

Functional Characterization of the *Sinorhizobium meliloti* Acetate Metabolism Genes *aceA*, SMc00767, and *glcB*^{∇†}

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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 knock-outs 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 N₂ 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 C₂ 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-

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TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant genotype or characteristics | Reference or source |
|----------------------------|---|---------------------|
| <i>S. meliloti</i> strains | | |
| Rm5000 | SU47 <i>rif-5</i> | 21 |
| RH190 | Rm5000 <i>aceA</i> :: Ω Sp/Sm | This study |
| RH198 | Rm5000 <i>aceA</i> :: Ω Sp/Sm, pBBR1MCS-5 <i>aceA</i> | This study |
| RH218 | Rm5000 <i>glcB</i> :: Ω Tc | This study |
| RH222 | Rm5000 <i>aceA</i> :: Ω Sp/Sm <i>glcB</i> :: Ω Tc | This study |
| RH302 | Rm5000 <i>glcB</i> :: Ω Tc, pBBR1MCS-5 <i>glcB</i> | This study |
| RH312 | Rm5000 <i>aceA</i> :: Ω Sp/Sm <i>glcB</i> :: Ω Tc, pBBR1MCS-5 <i>aceA-glcB</i> | This study |
| RH326 | Rm5000 <i>aceA</i> :: Ω Sp/Sm, pBBR1MCS-5 <i>aceA</i> -SMc00767 | This study |
| RH327 | Rm5000 <i>aceA</i> :: Ω Sp/Sm, <i>glcB</i> :: Ω Tc, pBBR1MCS-5, <i>aceA</i> -SMc00767 | This study |
| RH419 | Rm5000 <i>glcB</i> :: Ω Tc SMc00767:: Ω Sp/Sm | This study |
| RH421 | Rm5000 SMc00767:: Ω Sp/Sm | This study |
| RH429 | Rm5000 <i>glcB</i> :: Ω Tc SMc00767:: Ω Sp/Sm, pBBR1MCS-5 <i>glcB</i> | This study |
| RH435 | Rm5000 SMc00767:: Ω Sp/Sm, pBBR1MCS-5 SMc00767 | This study |
| RH436 | Rm5000 <i>glcB</i> :: Ω Tc, SMc00767:: Ω Sp/Sm, pBBR1MCS-5 SMc00767 | This study |
| RH442 | Rm5000 SMc00767:: Ω Sp/Sm, pBBR1MCS-5 <i>aceA</i> -SMc00767 with an internal 700-bp deletion in <i>aceA</i> that leaves wild-type SMc00767 and the intact <i>aceA</i> promoter region | This study |
| RH443 | Rm5000 <i>glcB</i> :: Ω Tc SMc00767:: Ω Sp/Sm, pBBR1MCS-5 <i>aceA</i> -SMc00767 with an internal 700-bp deletion in <i>aceA</i> that leaves wild-type SMc00767 and the intact <i>aceA</i> promoter region | This study |
| RH462 | Rm5000 <i>aceA</i> :: Ω Sp/Sm, pBBR1MCS-5 <i>aceA-glcB</i> | This study |
| RH465 | Rm5000 containing an <i>aceA</i> :: <i>gusA</i> fusion in pBBR1MCS-5 | This study |
| RH467 | RH421 containing an <i>aceA</i> :: <i>gusA</i> fusion in pBBR1MCS-5 | This study |
| <i>E. coli</i> strains | | |
| DH5 α | <i>recA1</i> ϕ 80 <i>dlacZ</i> Δ M15 <i>gyrA96</i> | Gibco BRL |
| HB101 | <i>recA13</i> <i>rspL20</i> (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 <i>sacB</i> Mob | 44 |
| pRK2013 | ColE1 replicon with RK2 transfer region, Km ^r Nm ^r | 20 |
| pHP45 | Ω Sp ^r /Sm ^r , vector Ap ^r | 19 |
| pHP45 | Ω Tc ^r , vector Ap ^r | 19 |
| pBBRMCS-5 | Broad-host-range cloning vector, Mob InsP Gm ^r | 34 |
| pWM6 | <i>QuidA2-aph</i> Km ^r /Nm ^r , vector Ap ^r | 38 |
| pHL76 | pCR2.1 <i>aceA</i> | This study |
| pHL79 | pCR2.1 <i>aceA</i> :: Ω Sp/Sm | This study |
| pHL85 | pBBR1MCS-5 <i>aceA</i> | This study |
| pHL86 | pCR2.1 <i>glcB</i> | This study |
| pHL87 | pBBR1MCS-5 <i>glcB</i> | This study |
| pHL88 | pBBR1MCS-5 <i>aceA-glcB</i> | This study |
| pHL89 | pBBR1MCS-5 <i>aceA</i> -SMc00767 | This study |
| pHL90 | pBBR1MCS-5 <i>aceA</i> -SMc00767 | This study |
| pHL91 | pBBR1MCS-5 SMc00767 | This study |
| pHL92 | pBBR1MCS-5 <i>aceA</i> -SMc00767 with an internal 720-bp deletion in <i>aceA</i> that leaves wild-type SMc00767 and the intact <i>aceA</i> promoter region | This study |
| pHL93 | <i>aceA</i> :: <i>gusA</i> fusion in pBBR1MCS-5 | This study |
| pHL95 | pJQ200mp18 <i>aceA</i> :: Ω Sp/Sm | This study |
| pHL96 | pCR2.1 with a 1,154-bp <i>glcB</i> fragment | This study |
| pHL97 | pCR2.1 with a 1,154-bp <i>glcB</i> fragment:: Ω Tc | This study |
| pHL98 | pJQ200mp18 with a 1,154-bp <i>glcB</i> fragment:: Ω Tc | This study |
| pHL99 | pJQ200mp18 <i>aceA</i> -SMc00767 | This study |
| pHL100 | pJQ200mp18 <i>aceA</i> -SMc00767:: Ω Sp/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 C₂ 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 μ g/ml), pantothenic acid (2 μ g/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 ³²P 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 Mannheim, 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'-GAGATCAAATAGGAAGGAG-3') and *aceA8* (5'-ACAGTCATCGGAGTGTCT-3'); and for *glcB*, *glcB210* (5'-CAAGGACGCTCGGGACA-3'), *glcB3973* (5'-GCTCACAGACCACGACCACG-3'), *msg387* (5'-CAATGCCCGTGGGGCTC GCT-3'), and *ms139* (5'-ATCGCCACATGCCCTTG-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 (OD₅₉₅) of 0.05. The cultures were incubated at 30°C at 200 rpm, and growth was followed by measuring the OD₅₉₅ every 24 h.

Preparation of cell extracts. To determine ICL activities, cells were grown to an OD₅₉₅ of 0.7, harvested by centrifugation, washed with a saline solution, and resuspended in breaking buffer [20 mM *N*-tris(hydroxymethyl)methyl-2-aminethanesulfonic acid (TES) (pH 7.0), 100 mM NaCl, 5 mM MgCl₂, 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 OD₅₉₅ 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 morpholinopropanesulfonic acid (MOPS) (pH 7.3), 5 mM MgCl₂, 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 phenylhydrazine 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 MgCl₂, 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 OD₅₉₅ 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 OD₅₉₅ 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

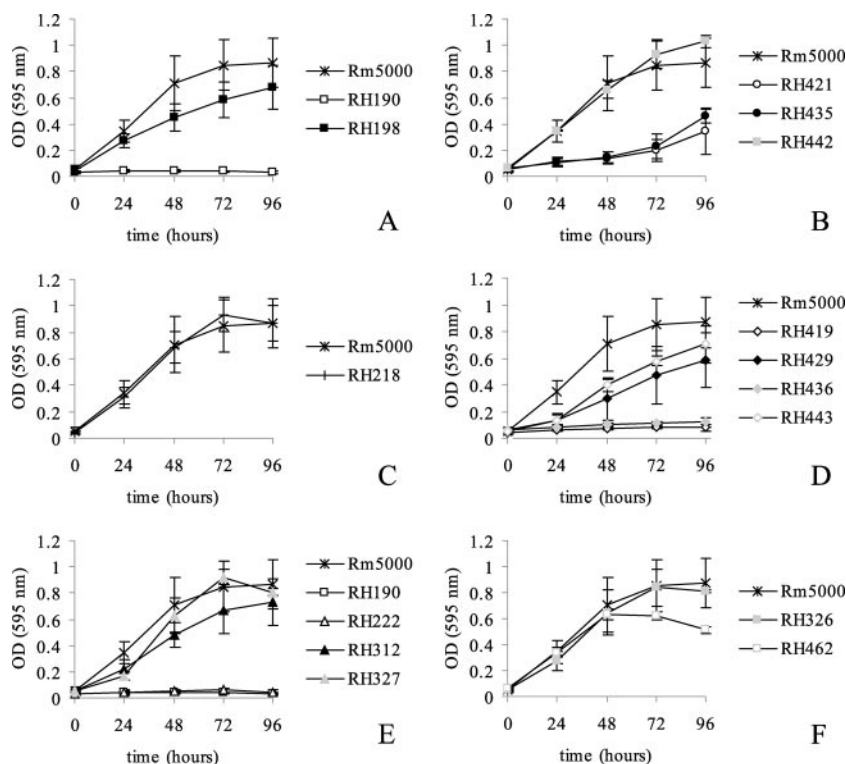


FIG. 1. Functional role of *aceA*, *SMc00767*, and *glcB* in acetate utilization by *S. meliloti*. Bacteria were grown in M9 minimal medium supplemented with 5 mM potassium acetate. The OD_{595} was measured every 24 h over a 96-h period. The bacterial strains used were wild-type strain Rm5000, *aceA* 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*-*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, $\text{mean}(R)$ is the mean log ratio, R_i is the log ratio for each element, and $\text{sd}(R)$ 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 grow 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 reestab-

lished 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 <i>aceA</i> ::ΩSp/Sm | 6.7 | 43 |
| RH198 | Rm5000 <i>aceA</i> ::ΩSp/Sm, pBBR1MCS-5 <i>aceA</i> | 1,080 ± 190 | 172 ± 21 |
| RH218 | Rm5000 <i>glcB</i> ::ΩTc | 297 ± 26 | ND |
| RH222 | Rm5000 <i>aceA</i> ::ΩSp/Sm <i>glcB</i> ::ΩTc | ND | ND |
| RH302 | Rm5000 <i>glcB</i> ::ΩTc, pBBR1MCS-5 <i>glcB</i> | 190 ± 18 | 230 ± 25 |
| RH312 | Rm5000 <i>aceA</i> ::ΩSp/Sm <i>glcB</i> ::ΩTc, pBBR1MCS-5 <i>aceA-glcB</i> | 1,030 ± 209 | 251 ± 26 |
| RH326 | Rm5000 <i>aceA</i> ::ΩSp/Sm, pBBR1MCS-5 <i>aceA-SMc00767</i> | 341 ± 22 | 115 ± 24 |
| RH327 | Rm5000 <i>aceA</i> ::ΩSp/Sm <i>glcB</i> ::ΩTc, pBBR1MCS-5 <i>aceA-SMc00767</i> | 443 ± 50 | ND |
| RH419 | Rm5000 <i>glcB</i> ::ΩTc <i>SMc00767</i> ::ΩSp/Sm | 12 ± 10 | ND |
| RH421 | Rm5000 <i>SMc00767</i> ::ΩSp/Sm | 685 ± 39 | 132 ± 19 |
| RH429 | Rm5000 <i>glcB</i> ::ΩTc <i>SMc00767</i> ::ΩSp/Sm, pBBR1MCS-5 <i>glcB</i> | 731 ± 37 | 907 ± 220 |
| RH435 | Rm5000 <i>SMc00767</i> ::ΩSp/Sm, pBBR1MCS-5 <i>SMc00767</i> | 657 ± 36 | 141 ± 2 |
| RH442 | Rm5000 <i>SMc00767</i> ::ΩSp/Sm, pBBR1MCS-5 <i>aceA-SMc00767</i> with an internal 700-bp deletion in <i>aceA</i> that leaves wild-type <i>SMc00767</i> and the intact <i>aceA</i> promoter region | 96 ± 18 | 116 ± 6 |
| RH443 | Rm5000 <i>glcB</i> ::ΩTc <i>SMc00767</i> ::ΩSp/Sm, pBBR1MCS-5 <i>aceA-SMc00767</i> with an internal 700-bp deletion in <i>aceA</i> that leaves wild-type <i>SMc00767</i> and the intact <i>aceA</i> promoter region | 216 ± 31 | ND |
| RH462 | Rm5000 <i>aceA</i> ::ΩSp/Sm, pBBR1MCS-5 <i>aceA-glcB</i> | 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 than 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-

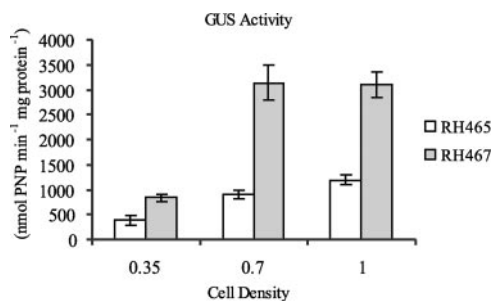


FIG. 2. β -Glucuronidase activities of RH465 (wild-type strain Rm5000 containing the *aceA::gusA* fusion) and RH467 (SMc00767 mutant RH421 harboring the *aceA::gusA* fusion) grown in M9 medium containing acetate. The cultures were collected at OD₅₉₅s of 0.35, 0.7, and 1. The values are the means of three independent experiments performed in duplicate. PNP, *p*-nitrophenyl; GUS, β -glucuronidase.

etate. The transcriptional assays revealed that at three different culture OD₅₉₅s (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 OD₅₉₅s (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 OD₅₉₅ 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), *Magnaporthe 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, <i>dnaB</i> | 3.5 |
| SMc00768 | ICL protein, <i>aceA</i> | 4.6 |
| SMc00769 | Conserved hypothetical protein | 2.6 |
| SMc00772 | Probable putrescine transport system permease protein, <i>potH</i> | 2.0 |

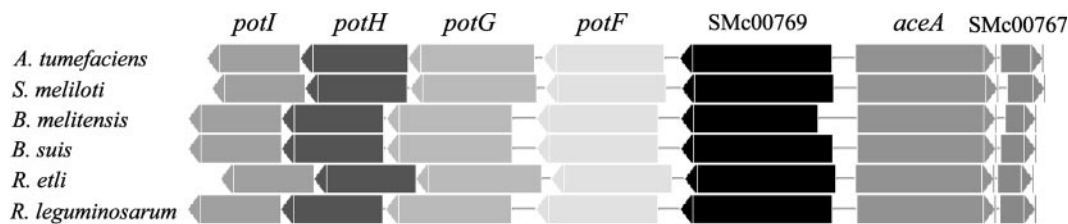


FIG. 3. Genome context of the SMc00767, *aceA*, SMc00769, *potF*, *potG*, *potH*, and *potI* genes in different members of the α -proteobacteria. In all cases, SMc00767 is clustered in this group of genes, suggesting that they are involved in a common process. Genes and intergenic distances are drawn 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 \cdot plant⁻¹ \cdot 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 C₂ 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 C₂ 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 C₂ 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.

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