JOURNAL OF BACTERIOLOGY, Dec. 1999, p. 7608–7613 Vol. 181, No. 24
0021-9193/99/$04.00+0

Citrate Synthase Mutants of *Sinorhizobium meliloti* Are Ineffective and Have Altered Cell Surface Polysaccharides

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Received 5 January 1999/Accepted 22 September 1999

The *gltA* gene, encoding *Sinorhizobium meliloti* 104A14 citrate synthase, was isolated by complementing an *Escherichia coli gltA* mutant. The *S. meliloti gltA* gene was mutated by inserting a kanamycin resistance gene and then using homologous recombination to replace the wild-type *gltA* with the *gltA*::*kan* allele. The resulting strain, CSDX1, was a glutamate auxotroph, and enzyme assays confirmed the absence of a requirement for glutamate. CSDX1 did not grow on succinate, malate, aspartate, pyruvate, or glucose. CSDX1 produced an unusual blue fluorescence on medium containing Calcofluor, which is different from the green fluorescence found with 104A14. High concentrations of arabinose (0.4%) or succinate (0.2%) restored the green fluorescence to CSDX1. High-performance liquid chromatography analyses showed that CSDX1 produced partially succinylated succinoglycan. CSDX1 was able to form nodules on alfalfa, but these nodules were not able to fix nitrogen. The symbiotic defect of a citrate synthase mutant could thus be due to disruption of the infection process or to the lack of energy generated by the tricarboxylic acid cycle.

The rhizobia are gram-negative, aerobic, rod-shaped organisms that have the ability to nodulate leguminous plants and fix nitrogen in a symbiotic relationship that involves the formation of nodules on the roots of legumes like alfalfa. The plant provides the bacteria with carbon compounds, which they oxidize to produce the energy required to reduce atmospheric dinitrogen to ammonia. Although sucrose is the major photosynthate transported into the nodules (24), dicarboxylic acids play a major role in determining the effectiveness of the symbiosis. For example, mutations in a gene coding for a dicarboxylic acid transport protein, DctA, do not generally disturb nodule development but block nitrogen fixation (1, 3, 9, 26). Since the dicarboxylates malate and succinate are found in high concentrations in the nodule (10, 27, 32) and are intermediates of the tricarboxylic acid (TCA) cycle, it is thought that the TCA cycle plays an important role in producing energy used by the bacteroids to fix nitrogen. McDermott and Kahn (20) found that mutants lacking the *Sinorhizobium meliloti* isocitrate dehydrogenase gene (*icd*) formed nodules but were unable to fix nitrogen. In fast-growing rhizobia, mutations that decrease the activity of other TCA cycle proteins, such as succinate dehydrogenase (11) and α-ketoglutarate dehydrogenase (8, 33), also cause the bacteria to be unable to fix nitrogen (Fix^-), but a recent report (12) shows that an α-ketoglutarate dehydrogenase mutant of *Bradyrhizobium japonicum* has a delayed Fix^+ phenotype.

Citrate synthase (CS) is the first enzyme of the TCA cycle and generally governs the entry of carbon into the pathway. *Rhizobium tropici* has two CS genes, one located on the chromosome and the second located on a plasmid (15, 22). Mutations in either of the CS genes did not block nitrogen fixation, but a strain carrying mutations in both genes was unable to fix nitrogen. Mutants with defects in the plasmid CS formed fewer nodules than the wild type, suggesting that the level of CS activity is important for normal infection of beans. While isolating mutants with mutations in the *S. meliloti* isocitrate dehydrogenase gene, *icd*, McDermott and Kahn (20) recovered a class of faster-growing *icd* mutants, such as A39L, that also lack CS activity. They speculated that citrate and isocitrate accumulation in an *icd* mutant background was inhibitory and provided a selective pressure for the recovery of an apparently spontaneous secondary mutation. Symbiotic tests revealed that these double mutants formed callous tissue and were essentially Nod^-. The symbiotic phenotype of these double mutants prompted us to create a defined CS mutation that might enable us to understand better the symbiotic differences between the *icd* mutant and the *icd* CS^- double mutant.

We report here the cloning of the only *S. meliloti* gene that encodes CS, the creation of a mutant unable to produce CS, and the examination of its free-living and symbiotic properties. Growth of strains without CS is relatively normal if a source of glutamate is available, but there is a defect in the modification of exopolysaccharides (EPS). However, a mutation in *gltA* does not itself lead to a nodulation defect as severe as that seen in A39L.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The strains and plasmids used in this study are listed in Table 1. *S. meliloti* strains were grown at 30°C on yeast extract-mannitol (YMB) (30), minimal mannitol medium (MM NH_4) (30), or mannitol glutamate salts medium at pH 7.3 (MGS) (34). CS and isocitrate dehydrogenase mutants were grown on MM NH_4 supplemented with arabinose (5.0 g/liter) except as indicated. *Escherichia coli* strains were grown at 37°C on either LB or M9 minimal salts (28) medium. The *E. coli* CS (*gltA*) mutant MOB154 was grown on M9 medium containing glucose as the carbon source and supplemented with glutamate (5 mg/ml), uracil (50 μg/ml), and thiamine (2 μg/ml). Antibiotics were filter sterilized and added to the medium at the following concentrations (in micrograms per milliliter): kanamycin, 40; tetracycline, 10 for *S. meliloti* and 25 for *E. coli*; gentamicin, 25; and penicillin G, 200. Sucrose was added to media at a concentration of 5% to select against plasmids carrying the *Bacillus subtilis* levansucrase gene. To examine acidic EPS production, Calcofluor (Fluorescence brightener 28; Sigma Chemical Co.) was added to agar

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TABLE 1. Plasmids and strains

Strain or plasmid	Relevant genotype or characteristics	Relevant phenotype[a]	Reference or source
S. meliloti strains			
1021		Wild type	34
104A14		Wild type	30
A39S	104A14 *icd*::Tn*5*	ICD$^-$ Glut$^-$ Kan^r	20
A39L	104A14 *icd*::Tn*5* CS$^-$	ICD$^-$ Glut$^-$ Kan^r CS$^-$	20
CSDX1	104A14 *gltA1*	CS$^-$ Glut$^-$ Kan^r	This study
CSDX426	CSDX1(pMK426)	CS$^+$ Kan^r Tet^r	This study
E. coli strains			
S17-1	*pro hsdR str mob*$^+$	Pro$^-$ Mob$^+$	29
MOB154	*gltA6 galK30 pyrD36 relA1 rpsL129 thi-1 supE44 hsdR4 recA*	CS$^-$ Tet^r	14
DH5α	*supE44 lacU169* (ϕ80 *lacZ*Δ M15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Rec$^-$ LacZ$^-$	28
Plasmids			
pBluescript		Amp^r	Stratagene
pJQ200mp18	*aacC1 sacB*	Gm^r Suc^s	23
pCPP30	*tetA*	Tet^r	16
pMK421	*S. meliloti* 104A14 *gltA* in pJQ200mp18	Gm^r Suc^s	This study
pMK424	*S. meliloti* 104A14 *gltA*::*nptI* in pJQ200mp18	Gm^r Kan^r Suc^s	This study
pMK426	*S. meliloti* 104A14 *gltA* in pCPP30	Tet^r	This study

[a] Kan^r, kanamycin resistance; Tet^r, tetracycline resistance; Amp^r, ampicillin resistance; Gm^r, gentamicin resistance; Glut$^-$, glutamate auxotroph; Suc^s, sucrose sensitive; CS$^-$, strain lacks CS; ICD$^-$, strain lacks isocitrate dehydrogenase.

media at a final concentration of 0.02%, and colonies were examined under long-wavelength (360-nm) UV light.

DNA manipulations. Restriction enzymes, T4 ligase, and calf intestine alkaline phosphatase were purchased from New England Biolabs and used according to the manufacturer's instructions. Genomic DNA was isolated by the method described by Ausubel et al. (2). Plasmids were isolated by the alkaline lysis method described by Sambrook et al. (28).

Mutagenesis and marker exchange of the *S. meliloti* CS gene. The *S. meliloti* gene library, constructed by inserting *Sau*3A partial digests of *S. meliloti* DNA into pUC18, has been described previously (20). Plasmids containing the *S. meliloti* CS gene (*gltA*) were isolated by their ability to complement the *E. coli gltA* mutant MOB154 for growth on media that lack glutamate. One of these plasmids, pL6F, contained a 9.0-kb insert. A 5.6-kb *Sac*I-*Pst*I fragment was subcloned from pL6F into pJQ200mp18 (23), resulting in pMK421. Mutagenesis of *gltA* was accomplished by inserting the kanamycin resistance gene (*nptI*) from pCP13 (4) into a *Bam*HI site within the pMK421 CS gene to yield pMK424. pMK424 was transformed into the *E. coli gltA* mutant MOB154 and tested for its ability to rescue glutamate auxotrophy. Recombinational mutagenesis was accomplished by mating wild-type *S. meliloti* 104A14 with *E. coli* S17-1(pMK424). Both strains were grown to mid-log phase and then washed twice in 0.85% NaCl. The strains were mixed and spotted on YMB agar. The mating proceeded overnight at 30°C. Bacteria from the mating were streaked onto MM NH_4 agar containing kanamycin, sucrose, and arabinose. In *S. meliloti*, arabinose is catabolized to α-ketoglutarate and can satisfy the glutamate requirement of strains with defects in the decarboxylating leg of the TCA cycle (6). Twenty colonies were restreaked onto the same medium. From these streaks, 200 colonies were chosen randomly, picked onto a control plate of MM NH_4 containing kanamycin, sucrose, and arabinose, and subsequently picked onto MM NH_4 agar containing only kanamycin and sucrose. Colonies unable to grow on plates lacking arabinose were immediately cultured and stored in glycerol at −80°C.

Southern hybridizations. DNA was digested with restriction enzymes, separated by electrophoresis in 0.8% agarose gels, and transferred to nylon membranes (GeneScreen Plus; Dupont) by capillary blotting. DNA probes were labeled with [α-^{32}P]dCTP (3,000 Ci/mmol) by random priming (T7 QuickPrime; Amersham Pharmacia). Prehybridization of the nylon membranes was done with QuikHyb (Stratagene, La Jolla, Calif.) according to the manufacturer's instructions. All hybridizations were done at 65°C, and filters were washed at 65°C in 0.3× SSC (450 mM sodium chloride, 45 mM sodium citrate)–5 mM EDTA–0.1% sodium dodecyl sulfate.

Enzyme assays. For measuring both CS and isocitrate lyase, cells were grown to late log phase in 25 ml of medium and washed twice by centrifugation in 100 mM Tris, pH 8.0. The cells were resuspended in 1 ml and then sonicated twice for 90 s on ice. Cell debris was removed by centrifugation at 15,000 × *g* for 10 min. The total protein concentration in the resulting supernatants was measured with the Bio-Rad (Hercules, Calif.) protein assay kit with bovine serum albumin as the standard. CS activity in the cell extracts was assayed by the method described by Srere (31), in which the free coenzyme A (CoA) formed during the reaction reacts with DTNB [5,5′-dithiobis-(2-nitrobenzoic acid)] to form a compound that absorbs strongly at 412 nm. Isocitrate lyase activity was assayed by measuring the production of glyoxylate from isocitrate by phenylhydrazine derivatization (5).

Plant growth, inoculation, and nodulation. Alfalfa (*Medicago sativa* cv. Champ) was used for all nodulation studies. Seeds were surface sterilized by wetting the seeds in 100% ethanol, decanting the ethanol, and subsequently soaking the seeds in 50% bleach (sodium hypochlorite) for 5 min. The seeds were rinsed several times in sterile distilled water to remove traces of bleach and then were germinated by spreading them evenly on a YMB agar plate. Seedlings showing no signs of contamination were moved to sterile growth box units (four seedling per box). Each growth box consisted of two Magenta (Sigma GA-7 vessel) plant tissue boxes with the top box inverted to act as an aseptic barrier.

Plants were cultured in a walk-in growth room as previously described (20). Six weeks after inoculation, plants were harvested and examined for root nodule formation. Nodules were evaluated morphologically and compared to those on plants inoculated with the wild-type strain. Nitrogen fixation was indirectly evaluated by scoring the plants Fix$^+$ or Fix$^-$ on the basis of color and plant dry matter production. To measure the latter, plant shoots were collected, dried in a vacuum oven at 50°C for 15 h, and weighed.

DNA sequencing and sequence analysis. The *S. meliloti* CS gene was sequenced by the DyeDeoxy terminator cycle sequencing protocol. Initial reactions were performed with M13 forward and reverse primers. Subsequent reactions used synthetic primers complementary to a previously sequenced segment. Reactions were run on an Applied Biosystems 373 DNA sequencer in the Laboratory for Biotechnology and Bioanalysis at Washington State University. Sequence alignments and database searches were carried out with the GAP and BLAST programs from the Genetics Computer Group (GCG package; University of Wisconsin, Madison).

EPS isolation and analysis. For preparation of succinoglycan, *S. meliloti* strains were cultivated in 500 ml of MGS liquid medium in 1-liter flasks for 7 days with constant shaking. Cultures were then diluted by addition of 500 ml of water, and cells were removed from cultures by centrifugation (4400 × *g*, 20 min). To reduce the volume of cell-free supernatants, samples were lyophilized and then dissolved in 200 ml of deionized water. High-molecular-weight EPS was precipitated and isolated from these samples by addition of 3 volumes of ethanol followed by centrifugation (4400 × *g*, 20 min); the remaining ethanolic supernatant was discarded. Precipitated EPS was dissolved in deionized water and then was desalted by exhaustive dialysis against deionized water.

Confirmation that EPS samples actually corresponded to succinoglycan was accomplished as follows. Succinoglycan depolymerase was purified from *Cytophaga arvensicola* as previously described (34). Succinoglycan samples were treated with this depolymerase in 100 mM potassium phosphate buffer (pH 5.8) at 37°C overnight. These treatments converted the EPS samples to forms that yield elution patterns that are consistent with monomers of the octasaccharide repeating unit, as determined by Bio-Gel P4 gel filtration chromatography (25).

HPLC analyses. Samples of Bio-Gel P4 column-purified octasaccharide were analyzed by high-performance liquid chromatography (HPLC) in order to quantify the succinyl and acetyl substituents. Specifically, octasaccharide samples were treated with potassium hydroxide, as described above, to release the acetyl and succinyl substituents from the octasaccharide and then were filtered by use of 0.22-μm syringe filters. Succinate and acetate were separated by passage of samples through an Aminex HPX-874 column (eluent, 8 mM sulfuric acid; flow rate, 0.6 ml/min; temperature, 40°C) and were quantified by use of a UV (220-nm wavelength) detection system.

Nucleotide sequence accession number. The *S. meliloti gltA* sequence has been submitted to GenBank and assigned accession no. U75365.

RESULTS

Cloning and mutagenesis of the *S. meliloti* CS gene. The *S. meliloti* 104A14 CS gene (*gltA*) was cloned by complementing *E. coli* MOB154, which is a glutamate auxotroph as the result of a defect in CS (14). A gene library constructed by inserting *Sau*3A partial digests of *S. meliloti* DNA into pUC18 (20) was transformed into MOB154 and plated on M9 agar medium without glutamate. Colonies that grew were subcultured on LB agar medium containing 200 μg of penicillin per ml. These colonies contained plasmids that had overlapping restriction fragments. Plasmid pL6F, which contained 9.0 kb of *S. meliloti* DNA in the pUC18 *Bam*HI site, was chosen for study. The CS gene was subcloned as a 5.6-kb *Pst*I-*Sac*I fragment by inserting it into the suicide vector pJQ200mp18 (23) to give pMK421. The cloned CS gene on pMK421 was mutagenized by inserting an *nptI* kanamycin resistance gene into a *Bam*HI site within *gltA* to give pMK424. The mutation of this site was confirmed by agarose gel electrophoresis, by the inability of pMK424 to complement the glutamate auxotrophy of *E. coli* MOB154, and by DNA sequencing of the cloned *gltA* gene. To obtain a plasmid that could carry *gltA* in *Sinorhizobium*, a 2.9-kb *Kpn*I fragment from pMK421 was inserted into the broad-host-range plasmid pCPP30 (16) to give pMK426.

Sequence analysis of the *S. meliloti gltA* gene. A 2.9-kb *Kpn*I fragment from pMK426 encoding CS was subcloned by *Bam*HI digestion and subsequent ligation into pBluescript in three parts, resulting in the plasmids pMK429, pMK432, and pMK433. The DNA sequence contained an open reading frame 1,290 bp long with strong homology to other *gltA* alleles. The DNA sequence in the coding region of the *S. meliloti gltA* gene was 82% identical to *pcsA* (the plasmid-borne CS gene of *R. tropici*), 70% identical to *gltA* of *Pseudomonas aeruginosa*, and 67% identical to that of *E. coli*. Alignments with the deduced amino acid sequence provided the following identities: 89% with *R. tropici* PcsA CS, 69% with *P. aeruginosa* CS, and 68% with *E. coli* CS.

R. tropici has two CS genes, *pcsA* and *ccs*, on the plasmid and chromosome, respectively. Because *S. meliloti* is the closest relative for which the CS sequence has been determined, a detailed comparison was made to determine the origin of the plasmid gene. The carboxyl termini of the three proteins are the most conserved. In the final 390 amino acids, there are 25 positions where the *S. meliloti gltA* sequence differs from those of both *pcsA* and *ccs*, 2 positions where the *S. meliloti gltA* sequence is the same as that of *pcsA* but not *ccs*, 1 position where the *gltA* sequence is the same as that of *ccs* but not *pcsA*, and 1 position where all are different. At the amino terminus, the first 40 amino acids are very divergent, with 22 identities between *gltA* and *pcsA* and only 12 between *gltA* and *ccs*. However, the DNA sequences in the region corresponding to amino acids 20 to 40 are more similar than this, and by inserting two frameshift mutations, the number of amino acid identities between *gltA* and *ccs* in the amino terminus can be raised to 22 and the number of identities between *ccs* and *pcsA* in this region changes from 4 to 13. A reasonable conclusion from this analysis might be that *pcsA* and *ccs* have been derived recently from the chromosome of *R. tropici* or a close relative—far more recently than the divergence of *S. meliloti* and *R. tropici*. The divergence at the amino terminus may be related to changes in the promoter regions that lead to differences in transcriptional regulation of the *R. tropici* genes (22).

Isolation of *S. meliloti gltA* mutants. Homologous recombination was used to generate a defined CS mutant of *S. meliloti* 104A14. *E. coli* S17-1(pMK424) was mated with *S. meliloti* 104A14 overnight at 30°C. Bacteria from the mating culture were streaked on several MM NH_4 plates containing kanamycin, sucrose, and arabinose and then incubated at 30°C for 4 days or until well-isolated colonies had formed. Twenty colonies were restreaked onto the same medium and incubated to isolate single colonies. From those plates, 200 colonies were transferred to medium that either contained or lacked arabinose. Arabinose is converted to α-ketoglutarate by *S. meliloti*, and preliminary experiments had shown that arabinose was more effective than glutamate in supporting the growth of *S. meliloti icd* mutants. Two isolates, designated CSDX1 and CSDX9, were unable to grow on medium that lacked arabinose, suggesting that homologous recombination had occurred and replaced *gltA* with the *gltA*::*kan* mutant allele. The colonies were checked by streaking onto minimal medium containing arabinose, sucrose (to check for the absence of pJQ200mp18), and kanamycin. CSDX1 and CSDX9 were also streaked onto minimal medium without arabinose to check for glutamate auxotrophy. Both strains were able to grow only when arabinose was present.

Recombination of the kanamycin cassette into the CS gene was confirmed by DNA hybridization. When total DNAs from CSDX1, CSDX9, and 104A14 were probed with labeled *S. meliloti gltA* DNA, CSDX1 and CSDX9 had identical banding patterns that were consistent with a double-recombination event and insertion of the 1.6-kb kanamycin resistance gene into the chromosome. CSDX1 was used for all subsequent enzyme assays and determination of symbiotic phenotype.

CS activity in the *S. meliloti gltA* mutant. The glutamate auxotrophy of CSDX1 suggested that the mutation in *gltA* inactivated CS, and this was confirmed by measuring CS activity. The specific activity of CS in 104A14 was 64 nmol/min/mg of protein, while CSDX1 had no detectable CS activity. Introduction of the copy of *gltA* carried on pMK426 into CSDX1 gave strain CSDX426, which was able to grow on medium that lacked arabinose and had a CS specific activity of 667 nmol/min/mg of protein.

The auxotrophic phenotype of CSDX1 probably occurs because it is unable to synthesize α-ketoglutarate, the precursor for glutamate and several other amino acids. Arabinose, glutamate, and α-ketoglutarate were tested for their ability to support growth of CSDX1, either as a sole carbon source or in combination with mannitol. Arabinose, which is converted to α-ketoglutarate in fast-growing rhizobia (6), supported good growth of CSDX1 in the presence or absence of mannitol. Surprisingly, growth on glutamate plus mannitol was poorer than growth on glutamate alone, and although α-ketoglutarate was able to support good growth of CSDX1, the addition of mannitol to medium containing α-ketoglutarate abolished growth entirely. We speculate that the inhibitory effect of mannitol is due to catabolite repression of oxoglutarate and glutamate transport.

Carbon sources used by the *S. meliloti gltA* mutant. The ability of CSDX1 to use various carbon compounds was evaluated. CSDX1 requires glutamate or arabinose for growth, and since both of these compounds can serve as carbon source, the minimum concentration of arabinose in solid medium that could satisfy the requirement of CSDX1 for glutamate was approximated by streaking CSDX1 on a series of 0.4% mannitol plates with concentration of arabinose ranging from 0.2 to 0.00625%. At 0.0125% arabinose, the colonies were as large as they were at higher concentrations, but lower concentrations gave smaller colonies (data not shown). When mannitol was omitted, leaving 0.0125% arabinose as the sole carbon source, growth of both CSDX1 and 104A14 was extremely slow. Thus, 0.0125% arabinose could satisfy the glutamate requirement of CSDX1 but was unable to support significant growth.

We determined which of various carbon compounds could

TABLE 2. Aerobic growth properties of CS mutants[a]

Carbon source	Growth[b] of strain:				
	104A14	CSDX1	CSDX426	A39S	A39L
Mannitol	++	++	++	++	++
α-Ketoglutarate	++	+	+	+	+
Succinate	++	−	++	++	+
Malate	++	−	++	+	±
Aspartate	++	−	++	++	++
Pyruvate	++	−	++	+	−
Glucose	++	−	++	−	−
Arabinose	++	±	++	+	+

[a] Growth was tested on minimal salts agar medium supplemented with 0.0125% arabinose and containing 0.4% of the indicated carbon source. Plates were incubated aerobically at 30°C for 6 days. The medium pH was 7.4.

[b] ++, growth similar to that of 104A14 on the carbon source (growth on mannitol, fructose, xylose, sorbitol, glycerol, and glutamate was similar for all five strains); +, growth notably poorer than that of 104A14; ±, visible but very poor growth; −, no growth.

be used for growth by CSDX1 by adding 0.4% of each to minimal agar medium containing 0.0125% arabinose. CSDX1, 104A14, CSDX426, and A39L (a *gltA icd* mutant that we had isolated previously [20]) were streaked onto separate plates and incubated at 30°C. After 6 days, the plates were examined and growth of the mutants was compared to growth of 104A14 on the same medium (Table 2). CSDX1 was unable to grow on several of these carbon sources, including succinate, malate, aspartate, pyruvate, and glucose, and grew slower on α-ketoglutarate and arabinose. Addition of pMK426 to give CSDX426 restored normal growth on all of these carbon sources except α-ketoglutarate. A39L had at least some growth on succinate, malate, and aspartate but did not grow on glucose and pyruvate.

Symbiotic phenotypes. CSDX1(pCPP30) formed ineffective nodules on alfalfa that were similar in shape and size to those induced by 104A14 or 104A14(pCPP30) (Table 3). The nodules were white, suggesting a lack of leghemoglobin, and microscopic examination of the nodule tissue showed the absence of bacteroids (data not shown). Plants inoculated with CSDX1 (pCPP30) resembled uninoculated controls, which were yellow and much smaller than plants nodulated by 104A14. After 6 weeks, the dry weight of shoots from plants inoculated with CSDX1(pCPP30) was only one-fifth of that of plants inoculated with 104A14. CSDX426 [CSDX1(pMK426)] formed nodules similar in size and shape to nodules formed by 104A14 or 104A14(pCPP30). These nodules were pink, suggesting the presence of leghemoglobin. Examination of the nodules by light microscopy revealed the presence of bacteroids (data not shown). Furthermore, the plants infected with CSDX426 appeared healthy and green, indicating that nitrogen was being fixed.

McDermott and Kahn (20) found that A39S, an isocitrate dehydrogenase mutant, induced nodules that were similar to those induced by CSDX1 and appeared to also lack leghemoglobin. The plants were also stunted and yellow. The dry weight of plants infected with A39S was similar to the dry weight found with plants infected with CSDX1. They also found that A39L, a strain lacking both CS and isocitrate dehydrogenase, was Nod$^-$. The phenotype of A39L was thus more severe than that of either of the single mutants. We compared the phenotypes of CSDX1, A39S, and A39L in the same experiment and confirmed that this difference was real (Table 3).

Calcofluor phenotypes. The Nod$^-$ phenotype of A39L suggested that there might be some alteration in the polysaccharides needed for successful nodule formation. The whitening compound Calcofluor binds to succinoglycan, an exopolysaccharide important in nodulation, and fluoresces when viewed under UV light (17). The relative fluorescence of the various strains was determined on minimal mannitol medium containing 0.02% Calcofluor and supplemented with either 0.2% arabinose, 0.0125% arabinose, 0.0125% arabinose plus 0.1% succinate, or 0.1% succinate (Table 4). CSDX1, CSDX426, A39L, and A39S grown on medium supplemented with 0.2% arabinose emitted a green fluorescence identical to that of 104A14. When the arabinose concentration was decreased to 0.0125%, 104A14 and CSDX426 still emitted a green fluorescence, but the two CS mutants, CSDX1 and A39L, had a bright blue fluorescence. When 0.1% succinate was added to plates containing 0.0125% arabinose, a green fluorescence initially appeared around the edges of the primary and secondary streak regions of CSDX1 and A39L, but this green fluorescence faded to bright blue within 48 h. Increasing the succinate concentration to 0.2% gave all strains the green fluorescence. The green fluorescence of 104A14 and CSDX426 was more intense on succinate minimal medium, but we could not test the effect of succinate alone on CSDX1, A39L, and A39S because these strains require a source of α-ketoglutarate. In an experiment where succinate was replaced by 0.2% α-ketoglutarate, no transient green fluorescence was observed with CSDX1 or A39L, nor was the green fluorescence enhanced in the wild type. These observations are consistent with the idea that CSDX1 or A39L has a reduced ability to succinylate succinoglycan because the TCA cycle route to succinyl-CoA is blocked and other pathways are not adequate. Increasing succinyl-CoA by providing substrates for α-ketoglutarate dehydrogenase or succinylthiokinase leads to normally modified succinoglycan.

Isocitrate lyase assays. Interestingly, the *icd* mutant, A39S, had a green fluorescence when the arabinose concentration was 0.0125%. Since A39S is also unable to carry out the TCA cycle reactions that lead to succinyl-CoA, persistence of the green color appeared to be inconsistent with the explanation above that the supply of succinyl-CoA was limiting the formation of the normal green fluorescence in the CS mutants. We speculated that A39S might be using isocitrate lyase to convert isocitrate, which we expected to accumulate in an *icd* background, into succinate and that this might contribute to the succinyl-CoA pool. 104A14, A39L, and A39S were assayed for isocitrate lyase activity. 104A14 and A39L had no detectable activity, while A39S was found to have a surprisingly high isocitrate lyase activity of 52 nmol of glyoxylate/min/mg of protein.

HPLC analysis of succinoglycan modifications. Succinyl and acetyl modifications were quantified by HPLC. The octasaccharide from 104A14 showed a succinyl concentration of 1.38 mM and an acetyl concentration of 1.32 mM, i.e., a succinyl-

TABLE 3. Nodulation phenotypes of wild-type strains and CS$^-$ mutants

Strain	Phenotype	Nodules/plant (mean ± SD)	Nodule color	Plant dry wt (mg/plant shoot)
104A14	Wild type	6.3 ± 3.5	Pink	23.0
104A14(pCPP30)	Wild type	7.1 ± 5.0	Pink	19.0
104A14(pMK426)	Wild type (CS$^+$)	7.9 ± 2.0	Pink	21.0
CSDX1(pCPP30)	CS$^-$	4.5 ± 2.1	White	4.0
CSDX1(pMK426)	CS$^-$ (CS$^+$)	8.8 ± 2.6	Pink	19.0
A39S	ICD$^-$	4.4 ± 2.1	White	4.0
A39L	ICD$^-$ CS$^-$	0.1 ± 0.2	White	5.0
No inoculum		0.0		3.0

TABLE 4. Calcofluor phenotypes of *Sinorhizobium* strains on various media supplements

Supplement to mannitol medium	Phenotype[a] of strain:					
	1021	A14	CSDX1	CSDX426	A39L	A39S
0.2% arabinose	Green	Green	Green	Green	Green	Green
0.0125% arabinose	Green	Green	Blue	Green	Blue	Green
0.0125% arabinose + 0.2% succinate	Green	Green	Green	Green	Green	Green
0.2% succinate	Green	Green	NG[b]	Green	NG	NG
0.0125% arabinose + 0.2% α-ketoglutarate	Green	Green	Blue	Green	Blue	Green
0.2% α-ketoglutarate	Green	Green	NG	Green	NG	NG

[a] Colony color under a long-wavelength UV light source.
[b] NG, no growth.

to-acetyl ratio of 1.05. By contrast, the CSDX1 succinyl and acetyl concentrations were 1.04 and 1.61 mM respectively, giving a ratio of 0.646. An *S. meliloti exoH* mutant, lacking the membrane-spanning protein involved in the succinylation of succinoglycan, was used as a control and produced undetectable levels of the succinyl group.

DISCUSSION

The CS (*gltA*) gene from *S. meliloti* 104A14 has been cloned on a 2.9-kb fragment that was selected through its ability to relieve the glutamate auxotrophy of an *E. coli gltA* mutant. The DNA sequence of *gltA* was also determined. By inserting *nptI* into the cloned CS gene and recombining the mutant allele into 104A14, we created a defined *gltA* mutant, CSDX1, which was a glutamate auxotroph and completely lacked CS activity. The absence of any CS activity in CSDX1, the appearance of only one hybridizing band in Southern blots, and our failure to find glutamate-independent pseudorevertants of CSDX1 suggest that this is the only CS gene in *S. meliloti* 104A14. *S. meliloti* thus differs from *R. tropici*, which contains both plasmid and chromosomal CS genes (15). By cloning *gltA* into the broad-host-range vector pCPP30 and mating the resulting plasmid, pMK426, back into CSDX1, we were able to complement the *gltA*::*kan* mutation. Enzyme assays of the complemented mutant, CSDX426, showed a 10-fold increase in the specific activity of CS relative to that in the wild-type strain. Despite the increase in activity, the number of nodules formed by CSDX426 did not increase. This result also differed from the situation in *R. tropici*, in which expression of the plasmid-borne copy of CS increases the number of nodules on *Phaseolus vulgaris* (22).

CSDX1 was unable to grow with succinate, malate, aspartate, pyruvate, or glucose as a carbon source under the condition in which a small amount of arabinose was included in the medium in an effort to relieve the glutamate auxotrophy of the strain. The previously isolated CS^- mutant, A39L, was also unable to use pyruvate or glucose as a sole carbon source but was able to grow to some extent on succinate, malate, and aspartate. In interpreting this data, it should be remembered that a lack of growth could result from an inability of the strain to use the carbon source or from some interference by the carbon source with use of arabinose to satisfy the glutamate requirement. For example, CSDX1 can grow on α-ketoglutarate and glutamate but does not grow if mannitol is added (21). We suspect that this is due to interference with α-ketoglutarate and glutamate transport.

The *S. meliloti icd* mutant, A39S, induces ineffective nodules (20). In the same selection, we isolated more rapidly growing variants, such as A39L, that were Nod^- and also lacked CS (20). One reason for generating a defined CS insertion mutant, CSDX1, was to evaluate its symbiotic phenotype. CSDX1 was Nod^+ Fix^- (Table 3). The nodules formed by CSDX1 were similar in size and shape to nodules induced by 104A14, but they were white and appeared to lack leghemoglobin. This result and the differences in carbon source utilization between CSDX1 and A39L suggested that there are differences between these strains that cannot be accounted for in a simple way. In order to investigate this further, we attempted to introduce a wild-type *gltA* gene into A39L, both by conjugating with S17-1(pMK426) and by integrating a single copy of *gltA* into the chromosome (21). Despite a significant effort, which included trying the construction in the presence of a wild-type *icd* gene, we were unsuccessful. One interpretation of this is that other mutations in A39L are incompatable with a functional citrate synthase. We speculate that these mutations are responsible for the greater symbiotic defect in A39L.

That TCA cycle mutations could affect nodulation by *S. meliloti* is not too surprising. It has been shown that EPS, specifically succinoglycan (EPSI), play a major role in root hair invasion and nodule development by *S. meliloti* (13) and that alterations in the succinoglycan substitutions can cause *S. meliloti* to become ineffective (7, 17–19). One possible explanation for the symbiotic phenotypes of CSDX1 and A39L is that the mutations present in those strains have altered carbon metabolism in a way that alters the modification of succinoglycan that is needed for normal nodule development.

Succinoglycan is both acetylated and succinylated, and inactivation of CS could increase acetyl-CoA pools and decrease succinyl-CoA pools. The experiments with Calcofluor provided preliminary evidence that the EPS was different in the *gltA* mutant, which was then confirmed by measuring the extent of succinylation and acetylation of EPS. Both strains that are missing CS, CSDX1 and A39L, have a blue fluorescence instead of the green fluorescence of 104A14 or CSDX426. A similarly altered fluorescence is seen with *exoH* mutants, which are unable to succinylate succinoglycan (18). Addition of high concentrations of succinate or arabinose restored the green fluorescence to both CSDX1 and A39L. This result is most easily explained by the potential of succinate to be converted to succinyl-CoA by succinate thiokinase and also the potential of arabinose to be directly converted to α-ketoglutarate, which is subsequently converted to succinyl-CoA by α-ketoglutarate dehydrogenase. Both of these additions could therefore lead to an increase in succinyl-CoA, a substrate for the ExoH succinyltransferase. The normal green fluorescence of the isocitrate dehydrogenase mutant, A39S, would appear to conflict with this explanation, since it also would be unable to generate succinyl-CoA via the TCA cycle. However, the very high isocitrate lyase activity of A39S might be able to generate a sufficient amount of succinate to allow normal succinylation. This pathway would not produce the reductant normally made by the TCA cycle, and this lack of reductant or of α-ketoglutarate could be responsible for the ineffectiveness of A39S.

In addition to providing new information about the genetics

of CS in rhizobia, this report shows that the effects of mutating this gene can be pleiotropic. The mutation had its expected effect in blocking glutamate synthesis and affected growth on several of the carbon sources we tested. The cell surface polysaccharide structure was altered, with a decrease in succinylation. The mutation blocked the development of effective nodules, but at this time we are unable to tell whether this is due to its effect on amino acid synthesis, on energy metabolism, or on the presentation by the bacteria of the appropriate EPS signals needed during nodule formation. Experiments to test some of these possibilities are under way.

ACKNOWLEDGMENTS

Aspects of the work presented here were supported by grants to M.L.K. from the U.S. Department of Energy Biosciences Program (DE-FG03-96ER20225 and DE-FG06-93ER20119) and the Department of Agriculture (NRI-CRGO 92-37305-7723) and by Public Health Service grant GM31030 from the National Institutes of Health to G.C.W.

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