacterias (*Burkholderia*, *Cupriavidus* y *Herbaspirillums*), son capaces de establecer simbiosis y fijación de nitrógeno con leguminosas (Weir, 2008; ICSP Subcommittee on the taxonomy of *Rhizobium* and *Agrobacterium* diversity, phylogeny and systematics, 2008).

Simbiosis

Los eventos iniciales de la simbiosis entre plantas leguminosas y *Rhizobium* se caracterizan por un continuo intercambio de señales. Este proceso culmina en la formación del nódulo, dentro del cual se realiza la fijación biológica de nitrógeno. Los flavonoides producidos por las leguminosas son la primera señal captada por *Rhizobium*, a través de la proteína NodD, un miembro de la familia LysR, que activa la expresión de los genes *nod*, *nol* y *noe*, los cuales codifican para enzimas requeridas en la síntesis y transporte del factor de nodulación (factor Nod) (Perret *et al*., 2000). El factor Nod es sintetizado por NodA, NodB y NodC. La enzima NodC cataliza la polimerización de oligómeros de N-acetil-D-glucosamina (usualmente cuatro o cinco residuos) unidos por

enlaces β1-4 (Spaink, 2000), mientras que NodB remueve el grupo acetilo del azúcar en el extremo no reductor, lugar donde NodA agrega un ácido graso. Las modificaciones al factor Nod pueden incluir la adición de un residuo de azúcar y/o de grupos acetato, sulfato, carbamato y metilo en varias posiciones del esqueleto. Dichas modificaciones son realizadas por los genes *nodH, nolO, nodPQ, nodS, nodX, nodZ,* lo cual resulta en diferentes estructuras del factor de nodulación imprimiendo especificidad al proceso simbiótico (Denarie *et al*., 1996; Long, 1996; Spaink, 2000).

El factor Nod, al ser secretado por las bacterias, induce la alteración en el citoesqueleto de los pelos radiculares, el enroscamiento del pelo radicular y la formación del primordio nodular. Las bacterias atrapadas en el pelo radicular inducen la formación del hilo de infección, un canal de origen vegetal por el cual las bacterias llegan hasta el nódulo en donde se diferencian a bacteroides y realizan la fijación de nitrógeno (Brewin, 2002).

Los bacteroides realizan la fijación de nitrógeno por medio de la enzima nitrogenasa, la cual transforma el N_2 en amoníaco (NH_3) como se muestra en la siguiente ecuación:

$$N_2 + 8H^+ + 8e^- + 16\text{Mg-ATP} \rightarrow 2NH_3 + H_2 + 16\text{Mg-ADP} + 16\text{Pi}$$

La enzima nitrogenasa está constituida por dos componentes protéicos, el componente I o dinitrogenasa (proteína-FeMo), un tetrámero de 220-240 kDa, el cual es producto de los genes *nifD* y *nifK*, contiene el sitio activo para la reducción del N_2. El componente II ó dinitrogenasa reductasa (proteína-Fe), es un dímero de aproximadamente 60 kDa codificado por el gene *nifH*. Este dímero realiza la hidrólisis del ATP así como la transferencia de electrones al componente I. La reacción catalizada por la nitrogenasa requiere de una gran cantidad de ATP y poder reductor. El primer producto estable que se obtiene de la fijación de N_2 es el amonio. Varias pruebas indican que la asimilación del amonio para formar compuestos de nitrógeno lo realiza principalmente la planta. (Waters *et al*., 1998; Allaway *et al*., 2000; Lodwig *et al*., 2003).

Metabolismo del Carbono en Simbiosis.

Durante la fijación simbiótica de nitrógeno, la planta provee a *Rhizobium* con fuentes de carbono necesarios para cubrir la demanda energética que requiere este proceso, mientras que la bacteria provee a la planta con el nitrógeno fijado. A continuación se presenta una sinopsis del metabolismo del carbono en *Rhizobium* durante la simbiosis.

Metabolismo de Carbohidratos

Se ha observado que en *Rhizobium* creciendo en vida libre pueden operar las rutas Entner-Doudoroff, Embden-Meyerhof y pentosa fosfato en el catabolismo de azúcares (Dilworth y Glenn, 1984; Irigoyen *et al.*, 1990; McDermot *et al*., 1989; Udvardi y Day, 1997). Sin embargo, a pesar de que los azúcares son muy abundantes en el nódulo, existe evidencia experimental que indica que no son esenciales para realizar una eficiente simbiosis, ya que los bacteroides de *B. japonicum* no pueden transportar glucosa y aparentemente no poseen una ruta glicolítica completa. Los bacteroides de *Rhizobium leguminosarum* no transportan ni metabolizan hexosas ni disacáridos. Además, varias de las enzimas necesarias para el metabolismo de azúcares en *Rhizobium sp.* NGR234 están ausentes en bacteroides (Glenn y Dilworth, 1981; Hudman y Glenn 1980). Adicionalmente, mutantes de *Rhizobium* afectadas en el metabolismo de azúcares retienen la habilidad de nodular y fijar nitrógeno simbióticamente (Cervenansky y Arias 1984; Glenn *et al*., 1984; Glenn *et al*., 1984).

Metabolismo de ácidos dicarboxílicos

Los ácidos dicarboxílicos tales como el malato y succinato se encuentran en concentraciones altas en el nódulo y son considerados la principal fuente de carbono para el bacteroide ya que estimulan la fijación de nitrógeno *in vitro* (Bergersen y Turner, 1967, Streeter, 1987; Rosendahl *et al*., 1990; Fougere *et al*., 1991). Mutantes de *Rhizobium* en el sistema transportador de estos compuestos forman nódulos inefectivos en chícharo, alfalfa y trébol (Glenn y Brewin, 1981; Ronson *et al*., 1981; Finan *et al*., 1983; Arwas *et al*., 1985; Arwas *et al*., 1986; Engelke *et al*., 1987; Engelke *et al*., 1989; Watson *et al*., 1988).

Sistema transportador de ácidos dicarboxílicos

El sistema transportador de ácidos dicarboxílicos es relevante en simbiosis y está codificado gen *dctA*. En respuesta a la presencia de los ácidos dicarboxílicos, las proteínas DctB y DctD codificados por *dctB* y *dctD* respectivamente, activan la trascripción del gen *dctA* que codifica para la proteína transportadora, permitiendo la entrada al bacteroide de compuestos como malato, succinato o fumarato (Ronson *et al*., 1984; Watson, 1990). Las mutantes en el gene *dctA* de *Rhizobium tropici* CFN899 son incapaces de crecer en malato y fumarato, sin embargo, poseen la habilidad de crecer en succinato, lo que indica la presencia de un sistema alterno para el transporte de este compuesto. Las plantas inoculadas con esta mutante forman nódulos con una baja fijación de nitrógeno (29% con respecto a la cepa silvestre) (Batista *et al*., 2001). En *S. meliloti* se sugiere que *dctA* se transcribe independientemente de *dctBD*, ya que mutantes en el sistema de dos componentes (*dctBD*) son capaces de fijar nitrógeno (Engelke *et al*., 1989; Yarosh *et al*., 1989; Watson, 1990).

Ciclo de Krebs.

El ciclo de Krebs se encuentra ampliamente distribuido en la escala filogenética (figura 1). Esta vía metabólica constituye una parte medular del funcionamiento celular. El ciclo de Krebs es la ruta central del metabolismo de los ácidos dicarboxílicos requeridos para la fijación de nitrógeno (Dunn, M., 1998). La oxidación de compuestos a través del ciclo de Krebs provee poder reductor, ATP, así como intermediarios para la biosíntesis de aminoácidos y otros metabolitos.

El ciclo de los ácidos tricarboxílicos inicia con la condensación de acetil-CoA y oxaloacetato para producir citrato, en una reacción enzimática catalizada por la citrato sintasa. En *Rhizobium tropici* CFN299 se identificaron dos genes, *pcsA* y *ccsA*, que codifican para la enzima citrato sintasa y se observó que la mutación en ambos genes induce la formación de nódulos desprovistos de bacteroides (Hernández-Lucas *et al*., 1995). Dado que las mutantes de *Sinorhizobium meliloti* y *S. fredii* en el gen que codifica para la enzima citrato sintasa (*gltA*) producen nódulos inefectivos (Mortimer *et al*., 1999; Krishnan *et al*., 2003), se concluye que la actividad de GltA es esencial para la fijación de nitrógeno en nódulo (Grzemski *et al*., 2005).

La enzima aconitasa es codificada por el gen *acnA* y cataliza la isomerización de citrato a isocitrato. Las mutantes de *Bradyrhizobium japonicum* en este gene presentan un 30% de actividad de aconitasa con respecto a la cepa silvestre (Thöny-Meyer y Künzler, 1996), lo que indicaría la presencia de un segundo gen *acnA*. Debido a que la mutante en aconitasa es capaz de fijar nitrógeno eficientemente en simbiosis con soya (Thöny-Meyer y Künzler 1996), no se ha podido esclarecer el verdadero papel de esta enzima en el metabolismo del bacteroide.

La isocitrato deshidrogenasa codificada por el gen *icd* cataliza la oxidación de isocitrato a α-cetoglutarato, la actividad de esta enzima es alta en bacteroides de *R. leguminosarum*, *S. meliloti* y *B. japonicum* (Mckay *et al*., 1989; Miller *et al*., 1991; Green *et al*., 1998). Mutantes en este gen en *S. meliloti* inducen bacteroides incapaces de fijar nitrógeno

(McDermott y Kahn, 1992). Sin embargo, mutantes de *B. japonicum* en este gen provocan un retraso en la nodulación, pero en cuanto a la fijación de nitrógeno no se observan alteraciones (Shah y Emerich, 2006).

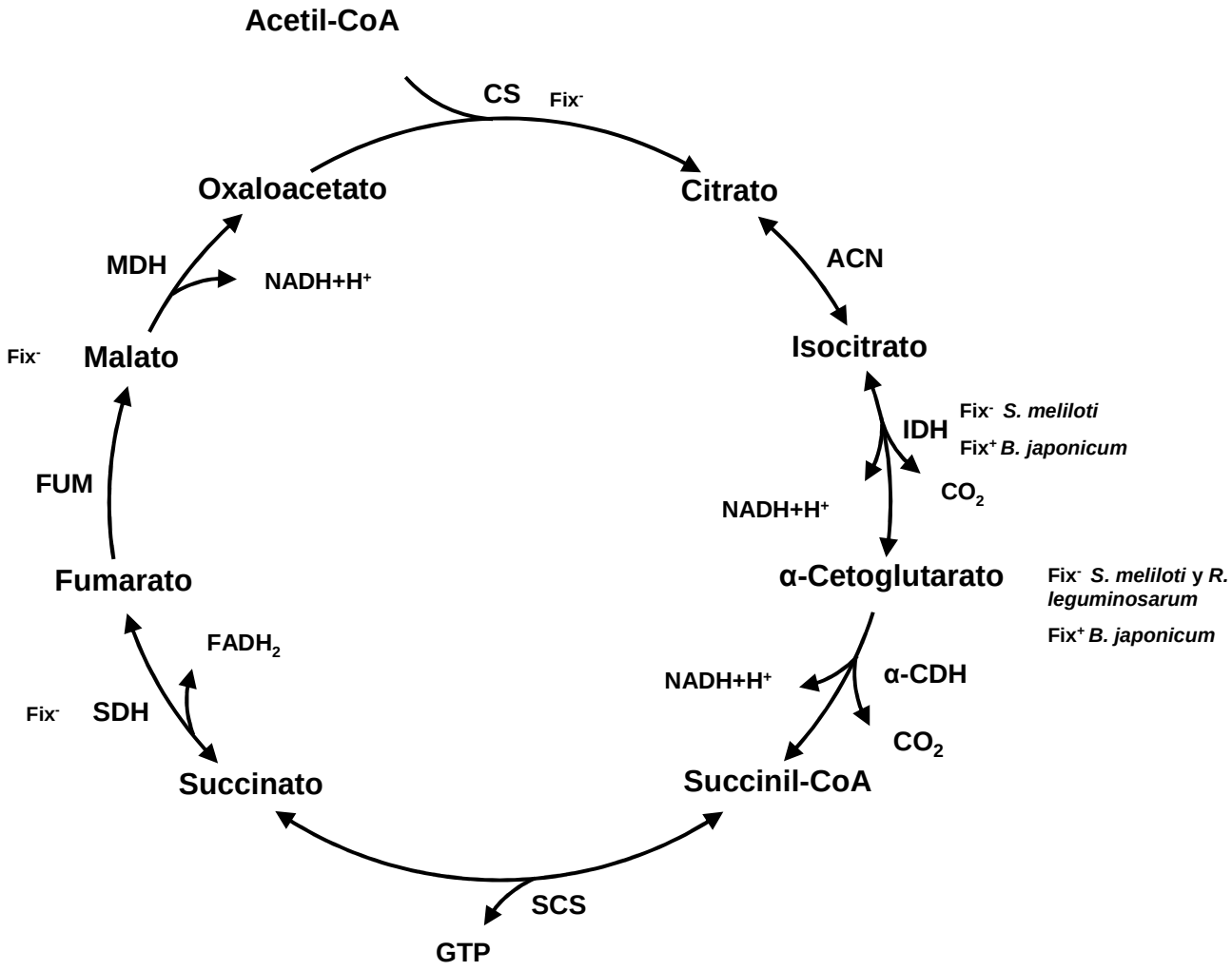

Figura 1. Reacciones enzimáticas del ciclo de Krebs. Abreviaturas: CS, citrato sintasa; ACN, aconitasa; IDH, isocitrato deshidrogenasa; α-CDH, α-cetoglutarato deshidrogenasa, SCS, succinil-CoA sintetasa; SDH, succinato deshidrogenasa; FUM, fumarasa; MDH, malato deshidrogenasa. Fix^+ y Fix^- representa los fenotipos simbióticos observados con diferentes mutantes en genes del ciclo de Krebs**.**

El complejo enzimático de la α-cetoglutarato deshidrogenasa, el cual está codificado por los genes *sucAB*, cataliza la descarboxilación oxidativa de α-cetoglutarato a succinil-CoA. En *R. leguminosarum*, *S. meliloti* y *M. loti* los genes *sucAB* están acoplados transcripcionalmente a los genes *mdh* y *sucCD* (Figura 2) (Poole *et al*., 1999; Kanecko *et al*., 2000; Galibert *et al*., 2001; Dymov *et al*., 2005) los cuales codifican para la malato deshidrogenasa y la succinil-CoA sintetasa, respectivamente. Este operón está controlado por un solo promotor corriente arriba del gen *mdh* (Poole *et al*., 1999; Dymov *et al*., 2005)

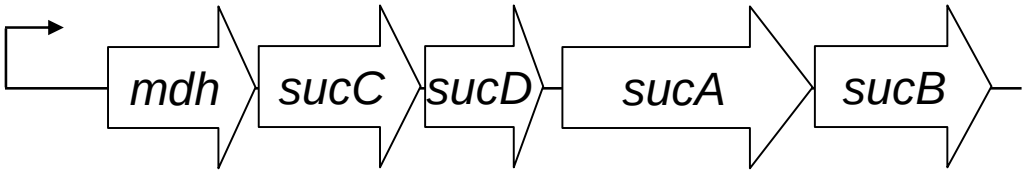

Figura 2. Organización genética de los genes que codifican para las enzimas malato deshidrogenasa (*mdh*), succinil-CoA sintetasa (*sucCD*) y α-cetoglutarato deshidrogenasa (*sucAB*).

Las mutantes de *S. meliloti* y *R. leguminosarum* en α-cetoglutarato deshidrogenasa (*sucA*) forman nódulos inefectivos en alfalfa y chícharo, respectivamente (Duncan y Fraenkel, 1979; Walshaw *et al*., 1997), indicando que este paso del ciclo de Krebs es necesario en simbiosis. Sin embargo, mutantes de *B. japonicum* en α-cetoglutarato deshidrogenasa forman nódulos funcionales en soya (Green y Emerich, 1997a; 1997b), sugiriendo que no se requiere un ciclo de Krebs completo para la fijación simbiótica de nitrógeno o que existe una ruta alternativa para la degradación del α-cetoglutarato en *B. japonuicum*. Green *et al*. (2000) sugieren que el fenotipo Fix^+ en *B. japonicum* es debido a que el α-cetoglutarato es metabolizado mediante la α-cetoglutarato descarboxilasa y la succinato semialdehído deshidrogenasa para formar succinil-CoA en nódulos.

El succinil-CoA se hidroliza a succinato mediante la acción de la enzima succinil-CoA sintetasa codificada por los genes *sucCD*. Una mutante de *R. leguminosarum* en *sucD* forma nódulos inefectivos en chícharo, probablemente debido al efecto polar en los genes corriente abajo *sucAB*. Esta mutante mantiene una actividad sustancial de succinil-CoA sintetasa (Walshaw *et al*., 1997) sugiriendo que *R. leguminosarum* posee un segundo gen

sucD. Basado en estos datos no es posible asignar un papel durante la simbiosis para el producto del gene *sucD*.

El complejo succinato deshidrogenasa (SDH) codificado por los genes *sdhC, sdhD, sdhA* y *sdhB* cataliza la oxidación del succinato a fumarato. Las mutantes de *S. meliloti* y *R. leguminosarum* en la succinato deshidrogenasa son incapaces de usar succinato como única fuente de carbono, pero crecen bien en fumarato y malato. Estas mutantes forman nódulos inefectivos en sus respectivas plantas huésped (Finan *et al*., 1981; Gardiol *et al*., 1982; Gardiol *et al*., 1987).

En la siguiente reacción del ciclo de Krebs el fumarato es hidratado para formar malato por medio de la enzima fumarasa codificada por el gene *fumC*. Accuna *et al.* (1991) clonaron e inactivaron un gen *fumC* de *B. japonicum*. Se encontró que la cepa mutante es capaz de nodular y fijar nitrógeno eficientemente, debido a la presencia de tres genes mas que codifican para fumarasas en *B. japonicum* (Kaneko *et al*., 2002).

El paso final en el ciclo de Krebs regenera oxaloacetato a partir del malato por medio de la enzima malato deshidrogenasa, codificada por el gene *mdh*. Como se mencionó anteriormente, los genes *mdh* y *sucCDAB* se encuentran formando un operón en *R. leguminosarum* y *S. meliloti* (Poole *et al*., 1999; Dymov *et al*., 2005), lo cual dificulta determinar la naturaleza de la mutación en *mdh* debido al efecto polar que ocasionaría en los genes *sucCDAB*. Sin embargo, Dymov *et al*. (2005) obtuvieron una mutante de *S. meliloti* en *mdh* usando un Tn5 que posee un promotor Tac, lo cual permite la transcripción de los genes *sucCDAB*. Se observó que el crecimiento en vida libre de esta mutante es lento y forma nódulos inefectivos en alfalfa (Dymov *et al*. 2005).

El ciclo de Krebs posee rutas anapleróticas que lo mantienen activo en diferentes condiciones. A continuación resumimos los estudios de estas vías metabólicas en la simbiosis *Rhizobium*-Leguminosa.

Enzima málica y piruvato deshidrogenasa.

El metabolismo de los ácidos dicarboxílicos requiere que se generen oxaloacetato y acetil-CoA a partir del malato. La enzima málica cataliza la descarboxilación oxidativa del malato a piruvato, el cual posteriormente es transformado en acetil-CoA por la piruvato deshidrogenasa (Figura 3). En *B. japonicum*, *R. leguminosarum* y *S. meliloti* se han identificado dos tipos de enzimas málicas, una dependiente de NAD^+ codificada por el gen *dme* (DME) y otra dependiente de $NADP^+$ codificada por el gen *tme* (TME). La regulación y la actividad de estos genes ha sido estudiada extensivamente en *S. meliloti* y *B. japonicum* (Kouchi *et al*., 1988; Copeland *et al*., 1989; Tomaszewska y Werner, 1995; McKay *et al*., 1988; Driscoll y Finan, 1993).

Driscoll y Finan (1997) caracterizaron mutantes sencillas y dobles en los genes *dme* y *tme*. En las plantas de alfalfa, la mutante sencilla *dme* así como la doble mutante *dme-tme* forman nódulos pequeños incapaces de fijar nitrógeno, la mutante sencilla *tme* no fue afectada en su funcionamiento simbiótico. Interesantemente la enzima málica dependiente de NAD^+ se induce en bacteroides. Al clonar el gen *tme* bajo el control del promotor *dme* no se restaura el fenotipo de la mutante en *dme*, por lo tanto DME es requerida en simbiosis. El piruvato formado por la enzima málica es transformado en acetil-CoA por el complejo enzimático piruvato deshidrogenasa, el cual está codificado por los genes *pdhAα*, *pdhAβ*, *pdhB*, y *lpd*. Una mutación en el gene *lpd* en *S. meliloti* presenta una disminución de 16 veces en la actividad de piruvato deshidrogenasa y presenta un fenotipo Fix^- (Soto *et al*., 2001).

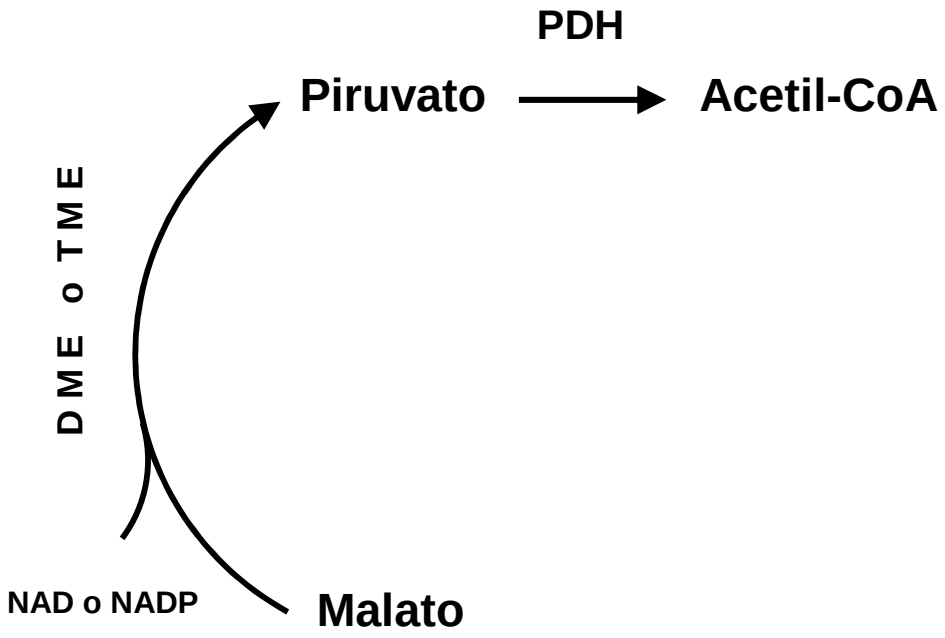

Figura 3. Reacciones enzimáticas de la ruta anplerótica para la generación de acetil-CoA a partir de malato. Abreviaturas: DME, enzima málica dependiente NAD; TME, enzima málica dependiente NADP; PDH, piruvato deshidrogenasa.

Metabolismo del PHB

El PHB o polihidroxibutirato es una excelente reserva de carbono. La ruta para la biosíntesis del PHB en *Rhizobium* inicia con la condensación de 2 moléculas de acetil-CoA para formar acetoacetil-CoA, paso catalizado por la enzima cetotiolasa codificada por el gen *phbA* (Figura 4). Posteriormente, el acetoacetil-CoA es transformado a β-hidroxibutiril-CoA por la enzima acetoacetil-CoA reductasa codificada por el gen *phbB*. Finalmente, el β-hidroxibutiril-CoA es transformado en PHB por la enzima PHB sintasa codificada por el gen *phbC*. En *Rhizobium* los genes *phbA* y *phbB* se encuentran formando un operón, mientras que el gene *phbC* se encuentra de manera independiente. Los bacteroides de nódulos formados por *B. japonicum* y *R. etli*, producen grandes cantidades de PHB durante la fase activa de fijación de nitrógeno (Wong y Evans 1971; Karr *et al*. 1984; Bergersen y Turner 1991). Una mutante de *R. etli* en el gene *phbC* es

más eficiente en simbiosis, ya que presenta una actividad de nitrogenasa mayor a la cepa silvestre. Las plantas inoculadas con la cepa deficiente en el gene *phbC* presentan mayor peso seco y las semillas contienen más nitrógeno, en comparación a las inoculadas con la cepa silvestre (Cevallos *et al*., 1996).

La degradación de PHB inicia con la acción de la enzima PHB depolimerasa (*phbZ*) y da por resultado la formación de monómeros de β-hidroxibutirato (Figura 4) (Trainer y Charles 2006). Posteriormente, el β-hidroxibutirato es oxidado a acetoacetato por la enzima β-hidroxibutirato deshidrogenasa (*bdhA*). En el siguiente paso el acetoacetato es transformado a acetoacetil-CoA por la acetoacetil-CoA sintetasa (*acsA2*). Finalmente, este compuesto es transformado en acetil-CoA por medio de la enzima cetotiolasa (Cai *et al*., 2000). Los nódulos de *Lupinus angustifolius* incubadas en oscuridad muestran niveles bajos de PHB, un incremento en la actividad de BdhA y una disminución de la fijación de nitrógeno, lo cual sugiere que las reservas de PHB en los nódulos pueden ser utilizadas en condiciones de poca disponibilidad de carbono (Gerson *et al*., 1978). Se ha especulado que el PHB proporciona el combustible necesario para realizar el proceso de infección en *S. meliloti* (Charles *et al.* 1997). Se piensa que en este organismo las reservas intracelulares de PHB pudieran servir como una fuente de energía para la división celular y el crecimiento durante la invasión e infección de la raíz (Charles et al. 1997). Sin embargo, el PHB no es crucial para el proceso simbiótico, ya que mutantes de *S. meliloti* en los genes *bdhA* y *acsA* pueden establecer una simbiosis eficiente (Aneja y Charles, 1999; Cai *et al*., 2000).

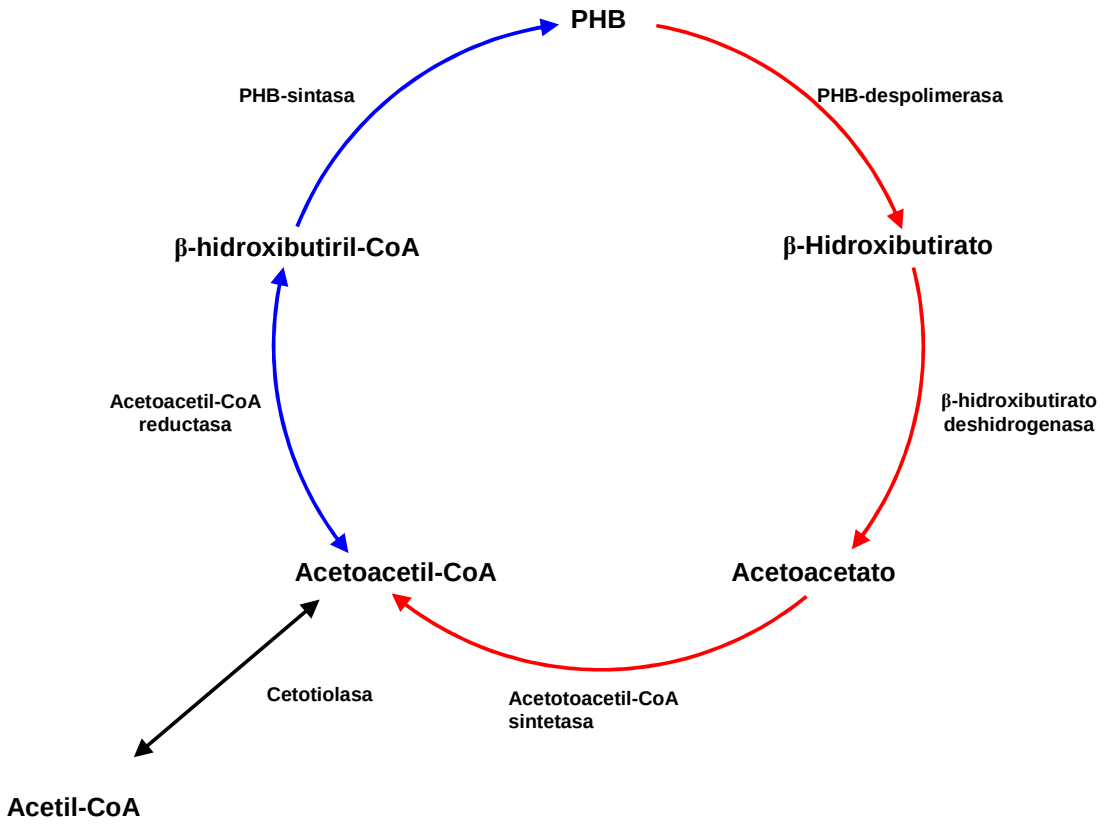

Figura 4. Reacciones enzimáticas del ciclo del PHB. En azul se muestran las reacciones de biosíntesis y en rojo se muestran las de degradación.

Biosíntesis y metabolismo de aminoácidos en el nódulo.

La ruta predominante para la asimilación de amonio en *Rhizobium* es a través de la glutamino sintetasa (GS) y la glutamato-α-cetoglutarato deshidrogenasa (GOGAT). El glutamato es aminado a glutamina por la enzima glutamino sintetasa. La glutamina es posteriormente desaminada por la GOGAT, que transfiere un grupo amino al α-cetoglutarato obteniendo dos moléculas de glutamato. Existen tres isoformas de la enzima GS en *S. meliloti*, *R. leguminosarum*, *R. etli* y *M. loti*: GSI, GSII y GSIII, codificadas por los genes *glnA*, *glnII* y *glnT*, respectivamente. La supresión de la actividad de GS originada por la mutación de los genes *glnA* y *glnII* en *S. meliloti* produce nódulos

efectivos; sin embargo, se ha detectado actividad residual de GS en bacteroides, debida a la presencia de la GSIII (de Bruijn *et al*., 1989). En contraste, las mutantes en *glnA* y *glnII* de *B. japonicum* son incapaces de nodular soya (Carlson *et al*., 1987). En *S. meliloti* la mutación en GOGAT no afecta el proceso simbiótico (Lewis *et al*., 1990).

La aspartato aminotransferasa (AatA) cataliza la transaminación de glutamato a oxaloacetato para obtener aspartato y α-cetoglutarato. La bacteria *S. meliloti* posee tres genes que codifican proteínas con actividad de aspartato aminotransferasa; sin embargo, solamente *aatA* está conservado en otros *Rhizobium* (Alfano y Kahn, 1993; Kaneko *et al*., 2000; Galibert *et al*., 2001; Kaneko *et al*., 2002; Gonzalez *et al*., 2006; Young *et al*., 2006). Las mutantes de *S. meliloti* y *R. leguminosarum* en el gen *aatA* nodulan pero no fijan nitrógeno, lo que sugiere que el aspartato podría ser una importante fuente de carbono en simbiosis (Rastogi y Watson, 1991; Lodwig *et al*., 2003).

La aspartasa cataliza la desaminación reversible del aspartato para obtener amonio y fumarato. Se ha observado que la actividad de aspartasa es alta en bacteroides de *R. etli* y *B. japonicum* indicando que en el nódulo podría existir cierta degradación de aspartato vía aspartasa. Sin embargo, la actividad de esta enzima no es esencial para realizar una simbiosis eficiente, ya que una mutante de *R. etli* en aspartasa, nodula y fija nitrógeno al igual que la cepa silvestre (Kouchi *et al*., 1991; Huerta-Zepeda *et al*., 1997).

El ciclo del glioxilato

Este ciclo fue descubierto en 1957 por Kornberg y Krebs, es una ruta alterna al ciclo del ácido cítrico que evita los pasos de descarboxilación, permitiendo la formación de esqueletos carbonados, sin la pérdida de carbono en forma de CO_2. Este ciclo comparte varias enzimas del ciclo de Krebs; sin embargo, posee dos enzimas exclusivas, la isocitrato liasa y la malato sintasa, cuya función es evitar los pasos de descarboxilación del ciclo de los ácidos tricarboxílicos. La isocitrato liasa transforma el isocitrato en succinato y glioxilato, la malato sintasa transforma el glioxilato y una molécula de acetil-

CoA en malato (Figura 5). Dos moléculas de acetil-CoA son introducidas en el ciclo del acido glioxílico, dando como resultando una molécula extra de malato la cual mantiene al ciclo operando, de tal manera que una molécula de malato sintetizada puede ser usada para gluconeogénesis. En diferentes microorganismos las dos enzimas específicas del ciclo del glioxilato se inducen cuando la bacteria crece en acetato o etanol como única fuente de carbono, mientras que en presencia de carbohidratos, el ciclo del ácido glioxílico es inhibido (Cozzone, 1998). En *E. coli* los genes estructurales de la isocitrato liasa y la malato sintasa (*aceA* y *aceB*, respectivamente) constituyen un operón junto con *aceK* que codifica para una isocitrato deshidrogenasa cinasa/fosfatasa la cual controla el flujo del isocitrato hacia el ciclo del glioxilato (Cozzone, 1998; Cozzone y El-Mansi, 2005).

El ciclo del glioxilato opera en aves, reptiles, arqueas, bacterias, hongos, plantas y nemátodos (Kondrashov *et al*., 2006; Davis *et al*., 1990; Morgunov *et al*., 2005; Davis *et al*., 1986). En mamíferos el gen de la malato sintasa es un seudogen y el de la isocitrato liasa se encuentra ausente (Kondrashov *et al*., 2006). Recientemente se ha observado que el ciclo del glioxilato es relevante en la patogénesis de organismos como *Mycobacterium tuberculosis* (McKinney *et al*., 2000), *Rhodococcus equi* (Wall *et al*., 2005), *Pseudomonas aeruginosa* (Son *et al*., 2007; Lindsey *et al*., 2008), *Candida albicans* (Lorenz y Fink, 2001), *Leptosphaeria maculans* (Idnurm y Howlett, 2002), *Magnaporthe grises* (Wang *et al*., 2003), *Colletotrichum lagenarium* (Asakura *et al*., 2006), *Rhodococcus fascians* (Vereecke *et al*., 2002; 2002a), *Xanthomonas campestres* (Tamir-Ariel *et al*., 2007) y *Stagonospora nodorum* (Solomon *et al*., 2004).

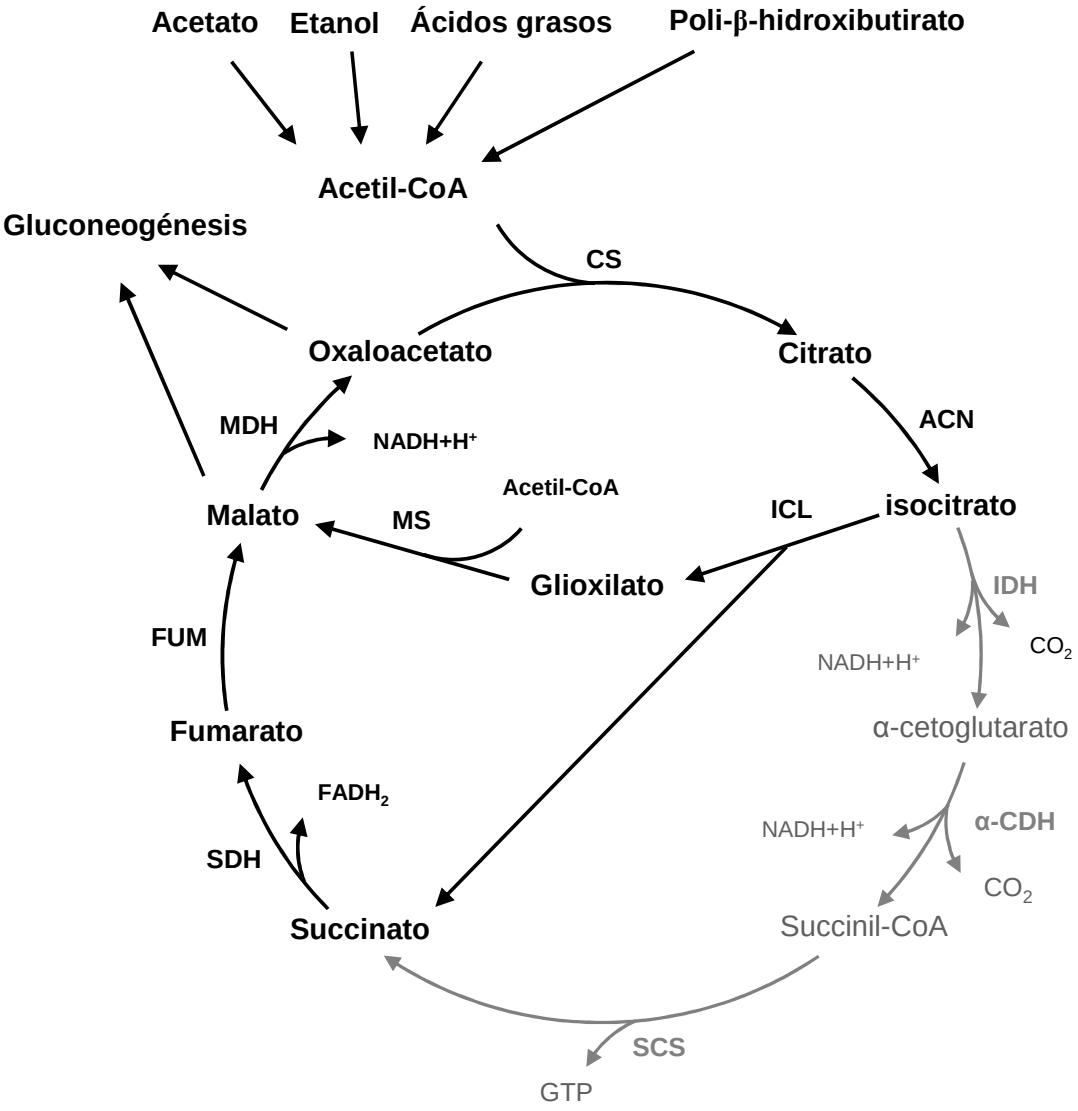

Figura 5. En negro reacciones del ciclo del glioxilato. Abreviaturas: CS, citrato sintasa; ACN, aconitasa; SDH, succinato deshidrogenasa; FUM, fumarasa; MDH, malato deshidrogenasa; ICL, isocitrato liasa; MS, malato sintasa. En gris se muestran reacciones exclusivas del ciclo de Krebs. IDH, isocitrato deshidrogenasa; α- CDH, α-Cetoglutarato deshidrogenasa; SCS, succinil-CoA sintetasa.

El ciclo del glioxilato en *Rhizobium*.

Rhizobium en su forma simbiótica depende completamente de la planta huésped por el suministro de carbono. Aunque se ha observado que los ácidos dicarboxílicos tales como, el malato, succinato y fumarato son una fuente importa de compuestos carbonados proporcionados por la planta como fuente de energía para la fijación de nitrógeno, la planta podría proveer al bacteroide con cantidades significantes de acetato. El acetato es uno de los ácidos orgánicos más abundantes en el nódulo (Jackson y Evans, 1966) y puede cruzar eficientemente la membrana peribacteroidal que separa a los bacteroides del citosol del nódulo (Udvardi y Day, 1989). Además, se ha observado que el acetato adicionado exógenamente a bacteroides de *B. japonicum* aislados, promueve la fijación de nitrógeno *ex planta* (Udvardi y Day, 1989). En bacteroides de *B. japonicum*, la tasa de incorporación de acetato y la actividad de las enzimas involucradas con la transformación del acetato en acetil-CoA (acetato cinasa, fosfotransacetilasa y acetil-CoA sintetasa) incrementa con la fijación de nitrógeno (Preston *et al*, 1989; Smith *et al*., 1994), implicando un incremento en la utilización de acetato.

En nódulos senescentes de soya y en nódulos de plantas de soya que fueron incubadas en la oscuridad, se observa un decremento en la cantidad de PHB y un incremento en la actividad de isocitrato liasa, lo que sugiere que el acetil-CoA producto de degradación del PHB es metabolizado por medio del ciclo del glioxilato (Wong y Evans, 1977). Sin embargo, en nódulos de plantas creciendo en condiciones favorables el funcionamiento del ciclo del glioxilato no se ha confirmado, ya que no se ha detectado la actividad de la isocitrato liasa en bacteroides de soya, chícharo, alfalfa ó trébol (Green *et al*., 1998; Jonson *et al*., 1966). En vida libre la actividad de isocitrato liasa solamente ha sido detectada en medios conteniendo acetato u oleato (Green *et al*., 1998; Duncan y Fraenkel, 1979; Mandal y Chakrabartty, 1992).

A diferencia de la isocitrato liasa, la actividad enzimática de malato sintasa se ha detectado en bacteroides de chícharo, alfalfa, trébol, frijol y soya (Jonson et al., 1966; Mandal y Chakrabartty, 1992). La actividad de malato sintasa parece ser constitutiva, ya

que se ha detectado en extractos de células creciendo en acetato, glucosa, arabinosa, piruvato y malato (Duncan y Fraenkel, 1979; Green *et al*., 1998; Mandal y Chakrabartty, 1992). Hasta el momento solo se ha estudiado en simbiosis una mutante de malato sintasa de *R. leguminosarum* la cual forma nódulos y fijan nitrógeno eficientemente (García-de los Santos *et al*., 2002) sin embargo no se hizo un análisis de esta mutante en vida libre creciendo en acetato como única fuente de carbono. Aunque los genes putativos *aceA* y *glcB,* están anotados en la secuencia del genoma de *S. meliloti* (Figura 6) y otros *Rhizobium,* el papel de estos genes en la utilización de acetato y la interacción *Rhizobium*-Leguminosa no ha sido evaluado. Para entender mejor el papel del ciclo del glioxilato en la interacción *Rhizobium*-Leguminosa es necesario el análisis de mutantes en los genes que codifican para las proteínas involucradas en dicho ciclo.

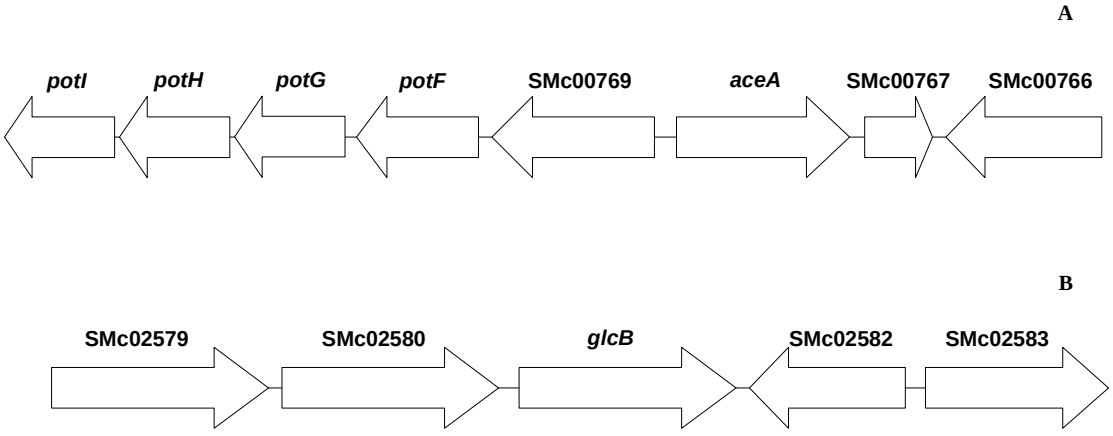

Figura 6.- A) organización genética de los genes *aceA* y SMc00767. B) organización genética del gen *glcB.*

HIPÓTESIS

Se conoce que en el nódulo existe una gran cantidad de acetato superado en concentración solo por malato y que en los bacteroides se encuentran activas las enzimas que transforman el acetato en acetil-CoA, así como la malato sintasa, además también se ha observado que los bacteroides pueden usar acetato como fuente de energía para soportar la fijación de nitrógeno, por lo tanto el ciclo del glioxilato podría estar operando en *Rhizobium* durante la simbiosis con leguminosas.

OBJETIVO GENERAL

Caracterización funcional de los genes que codifican para las enzimas isocitrato liasa y malato sintasa de *Sinorhizobium meliloti* en vida libre y en simbiosis.

OBJETIVOS PARTICULARES

Construcción de mutantes de *Sinorhizobium meliloti* en los genes que codifican para la isocitrato liasa (*aceA*) y malato sintasa (*glcB*).

Evaluar el papel de los genes del ciclo del glioxilato en vida libre mediante curvas de crecimiento de las diferentes mutantes.

Evaluar el papel de la isocitrato liasa y la malato sintasa en la simbiosis con alfalfa.

RESULTADOS

En esta sección de resultados se presenta un artículo publicado en la revista journal of bacteriology. En este trabajo se evaluó la contribución de los productos de los genes *aceA*, *glcB* y SMc00767 de *Sinorhizobium meliloti* en vida libre y simbiosis.

Ramírez-Trujillo J. A., Encarnación S., Salazar E., García-de los Santos A., Dunn M. F., Emerich D. W., Calva E., y Hernández-Lucas I. 2007. Functional characterization of *Sinorhizobium meliloti* acetate metabolism genes *aceA*, SMc00767 and *glcB*. *J Bacteriol* **189:**5875-5884.

JOURNAL OF BACTERIOLOGY, Aug. 2007, p. 5875–5884 Vol. 189, No. 16
0021-9193/07/$08.00+0 doi:10.1128/JB.00385-07

Functional Characterization of the *Sinorhizobium meliloti* Acetate Metabolism Genes *aceA*, SMc00767, and *glcB*▽†

J. A. Ramírez-Trujillo,[1] S. Encarnación,[2] E. Salazar,[2] A. García de los Santos,[2] M. F. Dunn,[2] D. W. Emerich,[3] E. Calva,[1] and I. Hernández-Lucas[1]*

Departamento de Microbiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos 62210, Mexico[1]; Centro de Ciencias Genómicas, Universidad Nacional Autónoma de Mexico, Cuernavaca, Morelos, México[2]; and Department of Biochemistry, University of Missouri, Columbia, Missouri 65211[3]

Received 15 March 2007/Accepted 17 May 2007

The genes encoding malate synthase (*glcB*) and isocitrate lyase (*aceA*) and a 240-bp open reading frame (SMc00767) located downstream of *aceA* were isolated and functionally characterized in *Sinorhizobium meliloti*. Independent and double interposon mutants of each gene were constructed, and the corresponding phenotypes were analyzed. *aceA* mutants failed to grow on acetate, and mutants deficient in SMc00767 were also affected in acetate utilization. In contrast, mutants deficient in *glcB* grew on acetate similar to wild-type strain Rm5000. Complementation experiments showed that *aceA* and SMc00767 gene constructs were able to restore the growth on acetate in the corresponding single mutants. *aceA-glcB*, *aceA*-SMc00767, and *glcB*-SMc00767 double knockouts were also unable to grow on acetate, but this ability was recovered when the wild-type *aceA-glcB* or *aceA*-SMc00767 loci were introduced into the double mutants. These data confirm the functional role of *aceA* and SMc00767 and show that *glcB*, in the absence of SMc00767, is required for acetate metabolism. Isocitrate lyase and malate synthase activities were measured in strain Rm5000, the mutant derivatives, and complemented strains. *aceA* and *glcB* were able to complement the enzymatic activity lacking in the corresponding single mutants. The enzymatic activities also showed that SMc00767 represses the activity of isocitrate lyase in cells grown on acetate. Gene fusions confirmed the repressor role of SMc00767, which regulates *aceA* expression at the transcriptional level. Comparison of the transcriptional profiles of the SMc00767 mutant and wild-type strain Rm5000 showed that SMc00767 represses the expression of a moderate number of open reading frames, including *aceA*; thus, we propose that SMc00767 is a novel repressor involved in acetate metabolism in *S. meliloti*. Genetic and functional analyses indicated that *aceA* and SMc00767 constitute a functional two-gene operon, which is conserved in other α-proteobacteria. Alfalfa plants infected with the *aceA* and *glcB* mutants were not impaired in nodulation or nitrogen fixation, and so the glyoxylate cycle is not required in the *Rhizobium*-legume symbiosis.

Bacteria of the genera *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, and *Bradyrhizobium* fix nitrogen within nodules that they form in symbiotic association with legumes. *Sinorhizobium meliloti*, the bacterium that interacts with alfalfa plants, has been used as a model to study the *Rhizobium* plant-microbe interaction. An aspect that has been investigated in *S. meliloti* is carbon metabolism, both in free-living cells and in the symbiotic state. Several rhizobial enzymes, such as citrate synthase (29), isocitrate dehydrogenase (37), succinate dehydrogenase (24), and malate dehydrogenase (17), are essential for N_2 fixation, indicating that a functional tricarboxylic acid (TCA) cycle is important in symbiosis (16). Anaplerotic pathways, such as the glyoxylate shunt, are essential for growth on C_2 substrates, such as acetate, allowing bacterial cells to replenish the pool of TCA cycle intermediates necessary for supporting gluconeogenesis and other biosynthetic processes (9, 10, 30). This bypass is widespread in prokaryotes and plants (2, 18) and is encoded by two principal genes, *aceA* (encoding isocitrate lyase [ICL]) and *glcB* (encoding malate synthase [MS]). ICL cleaves isocitrate to glyoxylate and succinate, and MS condenses glyoxylate with acetyl coenzyme A (acetyl-CoA) to produce malate. Two isoenzymes of MS have been described in *Escherichia coli*; MSA encoded by the *aceB* gene is part of the *ace* operon (91 min), which is required for growth on acetate, and MSG encoded by the *glcB* gene mapped in the *glc* locus (64.5 min), which is inducible by glycolate (9). The presence of large amounts of acetate and fatty acids in soybean nodules encouraged early studies on the glyoxylate cycle (30). The role of this pathway in symbiosis was also supported by radiorespirometric studies of *Bradyrhizobium japonicum* bacteroids which indicated that as much as 50% of the acetyl-CoA entering the TCA cycle is metabolized via MS (48). It was also shown that acetate can be used by isolated *B. japonicum* bacteroids to support ex planta nitrogen fixation (42, 43). The existence of the glyoxylate cycle in bacteroids is in doubt because the activity of ICL has not been detected in bacteroids isolated from soybean, pea, alfalfa, and clover nodules (27, 30). However ICL activity has been detected in bacteroids from senesced nodules formed by *B. japonicum* (53). *S. meliloti* and *B. japonicum* cells grown on acetate contain ICL activity as well (15, 27, 36). In contrast, MS activity was found in bacteroids isolated from pea, alfalfa, and clover nodules, and sub-

* Corresponding author. Mailing address: Departamento de Microbiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Av. Universidad 2001, Cuernavaca, Morelos 62210, México. Phone: (52) (777) 329-1645. Fax: (52) (777) 313-8673. E-mail: ismaelh@ibt.unam.mx.

† Supplemental material for this article may be found at http://jb.asm.org/.

▽ Published ahead of print on 25 May 2007.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or characteristics	Reference or source
S. meliloti strains		
Rm5000	SU47 *rif-5*	21
RH190	Rm5000 *aceA*::ΩSp/Sm	This study
RH198	Rm5000 *aceA*::ΩSp/Sm, pBBR1MCS-5 *aceA*	This study
RH218	Rm5000 *glcB*::ΩTc	This study
RH222	Rm5000 *aceA*::ΩSp/Sm *glcB*::ΩTc	This study
RH302	Rm5000 *glcB*::ΩTc, pBBR1MCS-5 *glcB*	This study
RH312	Rm5000 *aceA*::ΩSp/Sm *glcB*::ΩTc, pBBR1MCS-5 *aceA-glcB*	This study
RH326	Rm5000 *aceA*::ΩSp/Sm, pBBR1MCS-5 *aceA*-SMc00767	This study
RH327	Rm5000 *aceA*::ΩSp/Sm, *glcB*::ΩTc, pBBR1MCS-5, *aceA*-SMc00767	This study
RH419	Rm5000 *glcB*::ΩTc SMc00767::ΩSp/Sm	This study
RH421	Rm5000 SMc00767::ΩSp/Sm	This study
RH429	Rm5000 *glcB*::ΩTc SMc00767::ΩSp/Sm, pBBR1MCS-5 *glcB*	This study
RH435	Rm5000 SMc00767::ΩSp/Sm, pBBR1MCS-5 SMc00767	This study
RH436	Rm5000 *glcB*::ΩTc, SMc00767::ΩSp/Sm, pBBR1MCS-5 SMc00767	This study
RH442	Rm5000 SMc00767::ΩSp/Sm, pBBR1MCS-5 *aceA*-SMc00767 with an internal 700-bp deletion in *aceA* that leaves wild-type SMc00767 and the intact *aceA* promoter region	This study
RH443	Rm5000 *glcB*::ΩTc SMc00767::ΩSp/Sm, pBBR1MCS-5 *aceA*-SMc00767 with an internal 700-bp deletion in *aceA* that leaves wild-type SMc00767 and the intact *aceA* promoter region	This study
RH462	Rm5000 *aceA*::ΩSp/Sm, pBBR1MCS-5 *aceA-glcB*	This study
RH465	Rm5000 containing an *aceA*::*gusA* fusion in pBBR1MCS-5	This study
RH467	RH421 containing an *aceA*::*gusA* fusion in pBBR1MCS-5	This study
E. coli strains		
DH5α	*recA1* ϕ80d*lacZ*ΔM15 *gyrA96*	Gibco BRL
HB101	*recA13 rspL20* (Sm^r)	Gibco BRL
Plasmids		
pCR2.1	TA cloning vector for PCR products, Amp^r Km^r	Invitrogen
pJQ200mp18	Suicide vector for gene replacement, Gm^r *sacB* Mob	44
pRK2013	ColE1 replicon with RK2 transfer region, Km^r Nm^r	20
pHP45	$\Omega Sp^r/Sm^r$, vector Ap^r	19
pHP45	ΩTc^r, vector Ap^r	19
pBBRMCS-5	Broad-host-range cloning vector, Mob InsP Gm^r	34
pWM6	Ω*uidA2-aph* Km^r/Nm^r, vector Ap^r	38
pHL76	pCR2.1 *aceA*	This study
pHL79	pCR2.1 *aceA*::ΩSp/Sm	This study
pHL85	pBBR1MCS-5 *aceA*	This study
pHL86	pCR2.1 *glcB*	This study
pHL87	pBBR1MCS-5 *glcB*	This study
pHL88	pBBR1MCS-5 *aceA-glcB*	This study
pHL89	pBBR1MCS-5 *aceA*-SMc00767	This study
pHL90	pBBR1MCS-5 *aceA*-SMc00767	This study
pHL91	pBBR1MCS-5 SMc00767	This study
pHL92	pBBR1MCS-5 *aceA*-SMc00767 with an internal 720-bp deletion in *aceA* that leaves wild-type SMc00767 and the intact *aceA* promoter region	This study
pHL93	*aceA*::*gusA* fusion in pBBR1MCS-5	This study
pHL95	pJQ200mp18 *aceA*::ΩSp/Sm	This study
pHL96	pCR2.1 with a 1,154-bp *glcB* fragment	This study
pHL97	pCR2.1 with a 1,154-bp *glcB* fragment::ΩTc	This study
pHL98	pJQ200mp18 with a 1,154-bp *glcB* fragment::ΩTc	This study
pHL99	pJQ200mp18 *aceA*-SMc00767	This study
pHL100	pJQ200mp18 *aceA*-SMc00767::ΩSp/Sm	This study

stantially higher activities have been detected in bacteroids isolated from bean, cowpea, and soybean nodules (25, 30). The MS enzymatic activity seems to be constitutively expressed, since it has been detected in extracts of cells growing on acetate, glucose, arabinose, pyruvate, and malate (15, 27, 36). A better understanding of the role of the glyoxylate cycle in acetate metabolism and in the rhizobium-legume interaction could be achieved through analysis of mutants. Although putative *aceA* and *glcB* genes were annotated SMc00768 and SMc02581 in the complete genome sequence of *S. meliloti*, the role of these genes in C_2 utilization has not been evaluated. This paper reports the functional roles of *aceA*, *glcB*, and an open reading frame (ORF) encoding a 79-amino-acid protein (SMc00767) in acetate metabolism and in the *S. meliloti*-legume symbiosis.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and mating. The bacterial strains and plasmids used are listed in Table 1. *S. meliloti* was grown in LB (10 g tryptone per liter, 5 g yeast extract per liter, 10 g NaCl per liter) (45), in PY medium (5 g tryptone per liter, 3 g yeast extract per liter) (3), or in M9 (45) minimal medium supplemented with thiamine (1 μg/ml), pantothenic acid (2 μg/ml), biotin (0.1

μg/ml), and potassium acetate (2 mM or 5 mM) as a carbon source. To determine growth rates on acetate, *S. meliloti* cells were grown to saturation in PY medium and then transferred to minimal medium with acetate. When required, the following antibiotics were added: rifampin (50 μg/ml), spectinomycin (25 μg/ml), tetracycline (2 μg/ml), streptomycin (25 μg/ml), and gentamicin (30 μg/ml). *E. coli* strains were grown in LB supplemented with 25 μg/ml spectinomycin, 10 μg/ml tetracycline, 20 μg/ml gentamicin, and 100 μg/ml ampicillin when needed. *E. coli* and rhizobia were grown at 30°C. Conjugation experiments were performed in the presence of the helper strain *E. coli* HB101 containing pRK2013 (20) as previously described (6).

DNA manipulations. Plasmid purification and genomic DNA extraction were performed according to published protocols (45). For hybridization, DNA was digested with EcoRI or PstI and then transferred from agarose gels to nylon membranes. Probes were labeled with ^{32}P by polymerase extension using random primers, and hybridization was carried out under high-stringency conditions (47). For sequencing, double-stranded DNA was purified with a High Pure plasmid isolation kit (Boehringer Manheim, Germany), and sequencing was performed with an automatic Perkin-Elmer/Applied Biosystems 377-18 system.

Sequence analysis, primers, and PCR amplification. The *S. meliloti* ORFs designated SMc00768 and SMc02581 were annotated in the genome as the glyoxylate cycle genes *aceA* and *glcB*, respectively. For SMc00767, which encodes a small hypothetical protein, no functional homologs were identified. Sequence analysis was carried out with the GCG programs from the Genetics Computer Group program suite (12). For DNA manipulations the *aceA*, *glcB*, and SMc00767 genes were amplified by PCR using genomic DNA from wild-type strain Rm5000. The following primers were used: for *aceA*, aceA3 (5′-GAGAT TCAAATAGGAAGGAG-3′) and aceA8 (5′-ACAGTCATCGGAGTGCT-3′); and for *glcB*, glcB210 (5′-CAAGGACGGCTCGGGACA-3′), glcB3973 (5′-GC TCACAGACCACGACCACG-3′), msg387 (5′-CAATGCCCGCTGGGGCTC GCT-3′), and ms139 (5′-ATCGCCCACATGCCCTTG-3′). To amplify *aceA*-SMc00767, primers aceA3 and sm3 (5′-AATTCGGCATGAGCCTCCAG-3′) were used. PCR amplifications were performed in a 9700 thermocycler (Perkin-Elmer) with the following conditions: initial denaturation at 94°C for 3 min, followed by 34 cycles of denaturation (94°C, 2 min), annealing (55°C, 2 min), and extension (72°C, 3 min) and a final extension at 72°C for 5 min. PCR samples were electrophoresed through 0.8 to 1% agarose gels in Tris-acetate-EDTA buffer and stained with ethidium bromide.

Generation of mutants and complementation experiments. To generate recombinant plasmids for mutagenesis, 1,755-bp, 1,154-bp, and 2,162-bp PCR products corresponding to *aceA*, *glcB*, and *aceA*-SMc00767 were cloned in pCR2.1 (Invitrogen) to obtain plasmids pHL76, pHL96, and pHL89, respectively (see Fig. S1A.1, S1A.9, and S1B.1 in the supplemental material). The antibiotic resistance cassettes pHP45Ω Sp/Sm and pHP45Ω Tc (19) were inserted into the *aceA* and *glcB* genes to generate plasmids pHL79 and pHL97 (see Fig. S1A.3 and S1A.10 in the supplemental material), and the interrupted genes were subcloned into the pJQ200mp18 vector (44), generating plasmids pHL95 and pHL98 (see Fig. S1A.4 and S1A.11 in the supplemental material). To generate a construct for SMc00767 mutagenesis, the *aceA* and SMc00767 genes from plasmid pHL89 were subcloned into pJQ200mp18, generating plasmid pHL99 (see Fig. S1B.5 in the supplemental material). This plasmid was digested with MluI and filled in with the Klenow fragment, and then the antibiotic resistance cassette (Sp/St) previously digested with SmaI was inserted into the SMc00767 gene to generate plasmid pHL100 (see Fig. S1B.6 in the supplemental material).

Recombinant plasmids harboring the interrupted genes were introduced into Rm5000, and mutants generated by double crossover were selected for each gene. To generate the *aceA-glcB* double knockout, the interrupted *glcB* gene was transferred into the *aceA*-deficient mutant. To generate the *glcB*-SMc00767 mutant, the interrupted SMc00767 gene was transferred into the *glcB*-deficient strain, and then mutants generated by double recombination events were isolated. To obtain knockouts, we used the *sacRB* selection system (44) and the appropriate antibiotics. Gene replacement was confirmed by PCR using appropriate primers and Southern blot hybridization.

To complement the mutant phenotypes, a 3,783-nucleotide PCR fragment corresponding to *glcB* and flanking sequences was cloned in pCR2.1, generating plasmid pHL86 (see Fig. S1A.7 in the supplemental material). The *aceA*, *glcB*, and *aceA*-SMc00767 genes from plasmids pHL76, pHL86, and pHL89 were then subcloned into pBBR1MCS-5, generating plasmids pHL85, pHL87, and pHL90, respectively (see Fig. S1A.2, S1A.8, and S1B.2 in the supplemental material). To complement the *aceA-glcB* double mutant, plasmid pHL86 was digested with XbaI and SpeI, and the liberated *glcB* gene was cloned into plasmid pHL85, generating plasmid pHL88, which contains *aceA* and *glcB* (see Fig. S1A.6 in the supplemental material). Additionally, the *aceA-glcB* mutant was complemented with plasmid pHL90 carrying the *aceA* and SMc00767 genes (see Fig. S1B.2 in the supplemental material). To complement the SMc00767 mutant, the SMc00767 gene was excised from plasmid pHL89 with StuI and XbaI and then ligated into pBBR1MCS-5, generating plasmid pHL91 (see Fig. S1B.4 in the supplemental material). Alternatively, this mutant was complemented with plasmid pHL92 (see Fig. S1B.3 in the supplemental material). To obtain this plasmid, pHL90 was digested with StuI and NruI and relegated to obtain a 720-bp internal deletion of the *aceA* gene.

Growth curves. Bacterial strains were grown in PY medium overnight, and 4 ml was transferred to 50 ml of M9 medium with 2 mM acetate and cultivated overnight at 30°C at 200 rpm. Cells from the 2 mM acetate culture were used to inoculate 50 ml of M9 medium containing 5 mM acetate to an initial optical density at 595 nm (OD_{595}) of 0.05. The cultures were incubated at 30°C at 200 rpm, and growth was followed by measuring the OD_{595} every 24 h.

Preparation of cell extracts. To determine ICL activities, cells were grown to an OD_{595} of 0.7, harvested by centrifugation, washed with a saline solution, and resuspended in breaking buffer [20 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) (pH 7.0), 100 mM NaCl, 5 mM $MgCl_2$, 0.4 mM EDTA, 1.5 mM dithiothreitol, 2% (wt/vol) glycerol] (27). The cells were sonicated on ice five times for 45 s with 45-s rest periods using a Soniprep 150 (MSE). The homogenate was centrifuged, and the supernatant was used for activity measurement. To determine MS activities, cells were grown to an OD_{595} of 0.7, harvested by centrifugation, washed, and resuspended in 100 mM Tris-HCl (pH 7. 5). The cells were sonicated on ice three times for 15 s with 1-min rest periods (23). The lysate was centrifuged to remove cell debris, and the supernatant was used for MS activity measurement. The protein concentrations in cell extracts were determined by the Bradford method (4), using bovine serum albumin as the standard.

Enzyme assays. ICL activity was measured as described by Dixon and Kornberg (14). The assay mixtures (1 ml) contained 50 mM morpholinepropanesulfonic acid (MOPS) (pH 7.3), 5 mM $MgCl_2$, 1 mM EDTA, 4 mM phenylhydrazine HCl, and *S. meliloti* extract. Isocitric acid was added to a final concentration of 12.5 mM to initiate the reaction. The increase in the level of the phenylhydrazone derivative of glyoxylate was measured at 324 nm. Negative controls without isocitrate were included in each experiment. MS activity was monitored by determining the glyoxylate-dependent release of free CoA from acetyl-CoA (36). The assay mixtures (0.5 ml) contained 100 mM Tris HCl (pH 7.5), 10 mM $MgCl_2$, 2.5 mM glyoxylic acid, and *S. meliloti* extract. The reaction was initiated by addition of acetyl-CoA to a final concentration of 0.43 mM. After incubation for 5 min at room temperature, the reaction was stopped with 1 ml of 6 M urea. Color was developed by addition of 5,5′-dithiobis(2-nitrobenzoic acid) to a final concentration of 10 mM, and absorbance was determined at 412 nm. Negative controls without glyoxylate were included in each experiment. Enzymatic assays of ICL and MS activities were repeated four times.

Construction of transcriptional *aceA-gusA* reporter fusions. An *S. meliloti aceA-gusA* transcriptional gene fusion was constructed as follows. Plasmid pHL85 (see Fig. S1A.2 in the supplemental material) harboring the *aceA* regulatory region as well as the entire *aceA* gene was digested with StuI (the restriction site was located 490 bp downstream of the *aceA* start codon), and plasmid pWM6 (38) was digested with SmaI to obtain a 3,727-bp fragment containing the *gusA* reporter gene. The 3,727-bp fragment was inserted into the StuI restriction site of plasmid pHL85, generating plasmid pHL93 (see Fig. S1A.5 in the supplemental material). The pHL93 plasmid containing the *aceA::gusA* fusion was introduced into wild-type strain Rm5000, as well as into the SMc00767 mutant RH421, generating strains RH465 and RH467, respectively.

β-Glucuronidase activity measurement. To measure β-glucuronidase activity, separate cultures were grown in M9 containing 5 mM acetate to OD_{595}s of 0.35, 0.7, and 1. A 1-ml aliquot of culture was centrifuged and resuspended in a salt wash solution supplemented with chloramphenicol (100 μg ml^{-1}). Quantitative β-glucuronidase assays were performed with the *p*-nitrophenyl glucuronide substrate as described previously (52). Data were normalized to the total cell protein concentration by the Bradford method (4). The results presented below are the means of three independent experiments.

RNA isolation, synthesis of labeled cDNA, and microarray hybridization. *Sinorhizobium* strains were grown at 30°C in M9 medium containing acetate to an OD_{595} of 0.7. Bacterial cells (100 ml) were collected, and total RNA was isolated by acid hot-phenol extraction as described previously (13). The concentration of RNA was determined by measuring the absorbance at 260 nm. The integrity of RNA was determined by running a 1.5% agarose gel. Ten micrograms of RNA was labeled differentially with Cy3-dCTP and Cy5-dCTP using a CyScribe First-Strand cDNA labeling kit (Amersham Biosciences). Pairs of Cy3- and Cy5-labeled cDNA samples were mixed and hybridized to the array as described by Hegde et al. (28). After washing, the arrays were scanned using a pixel size of 10 μm with a Scan Array Lite microarray scanner (Perkin-Elmer, Boston, MA). The

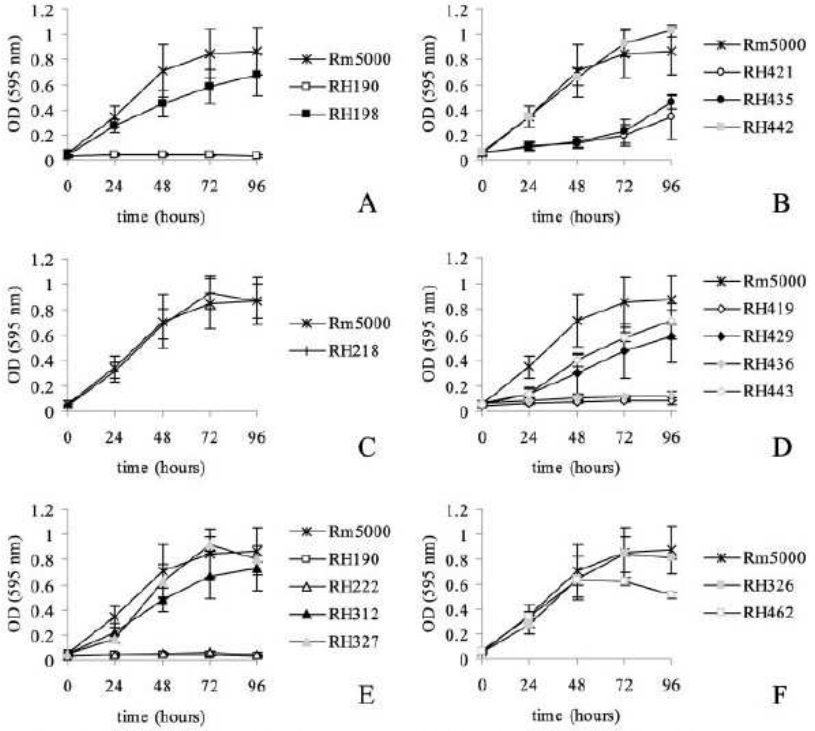

FIG. 1. Functional role of *aceA*, SMc00767, and *glcB* in acetate utilization by *S. meliloti*. Bacteria were grown in M9 minimal medium supplemented with 5 mM potassium acetate. The OD_{595} was measured every 24 h over a 96-h period. The bacterial strains used were wild-type strain Rm5000, *aceA* mutant RH190, SMc00767 mutant RH421, *glcB* mutant RH218, an *aceA* mutant complemented with the wild-type *aceA* gene (RH198), an SMc00767 mutant complemented with the SMc00786 gene (RH435), an SMc00767 mutant complemented with the wild-type SMc00767 gene and the intact *aceA* promoter region (RH442, *glcB*-SMc00767 double mutant RH419, a *glcB*-SMc00767 double mutant complemented with *glcB* (RH429), a *glcB*-SMc00767 double mutant complemented with SMc00767 (RH436), a *glcB*-SMc00767 double mutant complemented with the *aceA* promoter region and SMc00767 (RH443), *aceA-glcB* double mutant RH222, an *aceA-glcB* double mutant complemented with *aceA-glcB* (RH312), an *aceA-glcB* double mutant complemented with *aceA*-SMc00767 (RH327), an *aceA* mutant complemented with *aceA*-SMc00767 (RH326), and an *aceA* mutant complemented with *aceA-glcB* (RH462). Growth kinetics were determined at least four times, and the graphs show the averages of all experiments.

S. meliloti 6205 70-mer oligonucleotide set was acquired from QIAGEN (Hilden, Germany) (https://www.operon.com/arrays/oligosets_sinorhizobium.php). The oligonucleotide set was resuspended and spotted in duplicate on SuperAmine-coated slides (25 by 75 mm; TeleChem International, Inc.) by a high-speed robot at the microarray facility at the Cellular Physiology Institute (Universidad Nacional Autónoma de México).

DNA microarray analysis. Spot detection, mean signals, mean local background intensities, image segmentation, and signal quantification were determined for the microarray images using the Array-Pro Analyzer 4.0 software (Media Cybernetics, L.P). Microarray data analysis was performed with genArise software, developed in the Computing Unit of the Cellular Physiology Institute at Universidad Nacional Autónoma de México (http://www.ifc.unam.mx/genarise/). This software identifies differentially expressed genes by calculating an intensity-dependent z-score. It uses a sliding window algorithm to calculate the mean and standard deviation within a window surrounding each data point and defines a z-score where z measures the number of standard deviations that a data point is from the mean: $z_i = [R_i \cdot \text{mean}(R)]/\text{sd}(R)$, where z_i is the z-score for each element, mean(R) is the mean log ratio, R_i is the log ratio for each element, and sd(R) is the standard deviation of the log ratio. With this criterion, the elements in all experiments with a z-score of >2 standard deviations were considered significantly differentially expressed genes. DNA microarray experiments were performed three times with RNA isolated from independent cultures.

Plant nodulation experiments. Seeds of *Medicago sativa* were surface sterilized for 15 min in sulfuric acid and for 5 min in 1.5% sodium hypochlorite and washed in sterile distilled water. They were germinated for 48 h on 0.75% agar at 30°C in the dark. Fourteen seedlings were transplanted into a pot containing vermiculite. For nodulation experiments, each plant was inoculated with 1×10^5 bacteria. After inoculation, the plants were transferred to a growth chamber and incubated at 21°C with a photoperiod consisting of 16 h of light and 8 h of darkness. After 35 days, nitrogen fixation was determined by examining acetylene reduction by using a gas chromatograph (5). Nodules were surface sterilized and then crushed in a sterile saline solution and plated on PY medium. One hundred colonies were replica plated with the appropriate antibiotic to ensure that cross-contamination had not taken place.

RESULTS AND DISCUSSION

Growth rates of *aceA*, SMc00767, and *glcB* mutants on acetate. Mutants with mutations in the glyoxylate cycle genes were constructed and analyzed in liquid minimal medium supplemented with 5 mM potassium acetate as the carbon source (Fig. 1). The *aceA* mutant RH190 failed to grow on acetate, in contrast to wild-type strain Rm5000 and the complemented *aceA* mutant RH198, which contains the entire *aceA* gene (Fig. 1A). This result supports the essential role of *aceA* in the glyoxylate shunt. Downstream of and contiguous with *aceA* is a gene encoding a small hypothetical protein (79 amino acids) annotated SMc00767 (22) which appears to be part of a transcriptional unit with *aceA*. In order to evaluate if SMc00767 has a functional role in acetate metabolism, an SMc00767 mutant (RH421) was constructed. Growth profiles of mutant

RH421 show that it was able to grow on acetate, albeit at a very reduced rate (Fig. 1B), indicating that the SMc00767 gene has a role in acetate metabolism. Complementation experiments with SMc00767 mutant RH421 were performed. The intergenic region between *aceA* and SMc00767 is 111 bp long. Based on this organization, we complemented SMc00767 mutant RH421 with plasmid pHL91, which contains the entire SMc00767 ORF and sequences 800 bp upstream and 200 bp downstream (see Fig. S1B.4 in the supplemental material). The complemented SMc00767 mutant strain, designated RH435, had the same growth rate as the parental SMc00767 mutant RH421 strain on acetate (Fig. 1B). This result suggested that SMc00767 transcription may require the *aceA* promoter. To validate this assertion, pHL92, a derivative plasmid of pHL90, was constructed. pHL92 has an internal deletion (720 bp) in the *aceA* structural gene and contains 485 bp of the *aceA* putative promoter, 570 bp of the *aceA* structural gene, and the entire intergenic *aceA*-SMc00767 region, as well as the complete SMc00767 gene (see Fig. S1B.3 in the supplemental material). Conjugal transfer of pHL92 into the SMc00767 mutant RH421 generated complemented strain RH442. The growth rate of the complemented SMc00767 mutant RH442 on acetate was the same as that of the wild-type strain (Fig. 1B). This finding indicates that expression of SMc00767 requires the promoter region of *aceA* and that these two genes are organized in an operon. In contrast to the *aceA*- and SMc00767-deficient strains, the *glcB* mutant RH218 grew similar to the Rm5000 wild-type strain on acetate (Fig. 1C), indicating that single mutations in *glcB* do not affect acetate utilization.

Our results showed that the *aceA* mutant was unable to growth on acetate, while the growth rate of the SMc00767 mutant was reduced 65% in comparison to the growth rate of the wild- type strain. This observation indicated that another gene besides SMc00767 was involved in acetate utilization. Other data supporting the presence of an additional component for acetate utilization include the fact that the mutation in *aceA* had a polar effect on SMc00767, and so a second genetic component must be involved in the growth of the complemented *aceA* strain RH198 on acetate. In order to identify the additional genetic locus involved in acetate metabolism, an SMc00767-*glcB* double mutant, RH419, was constructed. *glcB* was chosen because it is involved in acetate metabolism in many bacterial species, although we have shown that the *glcB* single mutant was not affected in acetate utilization (Fig. 1C). The growth profiles of the SMc00767-*glcB* double mutant (Fig. 1D) show that this strain is unable to grow on acetate, indicating that *glcB* has a functional role in the absence of SMc00767. This result indicates that *aceA* itself is unable to restore growth on acetate and demonstrates the necessity of SMc00767 or *glcB* for growth on acetate. To support this hypothesis, we transferred plasmids pHL92 (harboring the *aceA* promoter and the SMc00767 structural gene), pHL87 (containing the *glcB* gene), and pHL91 (harboring the SMc00767 gene without the *aceA* promoter) (see Fig. S1B.3, S1A.8, and S1B.4 in the supplemental material) independently to the SMc00767-*glcB* double mutant RH419, obtaining the complemented strains RH443, RH429, and RH436, respectively. The growth of the SMc00767-*glcB* double mutant complemented with the *aceA* promoter and the SMc00767 structural gene (RH443) and the growth of the strain with *glcB* (RH429) were partially reestablished on acetate. In contrast, the SMc00767-*glcB* mutant complemented with SMc00767 without the *aceA* promoter was unable to restore growth (Fig. 1D). The results of these experiments support the hypothesis that SMc00767 and *glcB* have a functional role in acetate metabolism and provide further genetic evidence of the *aceA*-SMc00767 operon organization. To ascertain the requirement for SMc00767 and *glcB* for growth on acetate, an *aceA-glcB* double mutant (RH222) was constructed. The *aceA*-SMc00767 mutant strain RH190 (see above) and the *aceA-glcB* mutant strain RH222 were evaluated for growth on acetate. Neither mutant was able to grow on acetate as a carbon source (Fig. 1E). Complementation experiments with the *aceA-glcB* RH222 mutant strain were performed. Plasmid pHL88 (see Fig. S1A.6 in the supplemental material), which harbors the *aceA* and *glcB* genes, was transferred to the *aceA-glcB* RH222 mutant, generating the RH312 derivative. The complemented *aceA-glcB* mutant strain RH312 exhibited growth similar to that of the wild-type strain on acetate (Fig. 1E). In addition we generated the complemented strain RH327 by introducing plasmid pHL90 (see Fig. S1B.2 in the supplemental material), which contains *aceA* as well as SMc00767, into *aceA-glcB* mutant RH222. As shown in Fig. 1E, the *aceA-glcB* mutant, complemented with the wild-type *aceA* and SMc00767 genes, also reestablished growth on acetate. The *aceA*-SMc00767 double mutant RH190 was also complemented with plasmid pHL88, which contains *aceA* and *glcB*, and plasmid pHL90, which contains *aceA* and SMc00767 (see Fig. S1A.6 and Fig. S1B.2 in the supplemental material) (strains RH462 and RH326, respectively). The growth profiles on acetate (Fig. 1F) of the *aceA*-SMc00767 mutant complemented with *aceA-glcB* or *aceA* and SMc00767 are similar to those of wild-type strain Rm5000 (Fig. 1F). These results show that *aceA* and SMc00767, as well as *glcB*, are involved in acetate metabolism in *S. meliloti*. In order to determine if *aceA*, SMc00767, and *glcB* are functional with other carbon sources, growth rate experiments were performed with different carbon compounds. Previously, we reported (23) that *glcB* was induced on minimal medium supplemented with arabinose as the carbon source, and it has also been reported that GlcB activity was detectable in minimal medium supplemented with succinate, arabinose, or malate (15, 27). To evaluate if *glcB* has a functional role in the utilization of these carbon compounds, we performed growth rate experiments with the *glcB* mutant RH218 and the complemented *glcB* strain RH302 in minimal medium supplemented with arabinose, succinate, or glucose and in PY medium. With the different carbon sources tested, the growth of the *glcB* mutant and the complemented RH302 strain was identical to the growth of the wild-type Rm5000 strain (data not shown). This indicates that *glcB* is not essential for arabinose, succinate, glucose, or PY medium utilization. Growth rate experiments were also performed with the *aceA* mutant RH190 and the SMc00767 mutant RH421 in minimal medium supplemented with glucose and in PY medium. We found that the growth of the *aceA* mutant RH190 and the growth of the SMc00767 mutant RH421 were similar to the growth of the Rm5000 wild-type strain (data not shown), indicating that the *aceA* and SMc00767 loci are not involved in glucose or PY medium utilization.

Activity of glyoxylate cycle enzymes. A series of enzymatic assays were carried out with cell extracts of mutants and com-

TABLE 2. ICL and MS activities in cell extracts of *S. meliloti* strains[a]

Strain	Genotype	Enzyme sp act (nmol min^{-1} mg $protein^{-1}$)	
		ICL	MS
Rm5000	Wild type	138 ± 21	179 ± 27
RH190	Rm5000 *aceA*::ΩSp/Sm	6.7	43
RH198	Rm5000 *aceA*::ΩSp/Sm, pBBR1MCS-5 *aceA*	1,080 ± 190	172 ± 21
RH218	Rm5000 *glcB*::ΩTc	297 ± 26	ND
RH222	Rm5000 *aceA*::ΩSp/Sm *glcB*::ΩTc	ND	ND
RH302	Rm5000 *glcB*::ΩTc, pBBR1MCS-5 *glcB*	190 ± 18	230 ± 25
RH312	Rm5000 *aceA*::ΩSp/Sm *glcB*::ΩTc, pBBR1MCS-5 *aceA-glcB*	1,030 ± 209	251 ± 26
RH326	Rm5000 *aceA*::ΩSp/Sm, pBBR1MCS-5 *aceA*-SMc00767	341 ± 22	115 ± 24
RH327	Rm5000 *aceA*::ΩSp/Sm *glcB*::ΩTc, pBBR1MCS-5 *aceA*-SMc00767	443 ± 50	ND
RH419	Rm5000 *glcB*::ΩTc SMc00767::ΩSp/Sm	12 ± 10	ND
RH421	Rm5000 SMc00767::ΩSp/Sm	685 ± 39	132 ± 19
RH429	Rm5000 *glcB*::ΩTc SMc00767::ΩSp/Sm, pBBR1MCS-5 *glcB*	731 ± 37	907 ± 220
RH435	Rm5000 SMc00767::ΩSp/Sm, pBBR1MCS-5 SMc00767	657 ± 36	141 ± 2
RH442	Rm5000 SMc00767::ΩSp/Sm, pBBR1MCS-5 *aceA*-SMc00767 with an internal 700-bp deletion in *aceA* that leaves wild-type SMc00767 and the intact *aceA* promoter region	96 ± 18	116 ± 6
RH443	Rm5000 *glcB*::ΩTc SMc00767::ΩSp/Sm, pBBR1MCS-5 *aceA*-SMc00767 with an internal 700-bp deletion in *aceA* that leaves wild-type SMc00767 and the intact *aceA* promoter region	216 ± 31	ND
RH462	Rm5000 *aceA*::ΩSp/Sm, pBBR1MCS-5 *aceA-glcB*	832 ± 194	1,343 ± 303

[a] Bacteria were grown in M9 medium supplemented with 5 mM potassium acetate as the carbon source. In most cases the values are the averages of at least four separate experiments; the exception is the values for strain RH190, for which only one experiment was done. ND, not detected.

plemented strains growing on acetate as the carbon source. Table 2 shows the loss of ICL activity in the *aceA* mutant RH190. The complemented *aceA* mutant RH198, harboring the single *aceA* gene, had eightfold more ICL activity than the wild-type strain (Table 2). The *glcB* mutant RH218 lacked MS activity, while the *glcB* mutant complemented with *glcB* (RH302) exhibited a level of MS activity similar to that of the wild-type strain (Table 2). These results indicate that the inability of the *aceA* mutant to grow on acetate was due to the *aceA* mutation (Fig. 1A). The null activity of the *glcB* mutant and the restored activity of the complemented strain showed that a single MS gene was present in *S. meliloti*. The activities of the glyoxylate cycle enzymes ICL and MS were also measured in the *aceA-glcB* double mutant RH222. The double mutant was unable to grow on acetate (Fig. 1E) and had no ICL and MS activities (Table 2). The *aceA-glcB* mutant complemented with *aceA* and *glcB* (strain RH312) exhibited growth on acetate (Fig. 1E), as well as ICL and MS activities, and in the case of ICL, it had sevenfold more activity that the wild-type Rm5000 strain. The *aceA-glcB* double mutant complemented with *aceA*-SMc00767 (strain RH327) also exhibited ICL activity, but it showed only a threefold increase in ICL activity compared to the wild type (Table 2).

These results show that in the absence of SMc00767 ICL overexpression occurs, suggesting that SMc00767 is a repressor of the *aceA* gene. To validate this suggestion, ICL activities were measured. The *aceA*-SMc00767 mutant RH190, as mentioned above, has no ICL activity, but when it was complemented with the *aceA* and SMc00767 genes (strain RH326), it exhibited a twofold increase in ICL activity compared with the wild type (Table 2). In contrast, mutant RH421, lacking SMc00767, had five times as much in ICL activity as the wild type (Table 2). The SMc00767 mutant complemented with the SMc00767 gene without the *aceA* promoter (strain RH435) showed no reduction in ICL activity, while the SMc00767 mutant complemented with the *aceA* promoter and the SMc00767 gene (strain RH442) had a reduced level of ICL activity similar to the level of wild-type strain Rm5000. In addition, when *glcB*-SMc00767 mutant RH419, which showed no growth on acetate (Fig. 1D), was complemented with *glcB* (strain RH429), it showed a fivefold increase in ICL activity, while the *glcB*-SMc00767 mutant complemented with the *aceA* promoter and the SMc00767 gene (strain RH443) had ICL activity similar to that of the wild type. Together, these data indicate that SMc00767 repressed *aceA* expression. To determine if the glyoxylate cycle activities were present during grown on other carbon sources, ICL and MS enzymatic assays were performed. In PY medium, ICL activity was not detected in wild-type strain Rm5000 or in the SMc00767 mutant RH421 (which overexpressed ICL activity in the presence of acetate), supporting the specificity of ICL activity in acetate metabolism. To evaluate if MS activity was present during grown on other carbon sources, wild-type strain Rm5000 and the *glcB* mutant RH218 were grown with glucose, arabinose, or succinate as the sole carbon source. The *glcB* mutant RH218, as expected, had no MS activity on any carbon source tested. In contrast, on arabinose, succinate, and glucose, wild-type strain Rm5000 had MS activities that were 20, 15, and 17% of the activity on acetate. Together, the growth rate experiments and the MS activities suggest that this enzyme is not required for arabinose, succinate, or glucose metabolism.

SMc00767 regulates *aceA* expression on acetate. The enzymatic data indicate that SMc00767 represses *aceA* expression. To evaluate if this repression occurs at the transcriptional level, gene fusions were constructed in which the *gusA* reporter gene was inserted downstream of the *aceA* promoter of plasmid pHL85 to obtain plasmid pHL93 (see Fig. S1A.5 in the supplemental material). The resulting *aceA* promoter-*gusA* fusion plasmid was introduced into wild-type strain Rm5000 and the SMc00767 mutant to generate strains RH465 and RH467, respectively. β-Glucuronidase activity was determined for both strains in M9 minimal medium supplemented with 5 mM ac-

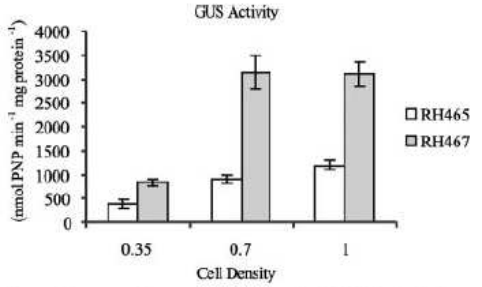

FIG. 2. β-Glucuronidase activities of RH465 (wild-type strain Rm5000 containing the *aceA::gusA* fusion) and RH467 (SMc00767 mutant RH421 harboring the *aceA::gusA* fusion) grown in M9 medium containing acetate. The cultures were collected at OD_{595}s of 0.35, 0.7, and 1. The values are the means of three independent experiments performed in duplicate. PNP, *p*-nitrophenyl; GUS, β-glucuronidase.

etate. The transcriptional assays revealed that at three different culture OD_{595}s (0.35, 0.7, and 1), the strain with the *aceA* promoter in the absence of SMc00767 had significantly greater β-glucuronidase activity than wild-type strain Rm5000 harboring the *aceA* promoter-*gusA* fusion plasmid (Fig. 2). Thus, we demonstrated, by using expression assays of the *aceA* promoter as well as by examining ICL enzymatic activity, that SMc00767 is a repressor of the *aceA* gene. To determine if SMc00767 repressed *aceA* expression in the presence of other carbon sources, β-glucuronidase assays were performed with these strains using succinate-, arabinose-, and glucose-grown cultures. At three different OD_{595}s (0.35, 0.7, and 1), *aceA* expression was not detected with arabinose, glucose, or succinate in RH465 and RH467 (data not shown), supporting the hypothesis that *aceA* and SMc00767 have a specific role in acetate metabolism.

Transcriptional profiling of the *S. meliloti* wild-type Rm5000 and SMc00767 mutant strains grown on acetate. The enzymatic and transcriptional results presented above indicate that SMc00767 is a novel regulator involved in acetate metabolism in rhizobia. To investigate the role of the SMc00767 gene in the global regulation of acetate metabolism and to identify further putative target genes of SMc00767, transcriptional profiles of wild-type strain Rm5000 and the SMc00767 mutant were compared. These strains were grown on 5 mM acetate to an OD_{595} of 0.7, the bacteria were collected, and the total RNA was isolated, labeled, and hybridized with slides that contained the genome oligonucleotide set acquired from QIAGEN. We found that in the SMc00767 mutant seven genes were overexpressed with a z-score of >2 standard deviations (Table 3). These genes correspond to four hypothetical proteins, *dnaB*, *potH*, and *aceA*. These genes are dispersed in the *S. meliloti* genome: SMb21456 and SMb21463 are encoded on pSymB, SMa2071 is encoded on pSymA, and *aceA*, SMc00769, *potH*, and *dnaB* are encoded on the chromosome. Interestingly, one of the genes encoding a hypothetical protein (SMc00769) and *potH* are located downstream of *aceA*. In *S. meliloti*, SMc00767 is clustered with *aceA*, SMc00769 *potF*, *potG potH*, and *potI*, and the microarray data show that three of these genes are overexpressed in the SMc00767 mutant, suggesting that this cluster of genes is involved in acetate utilization. The reason why the microarray experiments failed to detect all the genes of this cluster could be RNA degradation, RNA instability, or low mRNA levels. However, with the exception of *aceA*, the genes overexpressed in the microarray experiments represent novel genes for acetate metabolism in *S. meliloti*. Additional work is necessary to assign specific roles to these genes. The moderate number of overexpressed genes obtained with the microarray experiments shows that SMc00767 is a local repressor of acetate metabolism in *S. meliloti*. In agreement with the enzymatic data and expression analysis of the *aceA* gene, the microarray experiment also shows that SMc00767 represses *aceA* transcription; thus, we considered *aceA* a good internal control to validate the effect of the SMc00767 gene on the expression of other *S. meliloti* genes.

SMc00767 regulates the expression of a conserved cluster of genes in rhizobia. The enzymatic data and the transcriptional results show that SMc00767 regulates the expression of *aceA*, and the microarray experiments indicate that SMc00767 also regulates the expression of two genes located downstream of *aceA*. To determine if the genes repressed by SMc00767 are conserved in rhizobia, genomes of several α-proteobacteria were analyzed. The alignment in Fig. 3 shows that the transcriptional repressor SMc00767, as well as *aceA*, SMc00769, *potF*, *potG*, *potH*, and *potI*, are contiguous in *Agrobacterium tumefaciens* (26, 54), *Brucella melitensis* (11), *Brucella suis* (41), *Rhizobium etli* (25), *Rhizobium leguminosarum* (55), and *S. meliloti* (22). This finding, in light of the *S. meliloti* microarray data, suggests that these genes have a role in acetate metabolism in α-proteobacteria.

Symbiotic phenotypes of the *S. meliloti aceA and glcB* mutants. Recently, studies with *Candida albicans* (35), *Magnaphorte grisea* (51), *Mycobacterium tuberculosis* (39), *Rhodococcus equi* (50), *Rhodococcus fascians* (49), and *Stagonospora nodorum* (46) showed that the glyoxylate bypass is essential for virulence in both animal and plant pathogens. However, the functional role of this pathway in the *Rhizobium*-legume symbiosis has not been explored. To determine if the glyoxylate cycle has a role in symbiosis, plant nodulation experiments were performed. Fourteen alfalfa plants were inoculated independently with the *aceA* mutant, the *glcB* mutant, and the *aceA-glcB* double mutant and analyzed to determine the number of nodules, acetylene reduction, and dry weight 42 days postinoculation. The results show that the wild-type Rm5000 strain produced 18 nodules per plant and the *aceA*, *glcB*, and *aceA-glcB* mutants formed 19, 17, and 15 nodules per plant, respectively. Wild-

TABLE 3. Genes significantly induced in the SMc00767 mutant as detected by microarray analysis

ORF	Description and/or gene	Fold change in SMc00767 mutant vs wild type
SMa2071	Hypothetical protein	2.6
SMb21456	Hypothetical protein	2.4
SMb21473	Conserved hypothetical protein	2.9
SMc00561	Probable replicative DNA helicase protein, *dnaB*	3.5
SMc00768	ICL protein, *aceA*	4.6
SMc00769	Conserved hypothetical protein	2.6
SMc00772	Probable putrescine transport system permease protein, *potH*	2.0

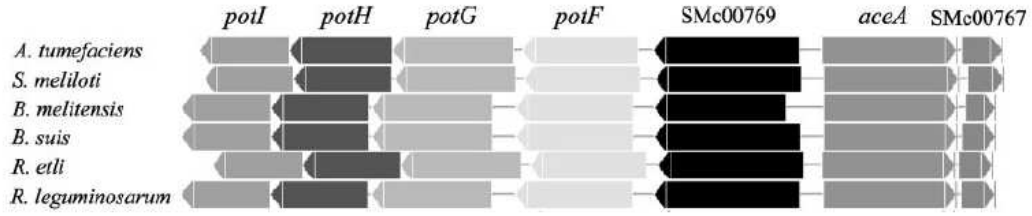

FIG. 3. Genome context of the SMc00767, *aceA*, SMc00769, *potF*, *potG*, *potH*, and *potI* genes in different members of the α-proteobacteria. In all cases, SMc00767 is clustered in this group of genes, suggesting that they are involved in a common process. Genes and intergenic distances are drawn in proportion. The figure was generated using GeCont (7).

type strain Rm5000, the *aceA* and *glcB* mutants, and the *aceA-glcB* double mutant had nitrogenase specific activities of 0.063, 0.096, 0.087, and 0.078 μmol of acetylene · plant^{-1} · h^{-1}, respectively. The dry weights of the 14 plants inoculated with wild-type strain Rm5000 and the *aceA*, *glcB*, and *aceA-glcB* mutants were 17.5, 19.5, 22, and 23.6 mg, respectively. Together, these data indicate that the glyoxylate cycle genes in *S. meliloti* are not involved in nodulation and nitrogen fixation.

The glyoxylate bypass has been established in several rhizobia (23, 27, 30, 36), but little research has focused on the functionality of this pathway in these organisms. The enzymatic activities of ICL and MS in several *Rhizobium* species indicated that the glyoxylate shunt was functional (15, 27, 30, 36). However, systematic studies of mutants with mutations in each of these genes have not been done until now. In this report we show that *aceA* and a 240-bp ORF designated SMc00767 are the principal genes for acetate metabolism in *S. meliloti*. The results obtained indicate that in rhizobia, as well as in other bacterial species, *aceA* is required in the glyoxylate cycle, since mutations in this gene completely abolish growth on acetate. A remarkable and interesting finding of this work was the identification of SMc00767, which is present only in symbionts, plant and animal pathogens such as *S. meliloti*, *R. etli*, *R. leguminosarum*, *A. tumefaciens*, and different *Brucella* spp. In all the organisms analyzed, SMc00767 is downstream of *aceA* in an operon, indicating that in rhizobia these genes operate in the same metabolic process. Our results support this hypothesis, since we show that the presence of SMc00767 is essential for optimal growth on acetate and that it regulates *aceA* transcription. Our results indicate that the C_2 metabolism in rhizobia is completely different from that in other bacteria, such as *E. coli* (31, 40). This assertion is also supported by the fact that *S. meliloti glcB* mutants are able to grow on acetate; this is an important result since until now most studies of acetate metabolism have shown that *glcB* mutants are unable to grow on acetate, while in *S. meliloti glcB* has a secondary role in the utilization of C_2 compounds. This result is similar to that obtained by Cornah et al. (8), who reported that *Arabidopsis* mutants lacking MS are capable of gluconeogenesis from acetate. These authors suggested that a new metabolic pathway to metabolize acetate to sugars in the absence of MS is present in *Arabidopsis* seedlings, and recent studies with *Rhodobacter sphaeroides* (1) and *Methylobacterium extorquens* (32, 33) provided evidence of alternative acetate assimilation pathways. Thus, the possibility of new metabolic pathways for C_2 compounds in rhizobia exists. In the case of *S. meliloti* we believe that the primary route for acetate utilization depends on *aceA* and SMc00767, but in the absence of SMc00767, *aceA* and *glcB* are able to support growth on acetate, indicating that *S. meliloti* is able to utilize acetate in these two ways.

The symbiotic performance of the *S. meliloti aceA* and *glcB* mutants shows that these genes are not involved in the interaction with alfalfa plants. Recently, we isolated and sequenced two *aceA* genes from *Rhizobium tropici*, one located on the chromosome and the other encoded on the symbiotic plasmid. An *R. tropici aceA* double mutant was constructed, and analysis of the symbiotic performance on bean plants (*Phaseolus vulgaris*) showed that this mutant was not affected in nodulation or nitrogen fixation (data not shown). Thus, *aceA* does not appear to be involved in the *S. meliloti*-alfalfa and *R. tropici-P. vulgaris* symbioses.

Previous reports showed that MS activity is present in bacteroids from pea, alfalfa, and clover, and substantially higher activities were detected in bacteroids from bean, cowpea, and soybean. However, our results show that MS is not involved in nodulation or nitrogen fixation in the interaction of *S. meliloti* with alfalfa plants, and we previously reported that pea plants inoculated with an *R. leguminosarum glcB* mutant showed no significant differences in nitrogen fixation (23). Thus, we have demonstrated that *glcB* is dispensable in plant-microbe interactions in two symbiotic systems.

ACKNOWLEDGMENTS

We thank G. Uribe-Figueroa, S. I. Fuentes, F. J. Santana, M. Fernández-Mora, A. Vazquez, J. Miranda-Ríos, H. Salgado, P. Mavingui, R. Oropeza, and J. Caballero-Mellado for technical help and useful scientific comments to improve the manuscript.

This research was supported by grants to I.H.-L. from DGAPA/UNAM (IN206802 and IN206705). J.A.R.-T. is a recipient of a Ph.D. studentship from the Consejo Nacional de Ciencia y Tecnología (Mexico).

REFERENCES

1. **Alber, B. E., R. Spanheimer, C. Ebenau-Jehle, and G. Fuchs.** 2006. Study of an alternate glyoxylate cycle for acetate assimilation by *Rhodobacter sphaeroides*. Mol. Microbiol. **62:**297–309.
2. **Beeckmans, S.** April 2001, posting date. Glyoxylate cycle. *In* Encyclopedia of life sciences. John Wiley & Sons, Ltd., Chichester, United Kingdom. http://www.els.net/.
3. **Beringer, J. E.** 1974. R-factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. **84:**188–198.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
5. **Burris, R. H.** 1972. Nitrogen fixation assay—methods and techniques. Methods Enzymol. **24:**415–431.
6. **Charles, T. C., and T. M. Finan.** 1990. Genetic map of *Rhizobium meliloti* megaplasmid pRmeSU47B. J. Bacteriol. **172:**2469–2476.
7. **Cirla, R., C. Abreu-Goodger, E. Morett, and E. Merino.** 2004. GeConT: gene context analysis. Bioinformatics **20:**2307–2308.

8. **Cornah, J. E., V. Germain, J. L. Ward, M. H. Beale, and S. M. Smith.** 2004. Lipid utilization, gluconeogenesis, and seedling growth in *Arabidopsis* mutants lacking the glyoxylate cycle enzyme malate synthase. J. Biol. Chem. **279:**42916–42923.
9. **Cozzone, A. J.** 1998. Regulation of acetate metabolism by protein phosphorylation in enteric bacteria. Annu. Rev. Microbiol. **52:**127–164.
10. **Cronan, J. E., and D. LaPorte.** 1996. Tricarboxylic acid cycle and glyoxylate bypass, p. 201–206. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, DC.
11. **DelVecchio, V. G., V. Kapatral, R. J. Redkar, G. Patra, C. Mujer, T. Los, N. Ivanova, I. Anderson, A. Bhattacharyya, A. Lykidis, G. Reznik, L. Jablonski, N. Larsen, M. D'Souza, A. Bernal, M. Mazur, E. Goltsman, E. Selkov, P. H Elzer, S. Hagius, D. O'Callaghan, J. J. Letesson, R. Haselkorn, N. Kyrpides, and R. Overbeek.** 2002. The genome sequence of the facultative intracellular pathogen *Brucella melitensis*. Proc. Natl. Acad. Sci. USA **99:**443–448.
12. **Devereaux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
13. **De Vries, S., H. Hoge, and T. Bisseling.** 1988. Isolation of total and polysomal RNA from plant tissues, p. 1–13. *In* S. B. Gelvin, R. A. Schilperoort, and D. P. S. Verma (ed.), Plant molecular biology manual. Kluwer Academic Publishers, Dordrecht, The Netherlands.
14. **Dixon, G. H., and H. L. Kornberg.** 1959. Assay methods for key enzymes of the glyoxylate cycle. Biochem. J. **72:**3P.
15. **Duncan, M. J., and D. G. Fraenkel.** 1979. Alpha-ketoglutarate dehydrogenase mutant of *Rhizobium meliloti*. J. Bacteriol. **137:**415–419.
16. **Dunn, M. F.** 1998. Tricarboxylic acid cycle and anaplerotic enzymes in rhizobia. FEMS Microbiol. Rev. **22:**105–123.
17. **Dymov, I. S., D. J. J. Meek, B. Steven, and B. T. Driscoll.** 2004. Insertion of transposon Tn5tac1 in the *Sinorhizobium meliloti* malate dehydrogenase (mdh) gene results in conditional polar effects on downstream TCA cycle genes. Mol. Plant-Microbe Interact. **17:**1318–1327.
18. **Escher, C. L., and F. Widmer.** 1997. Lipid mobilization and gluconeogenesis in plants: do glyoxylate cycle enzyme activities constitute a real cycle? A hypothesis. Biol. Chem. **378:**803–813.
19. **Fellay, R., J. Frey, and H. Krisch.** 1987. Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for in vitro insertional mutagenesis of Gram-negative bacteria. Gene **52:**147–154.
20. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci. USA **76:**1648–1652.
21. **Finan, T. M., E. Hartweig, K. LeMieux, K. Bergman, G. C. Walker, and E. R. Signer.** 1984. General transduction in *Rhizobium meliloti*. J. Bacteriol. **159:**120–124.
22. **Galibert, F., T. M. Finan, S. R. Long, A. Pühler, A. Abola, F. Ampe, F. Barloy-Hubler, M. J. Barnett, A. Becker, P. Boistard, M. Bothe, M. Boutry, L. Bowser, J. Buhrmester, E. Cadieu, D. Capela, P. Chain, A. Cowie, R. W. Davis, S. Dréano, N. A. Federspiel, R. F. Fisher, S. Gloux, T. Godrie, A. Goffeau, B. Golding, J. Gouzy, M. Gurjal, I. Hernandez-Lucas, A. Hong, L. Huizar, R. W. Hyman, T. Jones, D. Kahn, M. L. Kahn, S. Kalman, D. H. Keating, E. Kiss, C. Komp, V. Lelaure, D. Masuy, C. Palm, M. C. Peck, T. M. Pohl, D. Portetelle, B. Purnelle, U. Ramsperger, R. Surzycki, P. Thébault, M. Vandenbol, F. J. Vorhölter, S. Weidner, D. H. Wells, K. Wong, K. Chen Yeh, and J. Batut.** 2001. The composite genome of the legume symbiont *Sinorhizobium meliloti*. Science **293:**668–672.
23. **Garcia-de los Santos, A., A. Morales, L. Baldoma, S. R. Clark, S. Brom, C. K. Yost, I. Hernandez-Lucas, J. Aguilar, and M. F. Hynes.** 2002. The *glcB* locus of *Rhizobium leguminosarum* VF39 encodes an arabinose-inducible malate synthase. Can. J. Microbiol. **48:**922–932.
24. **Gardiol, A., A. Arias, C. Cerveñansky, and G. Martínez-Drets.** 1982. Succinate dehydrogenase mutant of *Rhizobium meliloti*. J. Bacteriol. **151:**1621–1623.
25. **Gonzalez, V., R. I. Santamaria, P. Bustos, I. Hernandez-Gonzalez, A. Medrano-Soto, G. Moreno-Hagelsieb, S. C. Janga, M. A. Ramirez, V. Jimenez-Jacinto, J. Collado-Vides, and G. Davila.** 2006. The partitioned *Rhizobium etli* genome: genetic and metabolic redundancy in seven interacting replicons. Proc. Natl. Acad. Sci. USA **103:**3834–3839.
26. **Goodner, B., G. Hinkle, S. Gattung, N. Miller, M. Blanchard, B. Qurollo, B. S. Goldman, Y. Cao, M. Askenazi, C. Halling, L. Mullin, K. Houmiel, J. Gordon, M. Vaudin, O. Iartchouk, A. Epp, F. Liu, C. Wollam, M. Allinger, D. Doughty, C. Scott, C. Lappas, B. Markelz, C. Flanagan, C. Crowell, J. Gurson, C. Lomo, C. Sear, G. Strub, C. Cielo, and S. Slater.** 2001. Genome sequence of the plant pathogen and biotechnology agent *Agrobacterium tumefaciens* C58. Science **294:**2323–2328.
27. **Green, L. S., D. B. Karr, and D. W. Emerich.** 1998. Isocitrate dehydrogenase and glyoxylate cycle enzyme activities in *Bradyrhizobium japonicum* under various growth conditions. Arch. Microbiol. **169:**445–451.
28. **Hegde, P., R. Qi, R. Abernathy, C. Gay, S. Dharap, R. Gaspard, J. E. Hughes, E. Snesrud, N. Lee, and J. Quackenbush.** 2000. A concise guide to cDNA microarray analysis. BioTechniques **29:**548–550, 552–554, 556.
29. **Hernández-Lucas, I., M. A. Pardo, L. Segovia, J. Miranda, and E. Martínez.** 1995. *Rhizobium tropici* chromosomal citrate synthase gene. Appl. Environ. Microbiol. **61:**3992–3997.
30. **Johnson, G. V., H. J. Evans, and T. Ching.** 1966. Enzymes of glyoxylate cycle in rhizobia and nodules of legumes. Plant Physiol. **41:**1330–1336.
31. **Kornberg, H. L.** 1966. The role and control of the glyoxylate cycle in *Escherichia coli*. Biochem. J. **99:**1–11.
32. **Korotkova, N., L. Chistoserdova, V. Kuksa, and M. E. Lidstrom.** 2002. Glyoxylate regeneration pathway in the methylotroph *Methylobacterium extorquens* AM1. J. Bacteriol. **184:**1750–1758.
33. **Korotkova, N., M. E. Lidstrom, and L. Chistoserdova.** 2005. Identification of genes involved in the glyoxylate regeneration cycle in *Methylobacterium extorquens* AM1, including two new genes, *meaC* and *meaD*. J. Bacteriol. **187:**1523–1526.
34. **Kovach, M. E., P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. Roop II, and K. M. Peterson.** 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene **166:**175–176.
35. **Lorenz, M. C., and G. R. Fink.** 2001. The glyoxylate cycle is required for fungal virulence. Nature **41:**83–86.
36. **Mandal, N. C., and P. K. Chakrabartty.** 1992. Regulation of enzymes of glyoxylate pathway in root-nodule bacteria. J. Gen. Appl. Microbiol. **38:**417–427.
37. **McDermott, T. R., and M. Kahn.** 1992. Cloning and mutagenesis of the *Rhizobium meliloti* isocitrate dehydrogenase gene. J. Bacteriol. **174:**4790–4797.
38. **Metcalf, W. W., and B. L. Wanner.** 1993. Construction of new β-glucuronidase cassettes for making transcriptional fusions and their use with new methods for allele replacement. Gene **129:**17–25.
39. **Muñoz-Elías, E. J., and J. D. McKinney.** 2005. *Mycobacterium tuberculosis* isocitrate lyases 1 and 2 are jointly required for in vivo growth and virulence. Nat. Med. **11:**638–644.
40. **Ornston, L. N., and M. K. Ornston.** 1969. Regulation of glyoxylate metabolism in *Escherichia coli* K-12. J. Bacteriol. **98:**1098–1108.
41. **Paulsen, I. T., R. Seshadri, K. E. Nelson, J. A. Eisen, J. F. Heidelberg, T. D. Read, R. J. Dodson, L. Umayam, L. M. Brinkac, M. J. Beanan, S. C. Daugherty, R. T. Deboy, A. S. Durkin, J. F. Kolonay, R. Madupu, W. C. Nelson, B. Ayodeji, M. Kraul, J. Shetty, J. Malek, S. E. Van Aken, S. Riedmuller, H. Tettelin, S. R. Gill, O. White, S. L. Salzberg, D. L. Hoover, L. E. Lindler, S. M. Halling, S. M. Boyle, and C. M. Fraser.** 2002. The *Brucella suis* genome reveals fundamental similarities between animal and plant pathogens and symbionts. Proc. Natl. Acad. Sci. USA **99:**13148–13153.
42. **Peterson, J. B., and T. A. LaRue.** 1981. Utilization of aldehydes and alcohols in soybean bacteroids. Plant Physiol. **68:**489–493.
43. **Peterson, J. B., and T. A. LaRue.** 1982. Soluble aldehyde dehydrogenase and metabolism of aldehydes by soybean bacteroids. J. Bacteriol. **151:**1473–1484.
44. **Quandt, J., and M. F. Hynes.** 1993. Versatile suicide vectors which allow direct selection for gene replacement in gram-negative bacteria. Gene **127:**15–21.
45. **Sambrook, J., E. F. Fritsch., and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
46. **Solomon, P. S., R. C. Lee, T. J. Wilson, and R. P. Oliver.** 2004. Pathogenicity of *Stagonospora nodorum* requires malate synthase. Mol. Microbiol. **53:**1065–1073.
47. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
48. **Stovall, I., and M. Cole.** 1978. Organic acid metabolism by isolated *Rhizobium japonicum* bacteroids. Plant Physiol. **61:**787–790.
49. **Vereecke, D., K. Cornelis, W. Temmerman, M. Jaziri, M. Van Montagu, M. Holsters, and K. Goethals.** 2002. Chromosomal locus that affects pathogenicity of *Rhodococcus fascians*. J. Bacteriol. **184:**1112–1120.
50. **Wall, D. M., P. S. Duffy, C. Dupont, J. F. Prescott, and W. G. Meijer.** 2005. Isocitrate lyase activity is required for virulence of the intracellular pathogen *Rhodococcus equi*. Infect. Immun. **73:**6736–6741.
51. **Wang, Z. Y., C. R. Thornton, M. J. Kershaw, L. Debao, and N. J. Talbot.** 2003. The glyoxylate cycle is required for temporal regulation of virulence by the plant pathogenic fungus *Magnaporthe grisea*. Mol. Microbiol. **47:**1601–1612.
52. **Wilson, K. J., S. G. Huges, and R. A. Jefferson.** 1992. The *Escherichia coli* gus operon, induction and expression of the gus operon in *E. coli* and the occurrence and use of GUS in other bacteria, p. 7–23. *In* S. R. Gallagher (ed.), Gus protocols, using the gus gene as a reporter of gene expression, vol. 1. Academic Press, San Diego, CA.
53. **Wong, P. P., and H. J. Evans.** 1971. Poly-β-hydroxybutyrate utilization by soybean (*Glycine max* Merr) nodules and assessment of its role in maintenance of nitrogenase activity. Plant Physiol. **47:**750–755.
54. **Wood, D. W., J. C. Setubal, R. Kaul, D. E. Monks, J. P. Kitajima, V. K. Okura, Y. Zhou, L. Chen, G. E. Wood, N. F. Almeida, Jr., L. Woo, Y. Chen, I. T. Paulsen, J. A. Eisen, P. D. Karp, D. Bovee, Sr., P. Chapman, J. Clendenning, G. Deatherage, W. Gillet, C. Grant, T. Kutyavin, R. Levy, M. J. Li, E. McClelland, A. Palmieri, C. Raymond, G. Rouse, C. Saenphimmachak, Z. Wu, P. Romero, D. Gordon, S. Zhang, H. Yoo, Y. Tao, P. Biddle,**

M. Jung, W. Krespan, M. Perry, B. Gordon-Kamm, L. Liao, S. Kim, C. Hendrick, Z. Y. Zhao, M. Dolan, F. Chumley, S. V. Tingey, J. F. Tomb, M. P. Gordon, M. V. Olson, and E. W. Nester. 2001. The genome of the natural genetic engineer *Agrobacterium tumefaciens* C58. Science **294:**2317–2323.

55. **Young, J. P., L. C. Crossman, A. W. Johnston, N. R. Thomson, Z. F. Ghazoui, K. H. Hull, M. Wexler, A. R. Curson, J. D. Todd, P. S. Poole, T. H. Mauchline, A. K. East, M. A. Quail, C. Churcher, C. Arrowsmith, I. Cherevach, T. Chillingworth, K. Clarke, A. Cronin, P. Davis, A. Fraser, Z. Hance, H. Hauser, K. Jagels, S. Moule, K. Mungall, H. Norbertczak, E. Rabbinowitsch, M. Sanders, M. Simmonds, S. Whitehead, and J. Parkhill.** 2006. The genome of *Rhizobium leguminosarum* has recognizable core and accessory components. Genome Biol. **7:**R34.

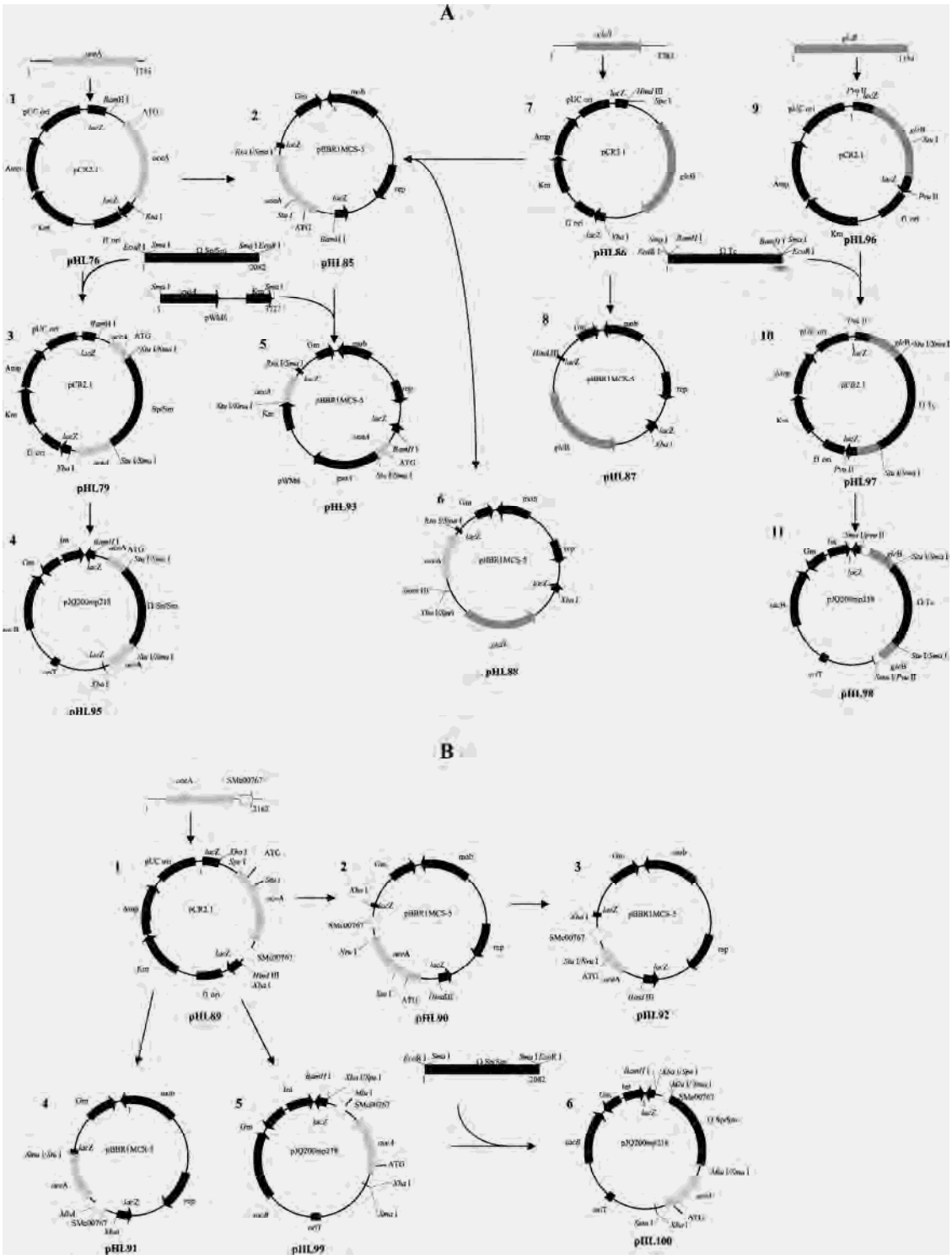

Figura S1. Material suplementario del articulo en donde se esquematiza la construcción de los plásmidos para la mutagénesis, complementación y las fusiones transcripcionales.

RESULTADOS ADICIONALES

Papel de los genes del ciclo del glioxilato en ayuno de carbono.

Una de las características de *S. meliloti* es la habilidad de acumular poli-β-hidroxibutirato en vida libre en condiciones de limitación de nutrientes. El PHB es convertido en acetoacetato, posteriormente es transformado en acetil-CoA, la cual puede ser utilizada mediante el ciclo de Krebs o por medio del ciclo del glioxilato. Para determinar si el ciclo del glioxilato está involucrado en el metabolismo del acetil-CoA producto de la degradación del PHB se realizaron experimentos de crecimiento en MM sin fuente de carbono. Las cepas silvestre (Rm5000), la mutante en la isocitrato liasa *aceA* (RH190), la mutante en malato sintasa *glcB* (RH218), la doble mutante *aceA-glcB* (RH222), la mutante en SMc00767 (RH421) y la doble mutante *glcB*-SMc00767 (RH419) fueron cultivadas bajo condiciones de acumulación de PHB en medio YM (manitol y extracto de levadura); posteriormente, los cultivos de estas cepas fueron transferidos a medio mínimo M9 sin fuente de carbono. La densidad óptica medida a lo largo de la curva de crecimiento indica que la cepa silvestre RM5000, así como las mutantes sencillas en SMc00767 (RH421) y *glcB* (RH218) poseen la habilidad de crecer en MM sin fuente de carbono (Figuras 7, 8 y 10); sin embargo, la mutante en *aceA* (RH190) y las dobles mutantes *aceA-glcB* (RH222) y *glcB*-SMc00767 (RH419) fueron incapaces de crecer en medio mínimo sin fuente de carbono (Figuras 7, 9 y 11).

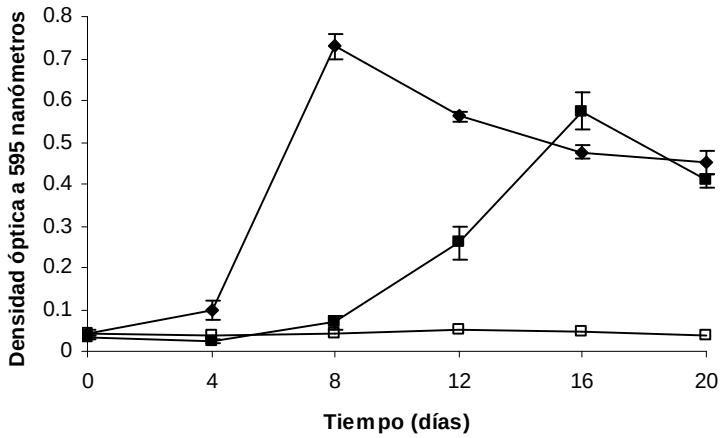

Figura 7. Curva de crecimiento en medio M9 sin fuente de carbono. *S. meliloti* Rm5000 —◆—; *S. meliloti* mutante *aceA* —□—; *S. meliloti* mutante *aceA* complementada con *aceA* —■—.

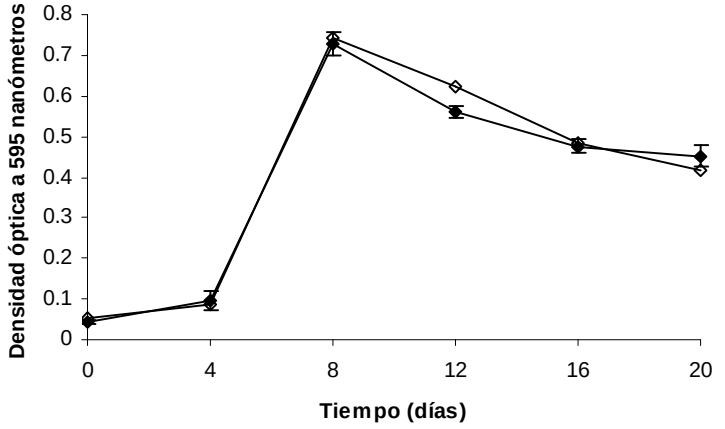

Figura 8. Curva de crecimiento en medio M9 sin fuente de carbono. *S. meliloti* Rm5000 —◆—; *S. meliloti* mutante *glcB* —◇—.

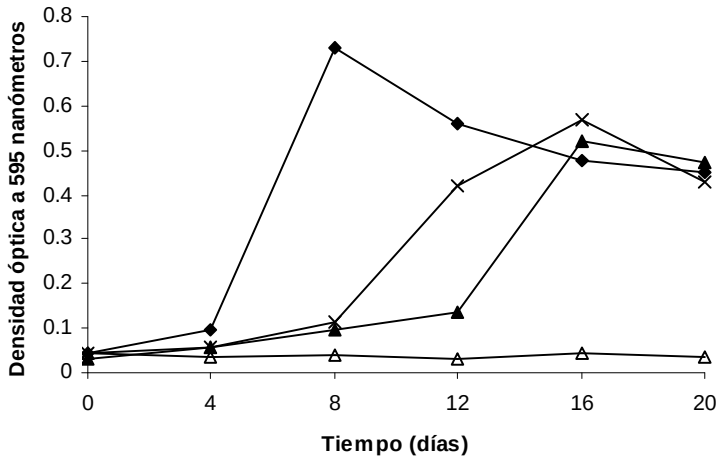

Figura 9. Curva de crecimiento en medio M9 sin fuente de carbono. *S. meliloti* Rm5000 —◆—; *S. meliloti* doble mutante *aceA-glcB* —△—; *S. meliloti* doble mutante *aceA-glcB* complementada con *aceA-glcB* —▲—; *S. meliloti* doble mutante *aceA-glcB* complementada con *aceA*-SMc00767—×—.

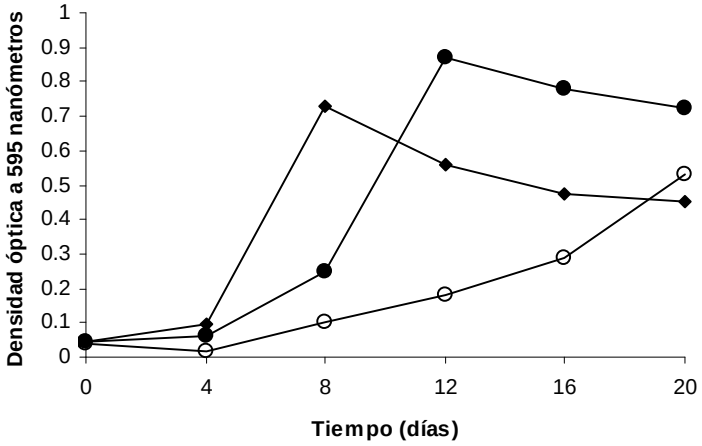

Figura 10. Curva de crecimiento en medio M9 sin fuente de carbono. *S. meliloti* Rm5000 —◆—; *S. meliloti* mutante SMc00767 —○—; *S. meliloti* mutante SMc00767 complementada con SMc00767 con el promotor de *aceA* —●—.

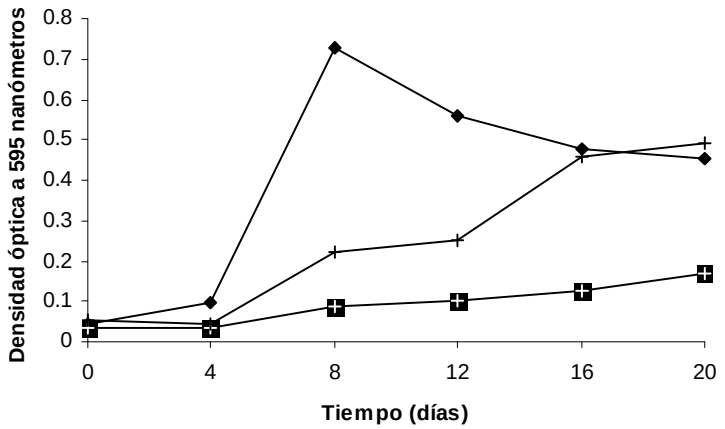

Figura 11. Curva de crecimiento en medio M9 sin fuente de carbono. *S. meliloti* Rm5000 —◆—; *S. meliloti* doble mutante *glcB*-SMc00767 —✚—; *S. meliloti* doble mutante *glcB*-SMc00767 complementada con SMc00767 con el promotor de *aceA* —+— .

Los resultados anteriores indican que bajo condiciones de movilización o degradación de PHB, *aceA*, SMc00767 y *glcB* se necesitan para asimilar el acetil-CoA liberado. Se realizaron experimentos de complementación, los cuales revelan que el crecimiento de la doble mutante *aceA-glcB* (RH222) se reestablece con los genes *aceA-glcB* (RH312) y *aceA*-SMc00767 (RH327) (figura 9). Por otra parte, la mutante SMc00767 (RH421) y la doble mutante *glcB*-SMc00767 (RH419) fueron complementadas con el gen SMc00767 generando las cepas RH442 y RH43, respectivamente. En todos los casos el fenotipo de crecimiento en MM mínimo se reestablece (Figuras 10 y 11). Estos datos muestran que los genes *aceA, glcB* y SMc00767 son necesarios para el crecimiento en PHB. Para determinar si realmente se acumula PHB y los genes *aceA* y *glcB* están involucrados en la asimilación del producto de degradación (el acetil-CoA) de este polímero, se realizaron experimentos de cuantificación y degradación de PHB a lo largo de una curva de crecimiento en MM sin fuente de carbono. Los resultados muestran que la mutante en *aceA,* así como la doble mutante *aceA*-SMc00767 y *aceA-glcB* no degradan completamente el PHB.

Las cepas anteriormente mencionadas al ser complementadas con los respectivos genes silvestres recuperan el fenotipo de crecimiento utilizando el PHB de reserva, indicando que los genes del

ciclo del ácido glioxílico *aceA*, *glcB* y SMc00776 están involucrados en la utilización del acetil-CoA producto de degradación de este polímero de reserva (Figuras 12 y 13). A continuación se muestran los experimentos de degradación de PHB. Las cepas fueron crecidas en medio YM (manitol y extracto de levadura) para la acumulación de PHB y posteriormente fueron subcultivadas en medio mínimo M9 sin fuente de carbono.

Experimentos de degradación de PHB en *Sinorhizobium meliloti*.

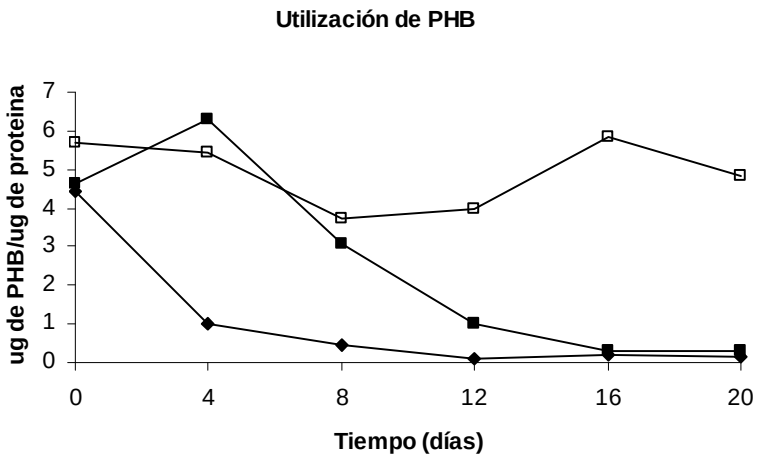

Figura 12.- Utilización de PHB. *S. meliloti* Rm5000 —◆—; *S. meliloti* mutante *aceA*—□—; *S. meliloti* mutante *aceA* complementada con *aceA* —■—.

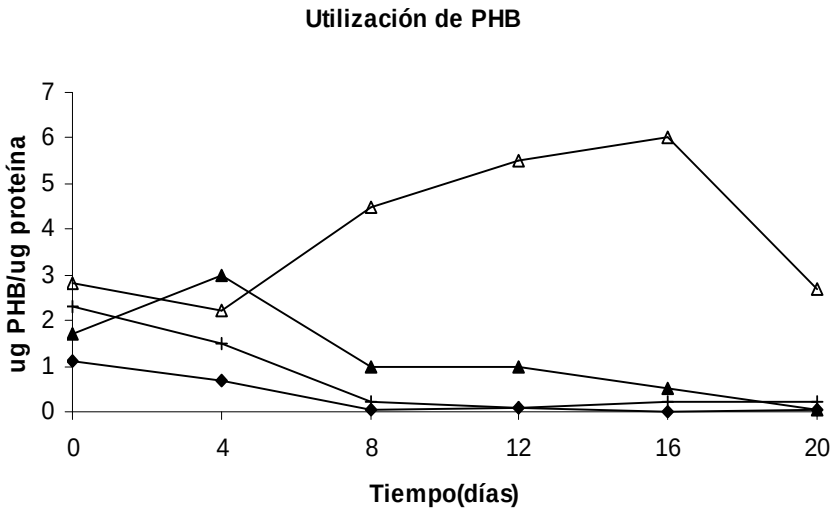

Figura 13.- Utilización de PHB. *S. meliloti* Rm5000 —◆—; *S. meliloti* doble mutante *aceA-glcB* —△—; *S. meliloti* doble mutante *aceA-glcB* complementada con *aceA-glcB* —▲—; *S. meliloti* doble mutante *aceA-glcB* complementada con *aceA*-SMc00767—×—.

DISCUSIÓN

El metabolismo del carbono juega un papel determinante en la vida libre y simbiótica de bacterias del género *Rhizobium*, estudios sobre rutas metabólicas centrales tales como la glicólisis, y el ciclo de Krebs han sido realizados, sin embargo, el conocimiento actual sobre el papel de las rutas anapleróticas del ciclo de Krebs es muy limitado. En este trabajo se estudió el ciclo del glioxilato en *Rhizobium*. Reportes previos han demostrado la funcionalidad en vida libre como en simbiosis de las principales enzimas del ciclo del glioxilato, isocitrato liasa y malato sintasa. Sin embargo, no se habían realizado estudios sistemáticos con mutantes en cada uno de los genes antes mencionados. Los resultados de este trabajo demuestran que *S. meliloti*, así como en la mayoría de las bacterias el gen que codifica para la isocitrato liasa, es necesario para el metabolismo del acetato. En esta investigación nosotros también demostramos que *aceA* es relevante para usar como fuente de carbono el PHB.

Una aportación relevante de este trabajo es la caracterización del gen SMc00767, el cual está presente en simbiontes, patógenos de plantas y animales. En todos los organismos analizados, el ORF SMc00767 se encuentra corriente abajo del gen *aceA*, formando un operón. En S. *meliloti* SMc00767 es relevante durante el crecimiento en acetato o en ayuno de carbono (utilización de PHB). Adicionalmente, SMc00767 regula negativamente la expresión de *aceA*. Datos de actividad enzimática así como fusiones transcripcionales y microarreglos validan el papel de SMc00767 como un regulador negativo en *S. meliloti*. Este regulador además de regular a *aceA* también regula negativamente a los genes SMa2071 (proteína hipotética), SMb21456 (proteína hipotética), SMb21473 (proteína hipotética), SMc00561 (Probable DNA helicasa, *dnaB*), SMc00769 (proteína hipotética) y SMc00772 (probable sistema transportador de putrecina, *potH*). Se propone que estos genes dependientes de SMc00767 podrían tener algún papel en el crecimiento de *Rhizobium* en acetato. El tercer componente descrito en esta investigación corresponde al gene *glcB* el cual codifica para la enzima malato sintasa. Las mutaciones en este gen no afectan el crecimiento en acetato en *S. meliloti*. Sin embargo, mutaciones en el gen de la malato sintasa de *Aspergillus fumigatus* (Olivas *et al*., 2008), *E. coli* (Pellicer *et al*., 1999), *Pseudomonas aeruginosa* PAO1 (Roucourt *et al*., 2009), *Rhodococcus fascians* (Vereecke *et al*., 2002) y *Rhizobium leguminosarum* Bv. viciae (Karunakaran *et al*., 2009) son incapaces de crecer

en acetato como única fuente de carbono. Estos datos indican que en *S. meliloti* existe otra ruta metabólica por la cual el glioxilato generado por isocitrato liasa es transformado en algún precursor biosintético que permite el crecimiento en ausencia de malato sintasa.

En *S. meliloti* se encuentran dos posibles rutas adicionales a la de malato sintasa por las cuales el glioxilato podría incorporarse al metabolismo central, la ruta de la serina glioxilato aminotransferasa, codificada por el gen *sgaA* (SMa2139), la cual transforma a la serina y al glioxilato en glicina e hidroxipiruvato. Posteriormente, una hidroxipiruvato reductasa, codificada por *ttuD3* (SMa1406) o *ttuD1* (SMc04389), transformaría el hidroxipiruvato en glicerato, este compuesto es fosforilado por la enzima glicerato cinasa, codificada por *ttuD2* (SMb20678) para producir 3-fosfoglicerato el cual es utilizado directamente en glicólisis. La segunda ruta alterna podría ser la vía del glicerato, en esta vía el glioxilato es transformado por la glioxilato carboligasa, codificada por el gen *gcl* (SMb20681), a tartronato semialdehído, el cual a su vez es metabolizado a glicerato por la tartronato semialdehído reductasa, codificada por *glxR* (SMb20679), el glicerato es fosforilado por la enzima glicerato cinasa para producir el intermediario glucolítico 3-fosfoglicerato. De manera interesante, todos los genes de las vías metabólicas propuestas para la utilización del glioxilato se encuentran presentes en *S. meliloti*. En la figura 14 se esquematizan las rutas propuestas para la utilización del ácido glioxílico en *S. meliloti*.

Como se mencionó anteriormente cepas deficientes en malato sintasa de *R. leguminosarum* son incapaces de crecer en acetato, probablemente debido a que las rutas antes mencionadas se encuentran ausentes en *R. leguminosarum*, sugiriendo que en *S. meliloti* las vías propuestas podrían funcionar para la utilización del glioxilato en ausencia de malato sintasa.

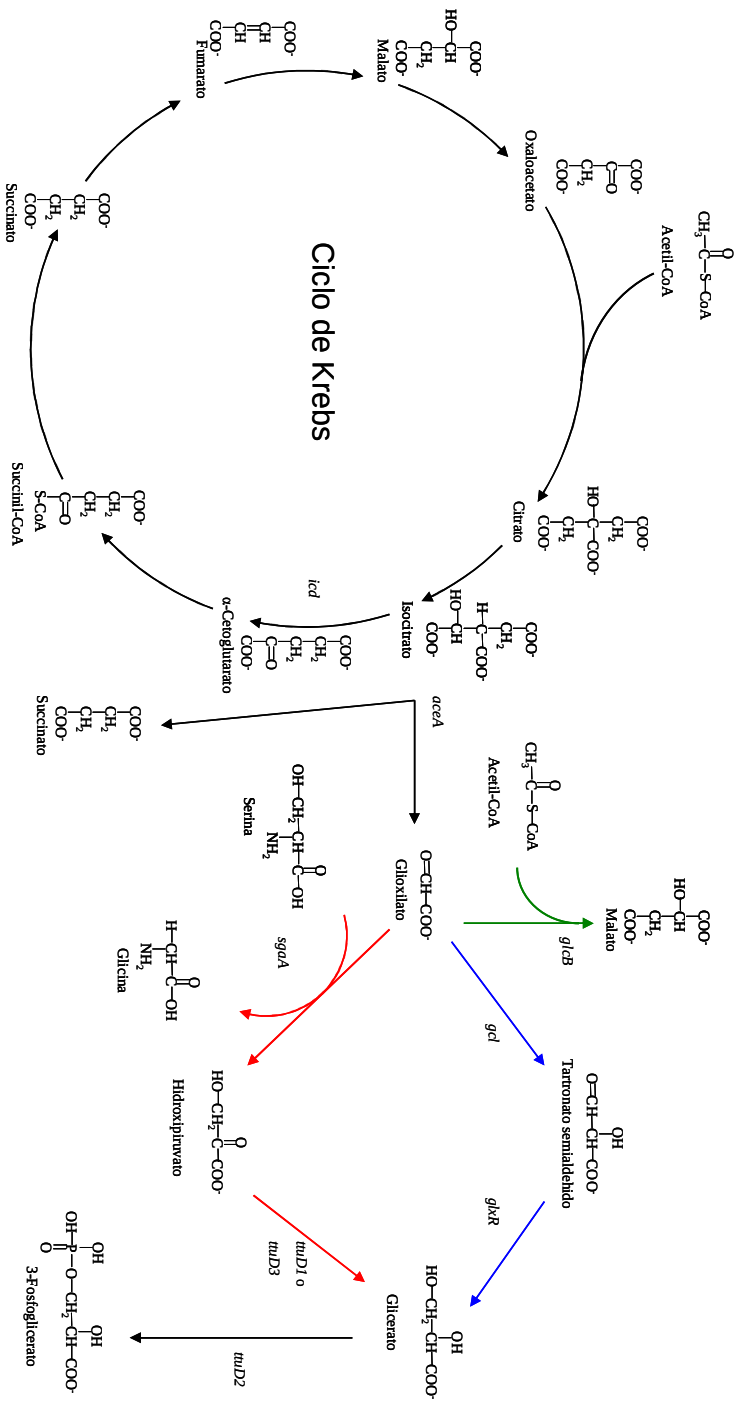

Figura 14. Rutas propuestas para la utilización del glioxilato, en cursiva se indican los genes involucrados, en colores se muestran las tres posible vías para la utilización del ácido glioxílico en *S. meliloti*; en verde, malato sintasa; en azul, vía del glicerato; y en rojo, vía de la serina glioxilato aminotransferasa.

Otra aportación relevante de esta investigación es la demostración de que los genes *aceA*, SMc00767 y *glcB* se requieren para la utilización de PHB en bacterias simbióticas. *Rhizobium* es habitante normal del suelo por lo tanto posee características metabólicas para sobrevivir en condiciones limitantes de carbono. En este sentido el acetil-CoA derivado del PHB podría ser utilizado por el ciclo del glioxilato en condiciones de ayuno de carbono para la sobrevivencia bacteriana en su ambiente natural. Por otra parte se ha observado que *S. meliloti* acumula PHB durante su paso por el hilo de infección, sin embargo, durante la diferenciación a bacteroide los gránulos de PHB desaparecen, implicando que el ciclo del glioxilato podría tener algún papel en esta etapa de la simbiosis. Adicionalmente, el ciclo del glioxilato podría estar operando con fuentes de carbono provistas por la raíz, ya que se conoce que el frijol secreta grandes cantidades de acetato. Finalmente, consideramos que el presente trabajo ha generado información relevante sobre una ruta anaplerótica del ciclo de Krebs y que sin duda este trabajo constituye un sólido marco de referencia para futuras investigaciones, las cuales nos ayudarán a entender de una forma global el metabolismo en bacterias del género *Rhizobium*

CONCLUSIONES

Los datos de este trabajo muestran que el gen *aceA* es necesario para *S. meliloti* durante el crecimiento en acetato y PHB. Contrario a *aceA*, el gen *glcB* no es necesario para *S. meliloti* durante el crecimiento en acetato. También se demostró que el gen *aceA* y el ORF SMc00767 de 240pb, forman una unidad transcripcional funcional y que el ORF SMc00767, esta involucrado en el metabolismo del acetato actuando como un regulador negativo de la transcripción de su propio operón, así como la de los ORF's SMa2071 (proteína hipotética), SMb21456 (proteína hipotética), SMb21473 (proteína hipotética conservada), SMc00561 (Probable proteína replicativa DNA helicasa, *dnaB*), SMc00769 (proteína hipotetica conservada) y SMc00772 (Probable permeasa, sistema transportador de putrescina, *potH*). Por otra parte también se observo que el ciclo del glioxilato no es necesario para *S. meliloti* durante la simbiosis con la leguminosa *Medicago sativa* ya que las mutantes en los genes *aceA* y *glcB* nodulan y fijan nitrógeno eficientemente.

PERSPECTIVAS

El presente trabajo de investigación revela información relevante sobre el metabolismo del carbono en bacterias del género *Rhizobium*; sin embargo, consideramos de importancia la identificación de la ruta metabólica mediante la cual se metaboliza el ácido glioxílico en ausencia de la enzima malato sintasa, este objetivo se puede cumplir mediante la mutación de los genes de la ruta del glicerato y de la serina glioxilato aminotransferasa, los resultados esperados serían que cepas deficientes en estos genes fueran incapaces de crecer en acetato como única fuente de carbono. Otra alternativa que podría indicarnos cuales son los genes relevantes para el crecimiento en acetato es la mutagénesis al azar en una cepa deficiente en malato sintasa. Adicionalmente, sería interesante realizar el metaboloma de *S. meliloti* en presencia de acetato, esto nos indicaría cuales son los compuestos sintetizados en esta condición de crecimiento y nos daría una idea de cuales son las enzimas involucradas. Consideramos relevante establecer si los genes reprimidos por SMc00767 (SMa2071, SMb21456, SMb21473, SMc00561, SMc00769 y SMc00772) identificados en los estudios transcripcionales pudieran tener algún papel en el crecimiento en acetato.

Como una segunda perspectiva de investigación sería interesante determinar el mecanismo de regulación que controla la expresión del operón *aceA*-SMc00767. Consideramos relevante determinar si SMc00767 interactúa de manera directa con la región promotora de *aceA*. Si la regulación fuera directa se podrían mutagenizar las zonas de pegado y se determinaría el sitio de interacción de SMc00767. Sería interesante determinar si las regiones reguladoras de los genes *aceA*, SMa2071, SMb21456, SMb21473, SMc00561, SMc00769 y SMc00772, los cuales son reprimidos por SMc00767, poseen algún consenso o algún sitio de reconocimiento para SMc00767. Consideramos también importante evaluar si realmente el ciclo del glioxilato opera de manera similar en patógenos de humanos o patógenos de plantas. Nuestros datos indican que esto podría ocurrir; sin embargo, esta hipótesis requiere ser validada en estudios posteriores.

REFERENCIAS

1. **Acuna, G., Ebeling, S. & Hennecke, H. (1991).** Cloning, sequencing, and mutational analysis of the *Bradyrhizobium japonicum fumC*-like gene: evidence for the existence of two different fumarases. *J Gen Microbiol* **137:**991-1000.
2. **Alfano, J. R. & Kahn, M. L. (1993).** Isolation and characterization of a gene coding for a novel aspartate aminotransferase from *Rhizobium meliloti*. *J Bacteriol* **175:**4186-4196.
3. **Allaway, D., Lodwig, E. M., Crompton, L. A., Wood, M., Parsons, R., Wheeler, T. R. & Poole P. S. (2000).** Identification of alanine dehydrogenase and its role in mixed secretion of ammonium and alanine by pea bacteroids. *Mol Microbiol* **36:**508-515.
4. **Aneja, P. and Charles, T. C. (1999).** Poly-3-hydroxybutyrate degradation in *Rhizobium* (*Sinorhizobium*) *meliloti*: Isolation and characterization of a gene encoding 3-hydroxybutyrate dehydrogenase. *J Bacteriol* **181:**849-857.
5. **Aneja, P., Dai, M., Lacorre, D. A., Pillon, B. & Charles, T. C. (2004).** Heterologous complementation of the exopolysaccharide synthesis and carbon utilization phenotypes of *Sinorhizobium meliloti* Rm1021 polyhydroxyalkanoate synthesis mutants. *FEMS Microbiol Lett* **239:**277–283.
6. **Arwas, R., Glenn, A. R., McKay, I. A. & Dilworth, M. J. (1986).** Properties of double mutants of *Rhizobium leguminosarum* which are defective in the utilization of dicarboxylic acids and sugars. *J Gen Microbiol* **132:**2743-2747.
7. **Arwas, R., McKay, I. A., Rowney, F. R. P., Dilworth, M. J. & Glenn, A. R. (1985).** Properties of organic acid utilization mutants of *Rhizobium leguminosarum* strain 300. *J Gen Microbiol* **131:**2059-2066.
8. **Asakura, M., Okuno, T. & Takano, Y. (2006).** Multiple contributions of peroxisomal metabolic function to fungal pathogenicity in *Colletotrichum lagenarium*. *Appl Environ Microbiol* **72**, 6345-6354.
9. **Batista, S., Catalán, A. I., Hernández-Lucas, I., Martínez-Romero, E., Aguilar, O. M. & Martínez-Drets, G. (2001).** Identification of a system that

allows a *Rhizobium tropici dctA* mutant to grow on succinate, but not on other C4-dicarboxylates. *Can J Microbiol* **47:**509–518.

10. **Bergersen, F. J. & Turner, G. L. (1967).** Nitrogen fixation by the bacteroid fraction of breis of soybean root nodules. *Biochim Biophys Acta* **141:**507-515.
11. **Bergersen, F. J. & Turner, G. L. (1990).** Bacteroids from soybean root nodules: accumulation of poly-3-hydroxybutyrate during supply of malate and succinate in relation to N2 fixation in flowchamber reactions. *Proc R Soc Lond B* **240:**39–59.
12. **Bergersen, F. J. & Turner, G. L. (1991).** A role for poly-3-hydroxybutyrate in bacteroids of soybean root nodules. *Proc R Soc Lond B* **245:**59–64.
13. **Brewin, J. N. (1991).** Development of legume root nodule. *Annu Rev Cell Biology* **7:**191-226.
14. **Cabanes, D., Boistard, P. & Batut, J. (2000 a).** Identification of *Sinorhizobium meliloti* genes regulated during symbiosis. *J Bacteriol* **182:**3632-3637.
15. **Cabanes, D., Boistard, P. & Batut, J. (2000 b).** Symbiotic induction of pyruvate dehydrogenase genes from *Sinorhizobium meliloti*. *Mol Plant Microbe Interact* **13:**483-493.
16. **Cai G, Driscoll BT, Charles TC (2000).** Requirement for the enzymes acetoacetyl coenzyme A synthetase and poly-3-hydroxybutyrate (PHB) synthase for growth of *Sinorhizobium meliloti* on PHB cycle intermediates. *J Bacteriol* **182:**2113-2118
17. **Carlson, T. A., Martin, G. B. & Chelm, B. K. (1987).** Differential transcription of the two glutamine synthetase genes of *Bradyrhizobium japonicum*. *J Bacteriol* **169:**5861 – 5866.
18. **Cervenansky, C. & Arias, A. (1984).** Glucose-6-phosphate dehydrogenase deficiency in pleiotropic carbohydrate negative mutant strains of *Rhizobium meliloti*. *J Bacteriol* **160:**1027-1030.
19. **Cevallos, M., Encarnacion, S., Leija, A., Mora, Y. & Mora, J. (1996).** Genetic and physiological characterization of a *Rhizobium etli* mutant strain unable to synthesize poly-beta-hydroxybutyrate. *J Bacteriol* **178:**1646–1654.

20. **Charles, T. C., Cai, G. & Aneja, P. (1997)** Megaplasmid and chromosomal loci for the PHB degradation pathway on *Rhizobium (Sinorhizobium) meliloti*. Genetics **146:**1211–1220.
21. **Copeland, L., Quinnell, R. G. & Day, D. A. (1989).** Malic enzyme activity in bacteroids from soybean nodules. *J Gen Microbiol* **135:**2005-2011.
22. **Copeland, L., Vella, J. & Hong, Z. Q. (1989).** Enzymes of carbohydrate metabolism in soybean nodules. *Phytochemistry* **28:**57-61.
23. **Cornah, J. E., Germain, V., Ward, J. L., Beale, M. H. & Smith, S. M. (2004).** Lipid utilization, gluconeogenesis, and seedling growth in *Arabidopsis* mutants lacking the glyoxylate cycle enzyme malate synthase. *J Biol Chem* **279:**42916-42923.
24. **Cozzone, A. J. & El-Mansi, M. (2005).** Control of isocitrate dehydrogenase catalytic activity by protein phosphorylation in *Escherichia coli*.
25. **Cozzone, A. J. (1998).** Regulation of acetate metabolism by protein phosphorylation in enteric bacteria. *Annu Rev Microbiol* **52,** 127-64.
26. **Davis, W. L., Jones, R. G. & Goodman, D. B. (1986).** Cytochemical localization of malate synthase in amphibian fat body adipocytes: possible glyoxylate cycle in a vertebrate. *J Histochem Cytochem* **34**:689-692.
27. **Davis, W. L., Jones, R. G., Farmer, G. R., Dickerson, T., Cortinas, E., Cooper, O. J., Crawford, L. & Goodman, D. B. (1990).** Identification of glyoxylate cycle enzymes in chick liver--the effect of vitamin D3: cytochemistry and biochemistry. *Anat Rec* **227**:271-284.
28. **de Bruijn, F. J., Rossbach, S., Schneider, M., Ratet, P., Messmer, S., Szeto, W. W., Ausubel, F. M. & Schell. J. (1989).** *Rhizobium meliloti* 1021 has three differentially regulated loci involved in glutamine biosynthesis, none of which is essential for symbiotic nitrogen fixation. *J Bacteriol* **171:**1673-1682.
29. **Dénarié, J., Debellé, F. & Promé, J. C. (1996).** *Rhizobium* lipo-chitooligosaccharide nodulation factors: signaling molecules mediating recognition and morphogenesis. *Annu Rev Biochem* **65:**503-535.
30. **Dilworth, M. & Glenn, A. (1984).** How does a legume nodule work?. *Trends Biol Sci* **9:**519–523.

31. **Driscoll, B. T. & Finan, T. M. (1993).** NAD+-dependent malic enzyme of *Rhizobium meliloti* is required for symbiotic nitrogen fixation. *Mol Microbiol* **7:**865-873.
32. **Driscoll, B. T. & Finan, T. M. (1996).** NADP+-dependent malic enzyme of *Rhizobium meliloti*. *J Bacteriol* **178**:2224-2231.
33. **Driscoll, B. T. & Finan, T. M. (1997).** Properties of NAD+- and NADP+-dependent malic enzymes of *Rhizobium* (*Sinorhizobium*) *meliloti* and differential expression of their genes in nitrogen fixing bacteroids. *Microbiology* **143:**489-498.
34. **Duncan, M. J. & Fraenkel, D. G. (1979).** α-Ketoglutarate dehydogenase mutant of *Rhizobium meliloti*. *J Bacteriol* **37:**415-419.
35. **Dunn, M. F. (1998).** Tricarboxylic acid cycle and anaplerotic enzymes in rhizobia. *FEMS Microbiol Rev* **22:**105-23.
36. **Dunn, M. F., Araiza, G., Encarnación, S., del Carmen-Vargas, M. & Mora, J. (2002).** Effect of *aniA* (carbon flux regulator) and *phaC* (poly-3-hydroxybutyrate synthase) mutations on pyruvate metabolism in *Rhizobium etli*. *J Bacteriol* **184:**2296–2299.
37. **Dymov, S. I., Meek, D. J., Steven, B. & Driscoll, B. T. (2005).** Insertion of transposon Tn5tac1 in the *Sinorhizobium meliloti* malate dehydrogenase (*mdh*) gene results in conditional polar effects on downstream TCA cycle genes. *Mol Plant Microbe Interact*. **17:**1318 – 1327.
38. **Encarnación, S., del Carmen-Vargas, M., Duna, M. F., Davalos, A., Mendoza, G., Mora, Y. & Mora, J. (2002).** AniA regulates reserve polymer accumulation and global protein expression in *Rhizobium etli*. *J Bacteriol* **184:**2287–2295.
39. **Engelke, T., Jagadish, M. N. & Pühler, A. (1987).** Biochemical and genetical analysis of *Rhizobium meliloti* mutants defective in C4-dicarboxylate transport. *J Gen Microbiol* **133:**3019-30**Engelke, T., Jording, D., Kapp, D. & Pühler, A. (1989).** Identification and sequence analysis of the *Rhizobium meliloti dctA* gene encoding the C4- dicarboxylate carrier. *J Bacteriol* **171:**5551-5560.

40. **Finan, T. M., Wood, J. M. & Jordan, C. (1981).** Succinate transport in *Rhizobium leguminosarum*. *J bacteriol* **148:**193-202.
41. **Finan, T. M., Wood, J. M. & Jordan, D. C. (1983).** Symbiotic properties of C4-dicarboxylic acid transport mutants of *Rhizobium leguminosarum*. *J Bacteriol* **154:**1403-1413.
42. **Forsyth, W. G. C., Hayward, A. C. & Roberts, I. B. (1958).** The occurence of poly-β-hydroxybutyrate in Gram-negative bacteria. *Nature* **182**, 800-801.
43. **Fougere, F., Le Rudulier, D. & Streeter, J, G. (1991).** Effects of salt stress on amino acid, organic acid, and carbohydrate composition of roots, bacteroids, and cytosol of alfalfa (*Medicago sativa* L.). *Plant Physiol* **96:**1228 – 1236.
44. **Galibert, F., Finan, T. M., Long, S. R., Pühler, A., Abola, P., Ampe, F., Balroy-Hubler, F., Barnett, M. J., Becker, A., Boistard, P., Bothe, G., Boutry, M., Bowser, L., Buhrmester, J., Cadieu, E., Capela, D., Chain, P., Cowie, A., Davis, R. W., Dréano, S., Federspiel, N. A., Fisher, R. F., Gloux, S., Godrie, T., Goffeau, A., Golding, B., Gouzy, J., Gurjal, M., Hernandez-Lucas, I., Hong, A., Huizar, L., Hyman, R. W., Jones, T., Kahn, D., Kahn, M. L., Kalman, S., Keating, D. H., Kiss, E., Komp, C., Lelaure, V., Masuy, D., Palm, C., Peck, M. C., Pohl, T. M., Portetelle, D., Purnelle, B., Ramsperger, U., Surzycki, R., Thébault, P., Vandenbol, M., Vorhölter, F.-J., Weidner, S., Wells, D. H., Wong, K., Yeh, K. C. & Batut, J. (2001).** The composite genome of the legume symbiont *Sinorhizobium meliloti*. *Science* **29:**668 – 672.
45. **García-de los Santos, A., Morales, A., Baldoma, L., Clark, S. R., Brom, S., Yost, C. K. Hernández-Lucas, I., Aguilar, J. & Hynes M. F. (2002).** The *glcB* locus of *Rhizobium leguminosarum* VF39 encodes an arabinose-inducible malate synthase. *Can. J. Microbiol* **48:**922–932.
46. **Gardiol, A. E., Truchet, G. L. & Dazzo, F. B. (1987).** Requirement of succinate dehydrogenase activity for symbiotic bacteroid differentiation of *Rhizobium meliloti* in alfalfa nodules. *Appl Environ Microbiol* **53:**1947-1950.
47. **Gardiol, A., Arias, A., Cervenansky, C. & Martinezdrets, G. (1982).** Succinate dehydrogenase mutant of *Rhizobium meliloti*. *Journal of Bacteriology* **151:**1621-1623.

48. **Gerson, T., Patel, J. J. & Wong, M. N. (1978).** The effects of age, darkness, and nitrate on poly-2-hydroxybutyrate levels and nitrogenfixing ability of *Rhizobium* in *Lupinus angustifolios*. *Physiol Plant* **42:**420–424.
49. **Glenn, A. R. & Brewin, N. J. (1981).** Succinate-resistant mutants of *Rhizobium leguminosarum*. *J Gen Microbiol* **126:**237-241.
50. **Glenn, A. R. & Dilworth, M. J. (1981).** The uptake and hydrolysis of disaccharides by fast-and slow-growing species of *Rhizobium*. *Arch Microbiol* **129:**238 – 239.
51. **Glenn, A. R., Arwas, R., McKay, I. A. & Dilworth, M. J. (1984).** Fructose metabolism in wild type, fructokinase negative and revertant strains of *Rhizobium leguminosarum*. *J Gen Microbiol* **130:**231-237.
52. **Glenn, A. R., McKay, I. A., Arwas, R. & Dilworth, M. J. (1984).** Sugar metabolism and the symbiotic properties of carbohydrate mutants of *Rhizobium leguminosarum*. *J Gen Microbiol* **130:**239-245.
53. **Glenn, A. R., Poole, P. S. & Hudman, J. F. (1980).** Succinate uptake by freeliving and bacteroid forms of *Rhizobium leguminosarum*. *J Gen Microbiol* **119:**267-271.
54. **Gonzalez, V., Santamaria, R. I., Bustos, P., Hernandez-Gonzalez, I., Medrano-Soto, A., Moreno-Hagelsieb, G., Janga, S. C., Ramirez, M. A., Jimenez-Jacinto, V., Collado-Vides, J. & Davila, G. (2006).** The partitioned *Rhizobium etli* genome: genetic and metabolic redundancy in seven interacting replicons. *Proc Natl Acad Sci USA* **103:**3834 – 3839.
55. **Green, L. S. & Emerich, D. W. (1997).** *Bradyrhizobium japonicum* does not require α-ketoglutarate dehydrogenase for growth on succinate or malate. *J Bacteriol* **179:**194-201.
56. **Green, L. S. & Emerich, D. W. (1997).** The formation of nitrogen fixing bacteroids is delayed but not abolished in soybean infected by an α-ketoglutarate dehydrogenase deficient mutant of *Bradyrhizobium japonicum*. *Plant Physiol* **114:**1359-1368.

57. **Green, L. S., Karr, D. B. & Emerich, D. W. (1998).** Isocitrate dehydrogenase and glyoxylate cycle enzyme activities in *Bradyrhizobium japonicum* under various growth conditions. *Arch Microbiol* **169:**445-451.
58. **Green, L. S., Li, Y. Z., Emerich, D. W., Bergersen, F. J. & Day, D. A. (2000).** Catabolism of α-ketoglutarate by a *sucA* mutant of *Bradyrhizobium japonicum*: Evidence for an alternative tricarboxylic acid cycle. *J Bacteriol* **182:**2838-2844.
59. **Grzemski, W., Akowski, J. P. & Kahn, M. L. (2005).** Probing the *Sinorhizobium meliloti*-alfalfa symbiosis using temperature-sensitive and impaired-function citrate synthase mutants. *Mol Plant Microbe Interact* **18:**134-141.
60. **Hahn, M. L., Meyer, D., Studer, B., Regensburger, B. & Hennecke, H. (1984).** Insertion and deletion mutations within the nif region of *Rhizobium japonicum*. *Plant Mol Biol* 3:159–168.
61. **Hernández-Lucas, I., Pardo, M. A., Segovia, L., Miranda, J. & Martínez-Romero, E. (1995).** *Rhizobium tropici* chromosomal citrate synthase gene. *Appl Environ Microbio* **61:**3992-3997.
62. **Hudman, J. F. & Glenn AR. (1980).** Glucose uptake by free living and bacteroid forms of *Rhizobium leguminosarum*. *Arch Microbiol* **128:**72–77.
63. **Huerta-Zepeda, A., Ortuno, L., Du Pont, G., Duran, S., Lloret, A., Merchant-Larios, H. & Calderon, J. (1997).** Isolation and characterization of *Rhizobium etli* mutants altered in degradation of asparagine. *J Bacteriol* **179:**2068-2072.
64. ICSP Subcommittee on the taxonomy of *Rhizobium* and *Agrobacterium* diversity, phylogeny and systematics. http://edzna.ccg.unam.mx/rhizobial-taxonomy/node/4.
65. **Idnurm, A. & Howlett, B. J. (2002).** Isocitrate lyase is essential for pathogenicity of the fungus *Leptosphaeria maculans* to canola (*Brassica napus*). *Eukaryot Cell* **1**, 719-724.
66. **Idnurm, A. & Howlett, B. J. (2002).** Isocitrate lyase is essential for pathogenicity of the fungus *Leptosphaeria maculans* to canola (*Brassica napus*). *Eukaryot Cell* **1:**719-724.

67. **Irigoyen, J. J., Sanchez-Diaz, M. & Emerich D. W. (1990).** Carbon metabolism enzymes of *Rhizobium meliloti* cultures and bacteroids and their distribution within alfalfa nodules. Applied and Environmental Microbiology **56:**2587-2589.
68. **Jackson, E. K. & Evans, H. J. (1966).** Propionate in heme biosynthesis in soybean nodules. *Plant Physiol* **41:**1673-1680.
69. **Jonson. G. V., Evans. H. J., and Ching T. (1966).** Enzimes of the glyoxylate cycle in rhizobia and nodules of legumes. *Plant Physiol* **41:**1330-1336.
70. **Kaneko T, Nakamura Y, Sato S, Minamisawa K, Uchiumi T, Sasamoto S, Watanabe A, Idesawa K, Iriguchi M, Kawashima K, Kohara M, Matsumoto M, Shimpo S, Tsuruoka H, Wada T, Yamada M, & Tabata S. (2002).** Complete genomic sequence of nitrogenfixing symbiotic bacterium *Bradyrhizobium japonicum* USDA110. DNA Res. **9:**189–197.
71. **Kaneko, T., Nakamura, Y., Sato, S., Asamizu, E., Kato, T., Sasamoto, S., Watanabe, A., Idesawa, K., Ishikawa, A., Kawashima, K., Kimura, T., Kishida, Y., Kiyokawa, C., Kohara, M., Matsumoto, M., Matsuno, A., Mochizuki, Y., Nakayama, S., Nakazaki, N., Shimpo, S., Sugimoto, M., Takeuchi, C., Yamada, M. & Tabata, S. (2000).** Complete genome structure of the nitrogen fixing symbiotic bacterium *Mesorhizobium loti*. *DNA Research* **7:**331–338.
72. **Karr, D. B., Waters, J. K., Suzuki, F. & Emerich, D. W. (1984).** Enzymes of the poly-β-hydroxybutyrate and citric acid cycles of *Rhizobium japonicum* bacteroids**.** *Plant Physiol* **75:**158-1162.
73. **Karunakaran, R., Ramachandran, V. K., Seaman, J. C., East, A. K., Mouhsine, B., Mauchline, T. H., Prell, J., Skeffington, A. & Poole, P. S. (2009).** Transcriptomic analysis of *Rhizobium leguminosarum* biovar viciae in symbiosis with host plants *Pisum sativum* and *Vicia cracca*. *J Bacteriol* **191:**4002-4014.
74. **Klucas, R. V. & Evans, H. J. (1968).** An electron donor system for nitrogenase-dependent acetylene reduction by extracts of soybean nodules. *Plant Physiol* **43:**1458–1460.

75. **Kondrashov, F. A., Koonin, E. V., Morgunov, I. G., Finogenova, T. V. & Kondrashova, M. N. (2006).** Evolution of glyoxylate cycle enzymes in Metazoa: evidence of multiple horizontal transfer events and pseudogene formation. *Biol Direct* **1**:31.
76. **Kornberg, H. L. & Krebs, H. A. (1957).** Synthesis of cell constituents from C2-units by a modified tricarboxylic acid cycle. *Nature* **179**, 988-91.
77. **Kouchi, H. & Nakaji, K. (1985).** Utilization and metabolism of photoassimilated ^{13}C in soybean roots and nodules. *Soil Sci Plant Nutr* **31:**323-334.
78. **Kouchi, H. & Yoneyama, T. (1986).** Metabolism of [^{13}C]-labelled photosynthate in plant cytosol and bacteroids of root nodules of *Glycine max*. *Physiol Plant* **68:**238-244.
79. **Kouchi, H., Fukai, K. & Kihara, A. (1991).** Metabolism of glutamate and aspartate in bacteroids isolated from soybean root nodules. *J gen Microbiol* **137:**2901-2910.
80. **Kouchi, H., Fukai, K., Katagiri, H., Minamisawa, K. & Tajima, S. (1988).** Isolation and enzymological characterization of infected and uninfected protoplasts from root nodules of *Glycine max*. *Physiol Plant* **73:**327-334.
81. **Krishnan, H. B., Kim, W. S., Sun-Hyung, J., Kim, K. Y. & Jiang G. (2003).** Citrate synthase mutants of *Sinorhizobium fredii* USDA257 form ineffective nodules with aberrant ultrastructure. *Appl Environ Microbiol* **69:**3561-3568.
82. **Lewis, T. A., Gonzalez, R. & Botsford, J. L. (1990).** *Rhizobium meliloti* glutamate synthase: cloning and initial characterization of the glt locus. *J bacteriol* **172:**2413 – 2420.
83. **Lindsey, T. L., Hagins, J. M., Sokol, P. A. & Silo-Suh, L. A. (2008).** Virulence determinants from a cystic fibrosis isolate of *Pseudomonas aeruginosa* include isocitrate lyase. *Microbiology* **154**, 1616-1627.
84. **Lindsey, T. L., Hagins, J. M., Sokol, P. A. & Silo-Suh, L. A. (2008).** Virulence determinants from a cystic fibrosis isolate of *Pseudomonas aeruginosa* include isocitrate lyase. *Microbiology* **154:**1616-1627.

85. **Lodwig, E. M., Hosie, A. H., Bourdès, A., Findlay, K., Allaway, D., Karunakaran, R., Downie. J. A. & Poole, P. S. (2003).** Amino-acid cycling drives nitrogen fixation in the legume-*Rhizobium* symbiosis. *Nature* **422:**722-726.
86. **Long, S. R. (1996).** *Rhizobium* symbiosis: nod factors in perspective. *Plant Cell* **8:**1885-1898.
87. **Lorenz, M. C. & Fink, G. R. (2001).** The glyoxylate cycle is required for fungal virulence. *Nature* **412:**83-86.
88. **Mandal, N. C. & Chakrabartty, P. K. (1992).** Regulatio of enzymes of glyoxylate pathway in root-nodule bacteria. *J. Gen. Appl. Microbiol* **174:**4790-4797.
89. **McDermott, T. R. & Kahn, M. L. (1992).** Cloning and mutagenesis of the *Rhizobium meliloti* isocitrate dehydrogenase gene. *J bacteriol* **174:**4790-4797.
90. **McDermott, T. R., Griffith, S. M., Vance, C. P. & Graham P. H. (1989).** Carbon metabolism in *Bradyrhizobium japonicum* bacteroids. *FEMS Microbiol Rev* **63:**327–340.
91. **McKay, I. A., Dilworth, M. J. & Glenn, A. R. (1988).** C4-dicarboxylate metabolism in free-living and bacteroid forms of *Rhizobium leguminosarum* MNF3841. *J Gen Microbiol* **134:**1433-1440.
92. **McKay, I. A., Dilworth, M. J. & Glenn, A. R. (1989).** Carbon catabolism incontinuous cultures and bacteroids of *Rhizobium leguminosarum* MNF3841. *Arch Microbiol* **152:**606-610.
93. **McKinney, J. D., Höner zu Bentrup, K., Muñoz-Elías, E. J., Miczak, A., Chen, B., Chan, W. T., Swenson, D., Sacchettini, J. C., Jacobs, W. R. Jr. & Russell, D. G. (2000).** Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature* **406**, 735-738.
94. **Miller, R. W., McRae, D. G. & Joy, K. (1991).** Glutamate and gammaaminobutyrate metabolism in isolated *Rhizobium meliloti* bacteroids. *Mol Plant Microbe Interact* **4:**37-45.

95. **Morgunov, I. G., Kondrashova, M. N., Kamzolova, S. V., Sokolov, A. P., Fedotcheva, N. I.& Finogenova, T. V. (2005).** Evidence of the glyoxylate cycle in the liver of newborn rats. *Med Sci Monit* **11:**BR57-60.
96. **Mortimer, M. W., McDermott, T. R., York, G. M., Walker, G. C. & Kahn, M. L. (1999).** Citrate synthase mutants of Sinorhizobium meliloti are ineffective and have altered cell surface polysaccharides. *J Bacteriol* **181:**7608-7613.
97. **Mylona. P., Pawlowski, K. & Bisseling, T. (1995).** Symbiotic Nitrogen Fixation. *Plant Cell*. **7:**869-885.
98. **Olivas, I., Royuela, M., Romero, B., Monteiro, M. C., Mínguez, J. M., Laborda, F. & De Lucas J. R. (2008).** Ability to grow on lipids accounts for the fully virulent phenotype in neutropenic mice of *Aspergillus fumigatus* null mutants in the key glyoxylate cycle enzymes. *Fungal Genet Biol* **45:**45-60.
99. **Paau, A. S. & Cowles, J. R. (1978).** Development of bacteroids in alfalfa (*Medicago sativa*) nodules. *Plant Physiol* **62:**526-530.
100. **Pardo, M. A., Lagunez, J., Miranda, J. & Martínez, E. (1994).** Nodulating ability of *Rhizobium tropici* is conditioned by a plasmid encoded citrate synthase. *Mol Microbiol* **11:**315-321.
101. **Pellicer, M. T., Fernandez, C., Badía, J., Aguilar, J., Lin, E. C. & Baldom, L. (1999).** Cross-induction of *glc* and *ace* operons of *Escherichia coli* attributable to pathway intersection. Characterization of the *glc* promoter. *J Biol Chem* **274:**1745-1752.
102. **Perret, X., Staehelin, C. & Broughton, W. J. (2000).** Molecular basis of symbiotic promiscuity. *Microbiol Mol Biol Rev* **64:**180–201.
103. **Peterson, J. B. & LaRue, T. A. (1982).** Soluble aldehyde dehydrogenase and metabolism of aldehydes by soybean bacteroids. *J bacteriol* **130:**881:886.
104. **Poole, P., Reid, C., East, A.K., Allaway, D., Day, M., & Leonard, M. (1999)**. Regulation of the mdh-sucCDAB operon in *Rhizobium leguminosarum*. *FEMS Microbiol Lett* **176:**247-255.
105. **Povolo, S. & Casella, S. (2000).** A critical role of *aniA* in energy-carbon flux and symbiotic nitrogen fixation in *Sinorhizobium meliloti*. *Arch Microbiol* **174:**42–49.

106. **Rastogi, V. K. & Watson, R. J. (1991).** Aspartate aminotransferase activity is required for aspartate catabolism and symbiotic nitrogen fixation in *Rhizobium meliloti*. *J Bacteriol* **173:**2879-2887.
107. **Reibach, P. H. & Streeter J. G. (1984).** Evaluation of active versus passive uptake of metabolites by *Rhizobium japonicum* bacteroids. *J Bacteriol* **159:**47–52.
108. **Reibach, P. H. & Streeter, J. G. (1983).** Metabolism of ^{14}C-labeled photosynthate and distribution of enzymes of glucose metabolism in soybean nodules. *Plant Physiol* **72:**634-640.
109. **Ronson, C. W., Astwood, P. M. & Downie, J. A.** (1984). Molecular cloning and genetic organization of C4-dicarboxylate transport genes from *Rhizobium leguminosarum*. *J bacteriol* **160:**903-909.
110. **Ronson, C. W., Lyttleton, P. & Robertson, J. G. (1981).** C4-dicarboxylate transport mutants of *Rhizobium trifolii* form ineffective nodules on *Trifolium repens*. *Proc Natl Acad Sci U S A* **78:**4284-4288.
111. **Rosendahl, L., Vance, C. P. & Pedersen, W. B. (1990).** Products of dark CO2 fixation in pea root nodules support bacteroid metabolism. *Plant Physiol* **93:**12-19.
112. **Roucourt, B., Minnebo, N., Augustijns, P., Hertveldt, K., Volckaert, G. & Lavigne, R. (2009).** Biochemical characterization of malate synthase G of *P. aeruginosa*. *BMC Biochem* **10:**20.
113. **Salminen, S. O. & Streeter, J. G. (1992).** Labeling of carbon pools in *Bradyrhizobium japonicum* and *Rhizobium leguminosarum* bv. viciae bacteroids following incubation of intact nodules with $^{14}CO_2$. *Plant Physiol* **100:**597-604.
114. **Shah, R. & Emerich, D. W. (2006).** Isocitrate dehydrogenase of *Bradyrhizobium japonicum* is not required for symbiotic nitrogen fixation with soybean. *J Bacteriol* **188:**7600-7608.
115. **Smith, M. T., Preston, G. G. & Emerich, D. W. (1994).** Development of acetate and pyruvate metabolic enzyme activities in soybean nodules. *Symbiosis* **17:**33–42.

116. **Solomon, P. S., Lee, R. C., Wilson, T. J. & Oliver, R. P. (2004).** Pathogenicity of *Stagonospora nodorum* requires malate synthase. *Mol Microbiol* **53**, 1065-1073.

117. **Son, M. S., Matthews, W. J. Jr., Kang, Y., Nguyen, D. T. & Hoang, T. T. (2007).** In vivo evidence of *Pseudomonas aeruginosa* nutrient acquisition and pathogenesis in the lungs of cystic fibrosis patients. *Infect Immun* **75**, 5313-5324.

118. **Soto, M. J., Sanjuan, J., & Olivares, J. (2001).** The disruption of a gene encoding a putative arylesterase impairs pyruvate dehydrogenase activity and nitrogen fixation in *Sinorhizobium meliloti*. *Mol Plant Microbe Interact* **14:**811-815.

119. **Spaink, H. P. (2000).** Root nodulation and infection factors produced by rhizobial bacteria. *Annu Rev Microbiol* **54:**257-288.

120. **Streeter, J. G. (1987).** Carbohydrate, organic acid, and amino acid composition of bacteroids and cytosol from soybean nodules. *Plant Physiol* **85:**768-773.

121. **Tamir-Ariel, D., Navon, N. & Burdman, S. (2007).** Identification of genes in *Xanthomonas campestris* pv. vesicatoria induced during its interaction with tomato. *J Bacteriol* **189**, 6359-6371.

122. **Thöny-Meyer, L. & Kunzler, P. (1996).** The *Bradyrhizobium japonicum* aconitase gene (*acnA*) is important for free-living growth but not for an effective root nodule symbiosis. *J Bacteriol* **178:**6166-6172.

123. **Thummler, P. & Verma D. P. (1987).** Nodulin-100 of soybean is the subunit of sucrose synthase regulated by the availability of free heme in nodules. *J Biol Chem* **262:**14730-14736.

124. **Tomaszewska, B. & Werner, D. (1995).** Purification and properties of NADdependent and NADP-dependent malic enzymes from *Bradyrhizobium japonicum* bacteroids. *J Plant Physiol* **146:**591-595.

125. **Tombolini, R. & Nuti, M. P. (1989).** Poly(β-hydroyxalkanoate) biosynthesis and accumulation by different *Rhizobium* species. *FEMS Microbiol Lett* **60:**299-304.

126. **Trainer, M. A. & Charles, T. C. (2006).** The role of PHB metabolism in the symbiosis of rhizobia with legumes. *Appl Microbiol Biotechnol* **71:**377-386.

127. **Udvardi, M. K. & Day, D. A. (1997).** Metabolite transport across symbiotic membranes of legume nodules. *Annu Rev Plant Physiol Plant Mol Biol* **48:**493-523.

128. **Udvardi, M. K., & Day, D. A. (1989).** Electrogenic ATPase activity on the peribacteroid membrane of soybean (*Glycine max* L.) root nodules. *Plant Physiol* **90:**982-987.

129. **van Slooten, J. C., Bhuvanasvari, T. V., Bardin, S. & Stanley, J. (1992).** Two C4-dicarboxylate transport systems in *Rhizobium* sp. NGR234: rhizobial dicarboxylate transport is essential for nitrogen fixation in tropical legume symbioses. *Mol Plant Microbe Interact* **5:**179–186.

130. **Van Straten, J. & Schmidt, E. L. (1974)**. Volatile compounds produced during acetylene reduction by dechated soybean nodules. *Soil Biol Biochem* **6:**374-351.

131. **Vance, C. P., Boylan, K. L. M., Maxwell, C. A., Heichel, G. H. & Hardman, L. L**. **(1985).** Transport and partitioning of CO_2 fixed by root nodules of ureide and amide producing legumes. *Plant Physiol* **78:**774-778.

132. **Vereecke, D., Cornelis, K., Temmerman, W., Holsters, M. & Goethals, K. (2002a).** Versatile persistence pathways for pathogens of animals and plants. *Trends Microbiol* **10**, 485-488.

133. **Vereecke, D., Cornelis, K., Temmerman, W., Jaziri, M., Van Montagu, M., Holsters, M. & Goethals, K. (2002).** Chromosomal locus that affects pathogenicity of *Rhodococcus fascians*. *J Bacteriol* **184:**1112-1120.

134. **Wall, D. M., Duffy, P. S., Dupont, C., Prescott, J. F. & Meijer, W. G. (2005).** Isocitrate lyase activity is required for virulence of the intracellular pathogen *Rhodococcus equi*. *Infect Immun* **73**, 6736-6741.

135. **Walshaw, D. L., Wilkinson, A., Mundy, M., Smith, M. & Poole, P. S. (1997)**. Regulation of the TCA cycle and the general amino acid permease by overflow metabolism in *Rhizobium leguminosarum*. *Microbiology* **143:**2209-2221.

136. **Wang, Y. P., Birkenhead, K., Boesten, B., Manian, S. & O'Gara, F. (1989).** Genetic analysis and regulation of the *Rhizobium meliloti* genes controlling C4-dicarboxylic acid transport. *Gene* **85:**135–144.

137. **Wang, Z. X., Brämer, C. O. & Steinbüchel, A. (2003).** The glyoxylate bypass of *Ralstonia eutropha*. *FEMS Microbiol Lett* **228:**63-71.

138. **Wang, Z. Y., Thornton, C. R., Kershaw, M. J., Debao, L. & Talbot, N. J. (2003).** The glyoxylate cycle is required for temporal regulation of virulence by the plant pathogenic fungus *Magnaporthe grisea*. *Mol Microbiol* **47**, 1601-1612.

139. **Waters, J. K., Hughes II, B. L., Purcel, L. C., Gerhardt, K. O., Mawhinney, T. P. & Emerich, D. W. (1998).** Alanine not ammnonia is excreted from N_2-fixing soybean nodule bacteroids. *Proc Natl Acad Sci U.S.A* **95:**12038-12042.

140. **Watson, R. J. (1990).** Analysis of the C4-dicarboxylate transport genes of *Rhizobium meliloti*: nucleotide sequence and deduced products of *dctA, dctB* and *dctD*. *Mol Plant Microbe Interact* **3:**174-181.

141. **Watson, R. J., Chan, Y. K., Wheatcroft, R., Yang, A. F. & Han, S. (1988).** *Rhizobium meliloti* genes required for C4-dicarboxylate transport and symbiotic nitrogen fixation are located on a megaplasmid. *J Bacteriol* **170:**927-934.

142. **Weir, B. S. (2008).** The current taxonomy of rhizobia. New Zealand rhizobia website. http://www.rhizobia.co.nz/taxonomy/rhizobia.html.

143. **Wong, P. P. & Evans, H. J. (1971).** Poly-3-hydroxybutyrate utilization by soybean (*Glycine max Merr*.) nodules and assessment of its role in maintenance of nitrogenase activity. *Plant Physiol* **47:**750–755.

144. **Yarosh, O. K., Charles, T. C. & Finan, T. M. (1989).** Analysis of C4dicarboxylate transport genes in *Rhizobium meliloti*. *Mol Microbiol* **3:**813–823.

145. **Young, J. P., Crossman, L. C., Johnston, A. W., Thomson, N. R., Ghazoui Z. F., Hull, K. H., Wexler, M., Curson, A. R., Todd, J. D., Poole, P. S., Mauchline, T. H., East, A. K., Quail, M. A., Churcher, C., Arrowsmith, C., Cherevach, I., Chillingworth, T., Clarke, K., Cronin, A., Davis, P., Fraser, A., Hance, Z., Hauser, H., Jagels, K., Moule, S., Mungall, K., Norbertczak, H., Rabbinowitsch, E., Sanders, M., Simmonds, M., Whitehead, S., Parkhill, J. (2006).** The genome of *Rhizobium leguminosarum* has recognizable core and accessory components. *Genome Biol* **7:**R34.