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Citrate Synthase Mutants of *Sinorhizobium fredii* USDA257 Form Ineffective Nodules with Aberrant Ultrastructure

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The tricarboxylic acid (TCA) cycle plays an important role in generating the energy required by bacteroids to fix atmospheric nitrogen. Citrate synthase is the first enzyme that controls the entry of carbon into the TCA cycle. We cloned and determined the nucleotide sequence of the *gltA* gene that encodes citrate synthase in *Sinorhizobium fredii* USDA257, a symbiont of soybeans (*Glycine max* [L.] Merr.) and several other legumes. The deduced citrate synthase protein has a molecular weight of 48,198 and exhibits sequence similarity to citrate synthases from several bacterial species, including *Sinorhizobium meliloti* and *Rhizobium tropici*. Southern blot analysis revealed that the fast-growing *S. fredii* strains and *Rhizobium* sp. strain NGR234 contained a single copy of the gene located in the bacterial chromosome. *S. fredii* USDA257 *gltA* mutant HBK-CS1, which had no detectable citrate synthase activity, had diminished nodulation capacity and produced ineffective nodules on soybean. Light and electron microscopy observations revealed that the nodules initiated by HBK-CS1 contained very few bacteroids. The infected cells contained large vacuoles and prominent starch grains. Within the vacuoles, membrane structures that appeared to be reminiscent of disintegrating bacteroids were detected. The citrate synthase mutant had altered cell surface characteristics and produced three times more exopolysaccarides than the wild type produced. A plasmid carrying the USDA257 *gltA* gene, when introduced into HBK-CS1, was able to restore all of the defects mentioned above. Our results demonstrate that a functional citrate synthase gene of *S. fredii* USDA257 is essential for efficient soybean nodulation and nitrogen fixation.

Legumes are exceptional in the ability to enter into symbiotic associations with soil-dwelling bacteria, which are collectively called rhizobia. This interaction leads to the formation of novel structures, the nodules, on the roots and, in certain cases, on the stems. The rhizobia inside the nodules are able to reduce atmospheric dinitrogen to ammonia, which is utilized by the plant for growth and development. The plant, in turn, provides the bacteria with a protective environment and carbon compounds necessary to generate the energy required to reduce atmospheric nitrogen. High concentrations of the dicarboxylic acids malate and succinate are found in the nodules (5, 25, 31). These tricarboxylic acid (TCA) cycle intermediate compounds are believed to play an important role in determining the effectiveness of symbiosis.

Previous studies have shown that rhizobia with mutations in the genes encoding TCA cycle enzymes form nodules that are unable to fix nitrogen. *Sinorhizobium meliloti* mutants lacking isocitrate dehydrogenase initiate nodules on alfalfa that are ineffective in nitrogen fixation (18). Similarly, rhizobia with mutations in genes encoding other TCA cycle enzymes, such as succinate dehydrogenase and α-ketoglutarate dehydrogenase, also induce ineffective nodules (3, 6, 33). However, it has been reported that an α-ketoglutarate dehydrogenase mutant of *Bradyrhizobium japonicum* is able to form nitrogen-fixing bacteroids in soybean, although the onset of nitrogen fixation is considerably delayed compared to the onset of nitrogen fixation in the wild type (7). The role of citrate synthase, the first enzyme of the TCA cycle, in symbiosis has been investigated in *S. meliloti* strain 104A14 (20) and *Rhizobium tropici* strain CFN299 (8, 21). *S. meliloti* has a single copy of the citrate synthase gene, and inactivation of this gene resulted in the Fix^- phenotype (20). *R. tropici* has two copies of the citrate synthase gene, one located on the *sym* plasmid and the other located on the chromosome. Mutation of the plasmid-borne citrate synthase gene resulted in the formation of fewer nodules by the mutant than by the wild-type strain. *R. tropici* carrying mutations in both copies of the citrate synthase gene was unable to fix nitrogen (8). These observations reveal the importance of TCA cycle enzymes for effective nitrogen fixation.

Sinorhizobium fredii USDA257 is a fast-growing bacterium that forms nitrogen-fixing nodules on soybeans and other legumes (9, 15, 22). Most of the nodulation (*nod*) and nitrogen fixation (*nif*) genes of this strain are located on a *sym* plasmid. Even though this strain forms nitrogen-fixing nodules on soybeans, the effectiveness of nitrogen fixation is considerably less than the effectiveness of nitrogen fixation by the classical soybean symbiont, *B. japonicum* (2). In contrast to the *B. japonicum* enzymes, we know very little about the TCA cycle enzymes in USDA257. Here, we describe molecular cloning and the sequence of the only USDA257 gene that encodes citrate synthase, the enzyme that governs the entry of carbon into the TCA cycle. Inactivation of the citrate synthase gene drastically reduced the ability of USDA257 to initiate nodules on soybean. In addition, the citrate synthase mutant produced inef-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
Sinorhizobium fredii USDA257	Nod^+ on soybean	USDA-ARS[a]
S. fredii USDA191	Nod^+ on soybean	USDA-ARS
S. fredii USDA192	Nod^+ on soybean	USDA-ARS
S. fredii USDA193	Nod^+ on soybean	USDA-ARS
S. fredii USDA196	Nod^+ on soybean	USDA-ARS
S. fredii USDA201	Nod^+ on soybean	USDA-ARS
S. fredii USDA206	Nod^+ on soybean	USDA-ARS
S. fredii USDA208	Nod^+ on soybean	USDA-ARS
S. fredii USDA214	Nod^+ on soybean	USDA-ARS
S. fredii USDA217	Nod^+ on soybean	USDA-ARS
SVQ192PC	Plasmid-cured derivative of USDA192	J. E. Ruiz-Sainz
IA728	Plasmid-cured derivative of USDA193	S. Shantharam
Rhizobium sp. strain NGR234	Broad host range	W. J. Broughton
ANU265	Plasmid-cured derivative of NGR234	W. J. Broughton
HBK-CS1	*gltA* mutant of USDA257, Km^r	This study
HBK-CS2	HBK-CS1 containing pMP220	This study
HBK-CS3	HBK-CS1 containing pHKCS-4	This study
Escherichia coli DH5α	ϕ80*lacZ*ΔM15Δ(*lacZYA-argF*) *U169 hsdR17 recA1 endA1 thi-1*	Gibco BRL
E. coli HB101	Restriction-minus, *recA* background	1
Plasmids		
pGEM-7zf(+)	Ap^r	Promega
pBluescript II SK (+)	Ap^r	Stratagene
pGEM-T Easy	Ap^r	Promega
pMP220	Tc^r	29
pJQ200uc1	Gm^r	23
pMK426	Tc^r, *S. meliloti* 104A14 *gltA* in pCPP30	20
pHKCS-1	Ap^r, 1.3-kb *S. meliloti gltA* PCR product in pGEM-T easy	This study
pHKCS-2	Tc^r, USDA257 *gltA* in pLAFR1	This study
pHKCS-3	Ap^r, 10.9-kb USDA257 *gltA* fragment in pGEM-7zf(+)	This study
pHKCS-4	Ap^r, 3.5-kb *Pst*I frament containing USDA257 *gltA* in pBluescript II SK(+)	This study
pHKCS-5	Tc^r, 3.5-kb *Pst*I frament containing USDA257 *gltA* in pMP220	This study
pHKCS-6	Ap^r, 2.4-kb *Pst*I/*Bam*HI fragment from pHKCS-4 in pBluescript II SK(+)	This study
pHKCS-6Ω	Ap^r Spc^r, 2.0-kb Ω fragment cloned into *SalI* site of pHKCS-6	This study
pHKCS-7Ω	Gm^r Spc^r, 4.4-kb *Pst*I/*Bam*HI fragment from pHKCS-6Ω cloned into the *Sma*I site of pJQ200uc1	This study

[a] USDA-ARS, U.S. Department of Agriculture Agricultural Research Service.

fective nodules on soybean, and the nodules had an aberrant ultrastructure.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Rhizobia were grown on a reciprocal shaker at 30°C in yeast extract mannitol (YEM) medium (32), and *Escherichia coli* was cultured in Luria-Bertani broth at 37°C (27). When appropriate, antibiotics were added at the following concentrations: tetracycline,10 μg/ml; kanamycin, 50 μg/ml; spectinomycin, 50 μg/ml; ampicillin, 100 μg/ml; gentamicin, 10 μg/ml; and trimethoprim, 10 μg/ml (for counterselection against *E. coli* donor strains).

Molecular techniques. Recombinant DNA techniques were performed by using standard methods (27). Rhizobial genomic DNA was isolated by the method of Jagadish and Szalay (12), and DNA probes were labeled with [^{32}P]dCTP by using a Multiprime DNA labeling system (Amersham LifeScience, Cleveland, Ohio). Restriction mapping, cloning of restriction fragments, and Southern blot analysis were performed by using standard protocols (27). A cosmid library of USDA257 in pLAFR1 was constructed as described previously (10).

Cloning and nucleotide sequence analysis of the citrate synthase gene. Two primers, 5′-ATGTCAGAGAAAAGCGCGACAGTAACATTC-3′ and 5′-TCA GCGCTTGGTGATCGGCACATAGTCGCG-3′, which correspond to the N- and C-terminal sequences of the *S. meliloti* citrate synthase gene (GenBank accession no. U75365), were utilized to amplify a 1.3-kb DNA fragment from pMK426 (20) DNA by PCR. The amplified PCR product was cloned into the pGEM-T Easy vector (Promega, Madison, Wis.) to produce pHKCS-1. The 1.3-kb *gltA* PCR fragment was labeled with ^{32}P and used to screen a cosmid library of USDA257. Three cosmid clones that yielded positive hybridization signals were identified. Cosmid DNA were isolated from these clones, restricted individually with *Eco*RI, and fractionated by agarose gel electrophoresis. The DNA were transferred to a nitrocellulose membrane and hybridized with the ^{32}P-labeled 1.3-kb *gltA* PCR fragment. All the positive cosmid clones exhibited strong hybridization with a 10.5-kb *Eco*RI fragment. The 10.5-kb *Eco*RI fragment from one of the cosmid clones (pHKCS-2) was cloned into pGEM 7zf(+) to obtain pHKCS-3. A 3.5-kb *Pst*I fragment from pHKCS-3 was subsequently cloned into pBluescript II SK(+) to produce pHKCS-4 and into pMP220 to produce pHKCS-5 (Table 1). A 2.5-kb region from plasmid pHKCS-4 was sequenced at the University of Missouri DNA Core Facility by utilizing appropriate primers synthesized by Integrated DNA Technologies (Coralville, Iowa).

Mutagenesis and marker exchange of the *S. fredii* USDA257 citrate synthase gene. A 2.4-kb *Pst*I/*Bam*HI fragment (Fig. 1) from pHKCS-4 was subcloned into pBluescript II SK(+) to produce pHKCS-6. For construction of the *gltA* mutant, a 2.0-kb Ω fragment was cloned into a *Sal*I site of pHKCS-6 to produce pHKCS-6Ω. The 4.4-kb *Pst*I/*Bam*HI fragment from pHKCS-6Ω was cloned into the suicide plasmid pJQ200uc1 to produce pHKCS-7Ω. This construct was mobilized into USDA257 by triparental mating with helper plasmid pRK2013 (4). Marker exchange was achieved by selection on YEM medium plates containing 5% (wt/vol) sucrose. Mutants were confirmed by Southern blot hybridization with the wild-type region.

Citrate synthase assays. Citrate synthase activity was measured by the method of Mortimer et al. (20). USDA257 and its derivatives were grown to the late log phase in 25 ml of YEM medium. The cells were pelleted by centrifugation at 15,000 × *g* for 10 min and washed twice with 100 mM Tris (pH 8.0). The cell pellet was resuspended in 100 mM Tris (pH 8.0) and sonicated twice for 90 s at 4°C. The cell debris was removed by centrifugation as described above. Citrate synthase activity was assayed spectrophotometrically at 412 nm by 5,5′-dithiobis-

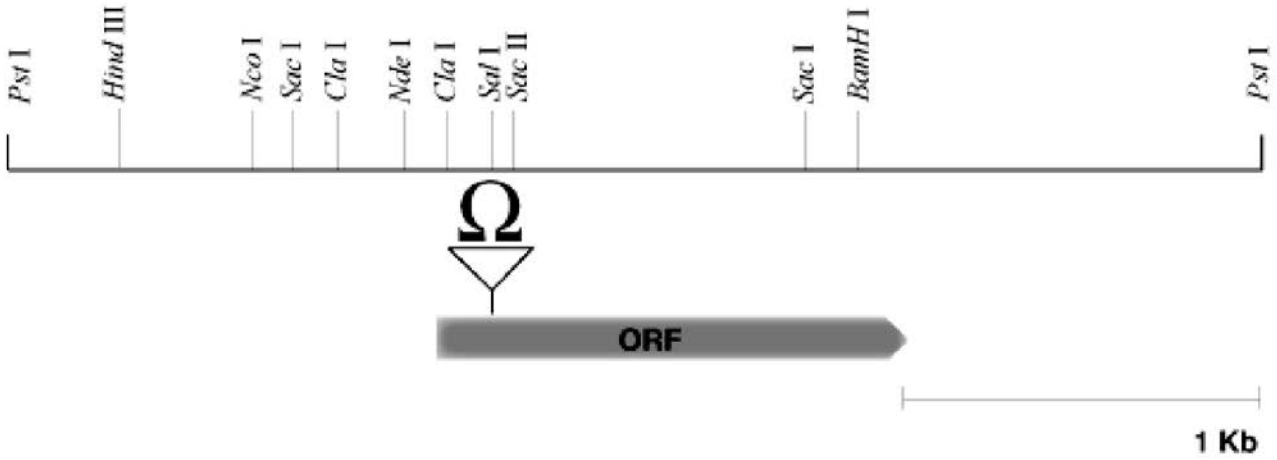

FIG. 1. Restriction map of the 3.5-kb DNA fragment that contains the citrate synthase gene of *S. fredii* USDA257. The orientation of the citrate synthase ORF and the location of the omega cassette used to inactivate the *gltA* gene are also shown.

2-nitrobenzoic acid reduction (30). Protein concentrations in the cell extracts were determined with a Bio-Rad (Hercules, Calif.) protein assay kit by using bovine serum albumin as the standard.

EPS isolation and quantification. For isolation of exopolysaccaride (EPS), *S. fredii* strains were cultivated in 25 ml of YEM medium in 250-ml flasks for 4 days with constant shaking (125 rpm). The bacterial cells were removed from the cultures by centrifugation (7,000 × *g*, 20 min). To each of the cell-free supernatants, 3 volumes of ice-cold 95% ethanol was added, and the preparations were incubated on ice for 2 h. Precipitated EPS was recovered by centrifugation (15,000 × *g*, 20 min) and dissolved in distilled water. Carbohydrates were quantified by the anthrone method (11) with glucose as the standard.

Assay for nodulation and nitrogen fixation. Soybean seeds were surface sterilized and germinated on water agar plates as described previously (16). Cells of USDA257 carrying the cloning vector pMP220 (HBK-CS2), a citrate synthase mutant (HBK-CS1), and a citrate synthase complemented strain (HBK-CS3) were harvested from liquid YEM medium cultures by centrifugation at 7,700 × *g* for 15 min. The cell concentrations were adjusted turbidimetrically to 10^8 cells/ml. Three-day-old soybean seedlings were dip inoculated with bacterial cells and transferred immediately to autoclaved plastic growth pouches that had been prewetted with distilled water. The position of the primary root tip of each seedling was marked on the surface of the pouch. Plants were placed in a growth chamber that was set at a constant temperature of 28°C with a light intensity of 500 μmol of photons/m^2/s and a 12-h day-night cycle. Nodules were counted on day 8 after inoculation and on alternate days thereafter until day 22. Acetylene reduction rates were determined by the method of Schwinghamer et al. (28).

Embedment of soybean nodules in paraffin. Soybean nodules harvested 15 and 25 days after inoculation were cut into two halves and immediately fixed in 50% ethyl alcohol–5% glacial acetic acid–10% formaldehyde for 24 h at 4°C. The nodules were dehydrated in a graded ethanol series, followed by a graded xylene series. Following this, the nodules were infiltrated with several changes of paraffin at 60°C over a 3-day period and embedded in Paraplast Plus tissue-embedding medium (Oxford Labware, St. Louis, Mo.). Paraplast-embedded nodules were sectioned (thickness, 10 μm) with a microtome and stained with hematoxylin and eosin.

Electron microscopy. Soybean nodules obtained 15 and 25 days after inoculation were dissected into 2- to 4-mm pieces with a double-edge razor blade and fixed immediately in 2.5% glutaraldehyde buffered at 7.2 with 50 mM sodium phosphate buffer. The nodule tissue was fixed at room temperature for 4 h and washed extensively with five changes of phosphate buffer. The nodule tissue was postfixed with 2% aqueous osmium tetroxide for 1 h. Following this, the tissue was rinsed with several changes of buffer and dehydrated in a graded acetone series and infiltrated with Spurr's resin. Thick sections were cut with a glass knife, stained with 1% toluidine blue for 2 min, and examined with bright-field optics. For electron microscopy, thin sections were cut with a diamond knife, collected on uncoated 200-mesh copper grids, and stained with 0.5% uranyl acetate and 0.4% lead citrate. Stained sections were examined with a 1200 EX transmission electron microscope (JEOL, Tokyo, Japan) at 80 kV.

Nucleotide sequence accession number. USDA257 *gltA* sequences have been deposited in the GenBank database under accession no. AY157738.

RESULTS

Cloning of USDA257 citrate synthase gene. The citrate synthase gene was isolated by screening a cosmid library of USDA257 with the ^{32}P-labeled 1.3-kb *S. meliloti gltA* PCR product. Three positive clones were identified by colony hybridization. Cosmid DNA were isolated from these positive clones and subjected to Southern blot analysis. All three cosmid clones contained a 10.5-kb *Eco*RI fragment that exhibited hybridization to the 1.3-kb *gltA* PCR probe. These cosmids had several overlapping *Eco*RI restriction fragments. The 10.5-kb *Eco*RI fragment from one of the cosmids (pHKCS-2) was purified from the agarose gel and was subcloned in pGEM-7zf(+) to produce plasmid pHKCS-3 (Table 1). Further restriction enzyme digestion analysis revealed that the *gltA* gene was localized in a 3.5-kb *Pst*I fragment. We subcloned this 3.5-kb *Pst*I fragment into pBluescript II SK(+) and pMP220 to obtain plasmids pHKCS-4 and pHKCS-5, respectively. A partial restriction map of the 3.5-kb *Pst*I fragment is shown in Fig. 1.

Sequence analysis of USDA257 citrate synthase gene. We determined the DNA sequence of a 2,595-bp region from plasmid pHKCS-4 (GenBank accession no. AY157738). Sequence analysis with the open reading frame (ORF) finder program resulted in identification of a 1,290-bp ORF. The predicted ORF encodes a 429-amino-acid protein with a molecular weight of 48,198. The theoretical isoelectric point of the protein was estimated to be 5.92. The amino acid sequence of USDA257 citrate synthase was similar to the sequences of citrate synthases from different bacteria. The USDA257 citrate synthase was 85% identical to the *S. meliloti* citrate synthase. Similarly, the USDA257 citrate synthase exhibited 72 and 70% identity to the plasmid and chromosome-borne citrate synthases from *R. tropici*.

***S. fredii* strains contain a single copy of the chromosomal citrate synthase gene.** *R. tropici* contains two copies of the citrate synthase gene, one located on the *sym* plasmid and the other located on the chromosome (8, 21). However, *S. meliloti* carries only a single copy of the citrate synthase gene (20). To examine the number of copies of the citrate synthase gene in *S. fredii*, we performed a Southern blot analysis with genomic

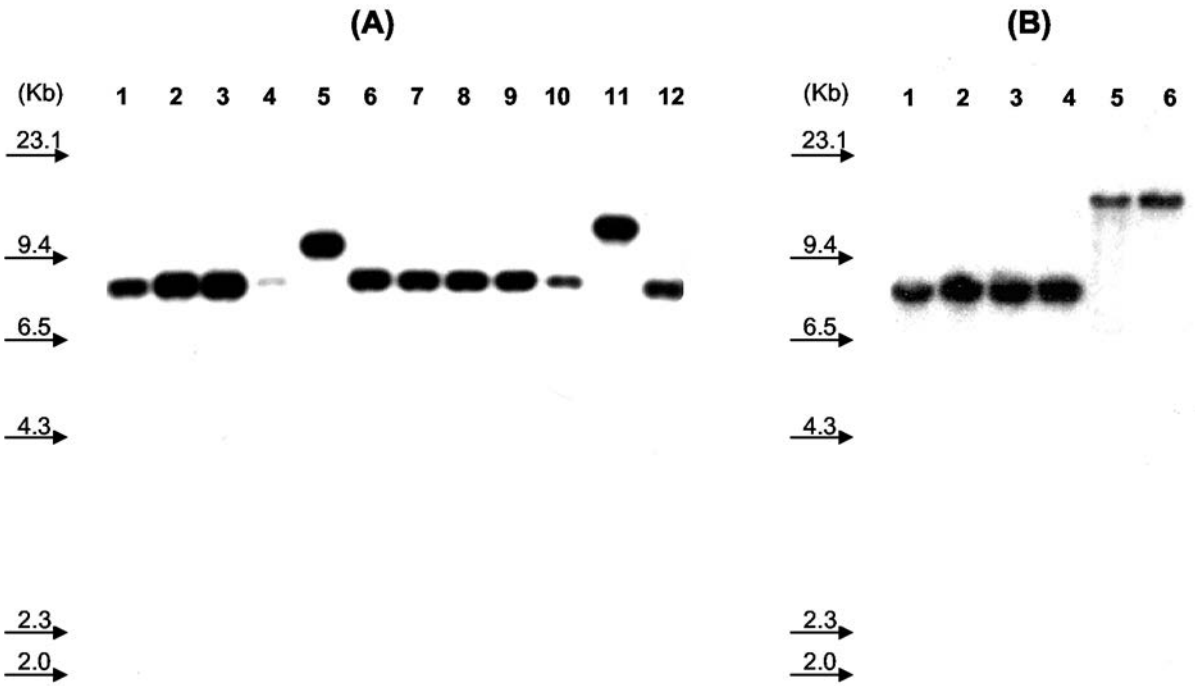

FIG. 2. (A) Southern blot analysis of the citrate synthase gene in the fast-growing strains of *S. fredii* and *Rhizobium* sp. strain NGR234. Genomic DNA from *S. fredii* USDA191 (lane 1), USDA192 (lane 2), USDA193 (lane 3), USDA196 (lane 4), USDA201 (lane 5), USDA205 (lane 6), USDA206 (lane 7), USDA208 (lane 8), USDA214 (lane 9), USDA217 (lane 10), USDA257 (lane 11), and *Rhizobium* sp. strain NGR234 (lane 12) were restricted with *Eco*RI and electrophoresed in 0.7% agarose. The gel was blotted onto nitrocellulose and probed with the ^{32}P-labeled USDA257 *gltA* gene. (B) Citrate synthase is located on the chromosome. Genomic DNA from *S. fredii* USDA192 and USDA193 and *Rhizobium* sp. strain NGR234 (lanes 1, 3, and 5, respectively) and their plasmid-cured derivatives (lanes 2, 4, and 6, respectively) were digested with *Pst*I and hybridized with the USDA257 *gltA* gene. The numbers on the left indicate the positions and sizes of molecular weight markers.

DNA that were isolated from different *S. fredii* strains and a close relative of USDA257, *Rhizobium* sp. strain NGR234. The coding region of the USDA257 citrate synthase gene was employed as the hybridizing probe. In most of the strains which we examined, we detected a single 8.7-kb *Eco*RI hybridizing fragment (Fig. 2A). In USDA201 and USDA257 the hybridizing *Eco*RI fragment was larger than the fragments in other fast-growing strains (Fig. 2A). To determine if the chromosome or the plasmid contains the *gltA* gene of *S. fredii*, we performed a Southern blot analysis with DNA from strains that have been cured of their plasmids. For this experiment we utilized plasmid-cured derivatives of *S. fredii* USDA193 (24), USDA205 (14), and *Rhizobium* sp. strain NGR234 (19). A hybridization signal was detected with both the wild-type and plasmid-cured strains, indicating that in these strains the *gltA* gene is located on the chromosome (Fig. 2B).

Citrate synthase activity in a USDA257 *gltA* mutant. We created a *gltA* mutant of USDA257 (HBK-CS1) by introducing an omega cassette into the *Sal*I site that is located near the N-terminal region of the protein. Recombination of the omega cassette into the citrate synthase gene was confirmed by Southern blot analysis. When genomic DNA from USDA257 and HBK-CS1 were probed with the ^{32}P-labeled USDA257 *gltA* gene, the mutant hybridized to a fragment that was 2 kb larger than the fragment that the wild type hybridized to, which is consistent with a double-recombination event. We first compared the citrate synthase activities in the wild type and the citrate synthase mutant. The specific activity of citrate synthase in USDA257 was 146 nmol/min/mg of protein, while the citrate synthase mutant had no detectable enzyme activity. Introduction of pHKCS-5, which contained the USDA 257 citrate synthase gene, into the mutant restored the citrate synthase activity. The citrate synthase activity in this complemented strain was 649 nmol/min/mg of protein and was substantially higher than that in the wild-type strain. Similarly, a plasmid that contained only the citrate synthase gene, when introduced into HBK-CS1, was able to completely restore the citrate synthase activity of the mutant (data not shown).

USDA257 *gltA* mutant overproduces EPS. Previous studies have revealed that citrate synthase mutants are defective in terms of growth in minimal media. An *S. meliloti* citrate synthase mutant requires glutamate or arabinose for growth (20). A citrate synthase mutant of *R. tropici* grows poorly in minimal media with sucrose as a carbon source (21). However, these growth rate deficiencies were not detected when the cells were grown in complete medium. Similarly, the USDA257 citrate synthase mutant also grew poorly in minimal media but grew normally in complete media. In addition, the colony morphology of the citrate synthase mutant was distinct from that of the wild type on YEM agar plates. The change in the colony morphology indicated that the citrate synthase mutant might have altered surface characteristics. This possibility was verified by quantifying the amounts of EPS produced by the citrate synthase mutant and wild-type strain. Wild-type strain USDA257 produced 2.2 $\pm$ 0.1 μg of EPS per ml, while the citrate synthase mutant produced 5.9 $\pm$ 0.46 μg of EPS per ml.

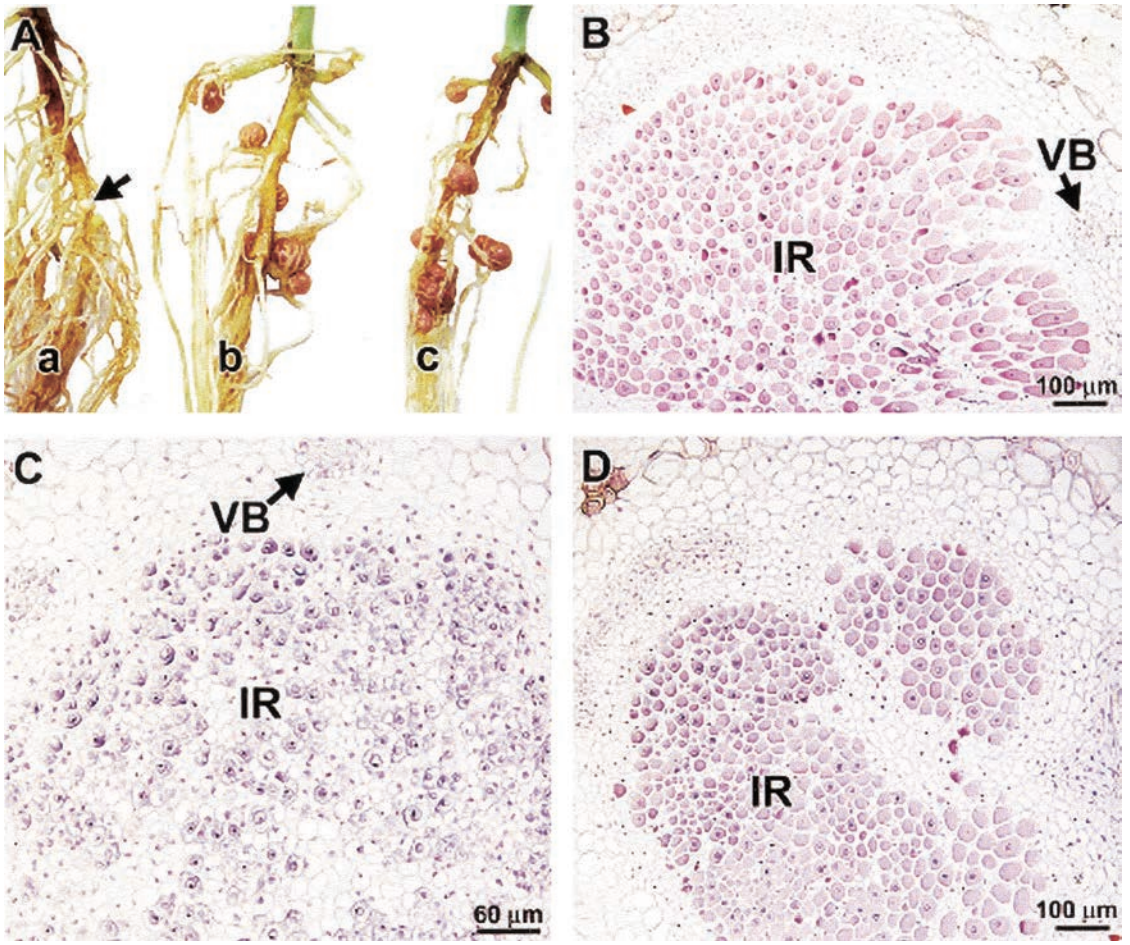

FIG. 3. (A) Responses of *V. unguiculata* to inoculation with citrate synthase mutant HBK-CS1 (plant a), USDA257 (plant b), and complemented strain HBK-CS3 (plant c). Note that the citrate synthase mutant produced a few nodules that are whitish (arrow), while the nodules produced by the wild type and the complemented strain are pinkish. (B to D) Light micrographs of nodules formed by the wild type and the *gltA* mutant. Nodules were collected at 15 days after inoculation and embedded in paraffin, and the sections were stained with hematoxylin and eosin. Note that the nodules initiated by the wild type (B) and the complemented strain (D) contain a central infected region (IR) that is occupied by bacteria. The citrate synthase mutant-initiated nodules contain infected cells with prominent nuclei and no visible bacteria (C). VB, vascular bundle.

Introduction of a cloned citrate synthase gene into this mutant lowered the EPS level to 2.6 ± 0.23 μg/ml, which is comparable to the level in the wild type.

USDA257 *gltA* mutant is impaired in nodulation and nitrogen fixation. We examined the ability of the USDA257 citrate synthase mutant to form nodules on *Glycine max* cv. Peking (soybean) and *Vigna unguiculata* (cowpea). With both hosts, the citrate synthase mutant formed drastically reduced numbers of nodules. We also examined the kinetics of nodule formation of the citrate synthase mutant and compared them to those of the wild type. USDA257 formed an average of seven nodules by 14 to 16 days after inoculation on cv. Peking soybean, while the citrate synthase mutant formed less than one nodule. We performed a complementation experiment in which a cloned citrate synthase gene was introduced into the citrate synthase mutant. The introduced gene restored the ability of the citrate synthase mutant to form nodules on soybean roots. The nodules produced on cv. Peking soybean and cowpea by the citrate synthase mutant were whitish. In contrast, the nodules produced by the wild type and the complemented strain were pinkish (Fig. 3A). The acetylene reduction rates for nodules harvested 20 days after inoculation were 13.4 and 17.2 μmol/h/g (fresh weight) of nodules for USDA257 and the complemented strain, respectively. Nodules produced by the citrate synthase mutant exhibited no detectable acetylene reduction activity.

USDA257 *gltA* mutant produces nodules with an aberrant ultrastructure. Light microscopic examination of nodules that were initiated by USDA257 revealed a structure that was typical of a determinate nodule (Fig. 3B). The nodules contained a central region that was filled with bacteria. The outer cortex contained several vascular bundles and a layer of sclerenchyma cells (Fig. 3B). The structure of the nodules initiated by the citrate synthase mutant was similar to the structure of the nodules initiated by USDA257, except that they contained fewer infected cells (Fig. 3C). The cells in these nodules also had prominent nuclei (Fig. 3C). The nodules initiated by the complementing strain, however, had anatomical features that were indistinguishable from those of USDA257-initiated nodules (Fig. 3D).

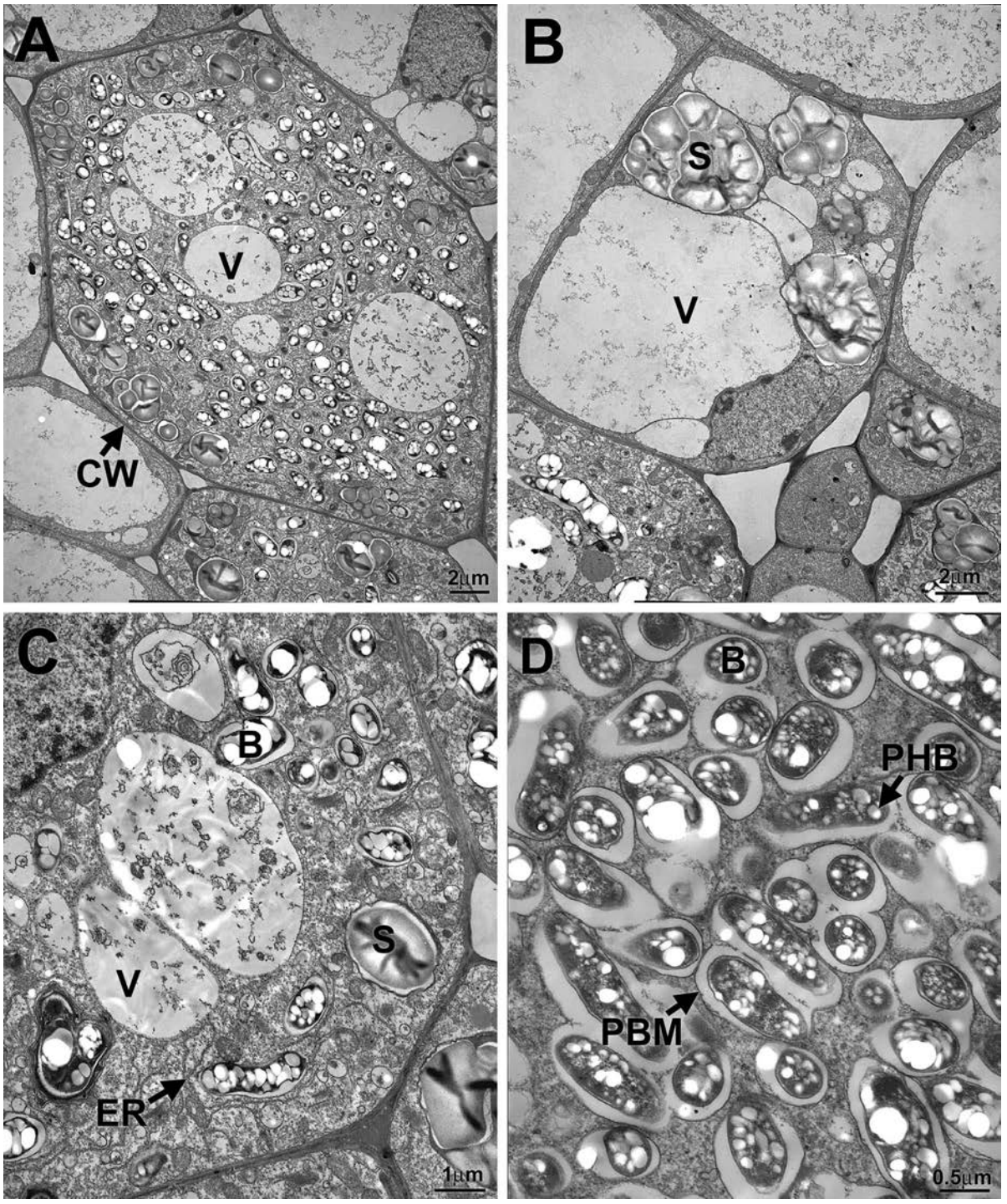

FIG. 4. Transmission electron micrographs of cv. Peking soybean nodules. The *gltA* mutant-initiated nodules contain bacteroids, large vacuoles, and starch grains (A and C). The uninfected cells contain prominent starch grains (B). The bacteroids are surrounded by symbiosomes, and the cell has extensive endoplasmic reticulum (C). The vacuoles contain large amounts of membrane structures (C). USDA257-initiated nodules contain densely packed bacteroids and no starch grains (D). CW, cell wall, B, bacteroids; S, starch; PBM, peribacteroid membrane; PHB, poly-β-hydroxybutyrate; V, vacuole; ER, endoplasmic reticulum.

The ultrastructure of the nodules initiated by the citrate synthase mutant was examined by transmission electron microscopy (Fig. 4A to C). In contrast to the nodules initiated by the wild type, the citrate synthase mutant-initiated nodules (15 days after inoculation) contained low numbers of bacteroids (Fig. 4A). The infected cells also had prominent vacuoles that contained membranous structures. In addition, these cells also contained numerous starch grains that lined the cell walls (Fig. 4A). The starch grains were also prominent in the uninfected cells and occupied major portions of the uninfected cells (Fig.

4B). Nodules harvested at 25 days after inoculation also had ultrastructural features similar to those seen at 15 days after inoculation (Fig. 4C). However, the infected cells contained even fewer bacteroids than the cells observed at 15 days after inoculation. The infected cells also contained an extensive endoplasmic reticulum (Fig. 4C). The vacuoles contained dense membrane structures and, in a few cases, contained disintegrating bacteroids. The bacteroids that were present in these cells were enclosed in symbiosomes (peribacteroid membranes). The symbiosomes generally contained a single bacterium. In contrast, the cells infected by USDA257 had symbiosomes that contained more than one bacteroid (Fig. 4D). Moreover, these cells did not contain any vacuoles or starch grains. Prominent polyhydroxybutyrate inclusions were present in the bacteroids of USDA257 and the citrate synthase mutant (Fig. 4C and D).

DISCUSSION

We cloned and characterized the citrate synthase gene from *S. fredii* USDA257. There is a single copy of *gltA* in *S. fredii*, and this gene is not located on the *sym* plasmid. This observation was confirmed by Southern blot analysis by using plasmid-cured strains of fast-growing *S. fredii*. In this regard, the citrate synthase gene of *S. fredii* is very similar to that of *S. meliloti* (20). We also examined the number of copies of the citrate synthase gene in *Rhizobium* sp. strain NGR234. This strain is very closely related to *S. fredii* USDA257 both at the genetic level and in terms of its broad host range (22). A previous study suggested that there could be two copies of the citrate synthase gene in *Rhizobium* sp. strain NGR234 (8). The Southern blot analysis performed in our study, however, revealed that NGR234 contained a single hybridizing band. Like USDA257, the citrate synthase gene was also localized on the chromosome. In this regard, *S. fredii* strains, *S. meliloti*, and NGR234 differ from *R. tropici*, which contains two copies of the citrate synthase gene, one located on the *sym* plasmid and the other located on the chromosome (8, 21). The occurrence of multiple copies of the citrate synthase gene is not unique to *R. tropici* since two copies of this gene have also been reported in *Bacillus subtilis* (13) and *Saccharomyces cerevisiae* (26). It is not known if the presence of multiple copies of the citrate synthase gene provides any advantage for these microorganisms.

The USDA257 citrate synthase mutant was impaired in the ability to initiate nodules on soybeans. Similar results have been reported for citrate synthase mutants of *S. meliloti* and *R. tropici*. In the case of *S. meliloti*, the citrate synthase mutant formed ineffective nodules, and the number of nodules was 30% less than the number of nodules formed by the wild type (20). However, the kinetics of nodulation were not examined, and it is not known if the citrate synthase mutant had a delayed-nodulation phenotype. In *R. tropici*, which contains two copies of the citrate synthase gene, inactivation of the *sym* plasmid copy did not result in delayed nodulation even though a 30 to 40% reduction in the number of nodules was observed (21). In contrast, inactivation of the chromosomal citrate synthase gene resulted in delayed nodulation and a 60 to 70% reduction in the number of nodules (8). In both cases, the nodules were effective in nitrogen fixation. However, when both copies of the citrate synthase gene were mutated, the resulting mutant produced ineffective nodules on *Phaseolus vulgaris* (8). An examination of the kinetics of soybean nodulation by the USDA257 citrate synthase mutant clearly showed that this strain was delayed in terms of initiation of nodules on soybean roots. The delayed-nodulation phenotype could be related to the poor growth rate of HBK-CS1. Citrate synthase mutants of *S. meliloti* are glutamate auxotrophs and do not utilize several carbon sources (20). We also noticed that the USDA257 citrate synthase mutant was unable to utilize several carbon sources for growth. The poor growth rate of HBK-CS1 could be a contributing factor to the observed reduction in nodulation. Another reason for the reduction in nodulation could be related to changes in the surface characteristics of the mutant. It is well established that EPS plays an important role in infection and nodule development. The USDA257 citrate synthase mutant produces three times more EPS than the wild type produces. However, we do not know if the composition of the EPS produced by the citrate synthase mutant of USDA257 is different from the composition of EPS produced by the wild type. Interestingly, inactivation of the citrate synthase gene of *S. meliloti* results in altered EPS production, which results in partially succinylated succinoglycan (20). This altered succinoglycan is believed to induce ineffective nodules on alfalfa (*Medicago sativa* cv. Champ). Similarly, *exo* mutants of *Rhizobium* sp. strain GRH2, which produces structurally different EPS than the wild type produces, formed ineffective nodules on *Acacia* spp. (17). Another possible reason that the citrate synthase mutants are unable to fix atmospheric nitrogen may be related to the inability of these organisms to generate sufficient energy by the TCA cycle.

Alfalfa nodules initiated by citrate synthase mutants of *S. meliloti* were reported to lack bacteroids (20). Similarly, a citrate synthase double mutant of *R. tropici* was reported to form ineffective nodules that were devoid of bacteroids (21). However, no microscopic evidence was provided in these studies. Light microscopic observation of soybean nodules initiated by the citrate synthase mutant of USDA257 revealed that the central infected region contained cells that were highly vacuolated and had prominent nuclei. Under low magnification, it was difficult to detect the presence of bacteroids in these cells. However, electron microscopic examination clearly revealed the presence of bacteroids, albeit at a low concentration. The ultrastructure of nodules initiated by the USDA257 citrate synthase mutant resembles the ultrastructure of nodules formed by a *B. japonicum* α-ketoglutarate dehydrogenase mutant (7). The α-ketoglutarate dehydrogenase mutant had a delayed-nodulation phenotype, and the nodules (19 days after inoculation) contained substantially lower numbers of bacteroids than the nodules initiated by the wild type contained. Just as we observed in our study, the infected cells contained prominent starch grains. However, examination of older nodules (32 days after inoculation) revealed that the entire cytoplasm was filled with bacteroids and that the starch grains had disappeared. This situation, however, does not occur in soybean nodules initiated by the USDA257 citrate synthase mutant. We examined older soybean nodules (25 days after inoculation) and found that the infected cells still contained prominent vacuoles that had membranous inclusions in them and the cell cytoplasm contained bacteroids that appeared to be disintegrating. The breakdown of the internal organization of

infected cells may be another reason for the inability of USDA257 citrate synthase mutants to fix nitrogen.

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