

Disruption of the Glycine Cleavage System Enables *Sinorhizobium fredii* USDA257 To Form Nitrogen-Fixing Nodules on Agronomically Improved North American Soybean Cultivars[∇]

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The symbiosis between *Sinorhizobium fredii* USDA257 and soybean [*Glycine max* (L.) Merr.] exhibits a high degree of cultivar specificity. USDA257 nodulates primitive soybean cultivars but fails to nodulate agronomically improved cultivars such as McCall. In this study we provide evidence for the involvement of a new genetic locus that controls soybean cultivar specificity. This locus was identified in USDA257 by Tn5 transposon mutagenesis followed by nodulation screening on McCall soybean. We have cloned the region corresponding to the site of Tn5 insertion and found that it lies within a 1.5-kb EcoRI fragment. DNA sequence analysis of this fragment and an adjacent 4.4-kb region identified an operon made up of three open reading frames encoding proteins of deduced molecular masses of 41, 13, and 104 kDa, respectively. These proteins revealed significant amino acid homology to glycine cleavage (*gcv*) system T, H, and P proteins of *Escherichia coli* and other organisms. Southern blot analysis revealed the presence of similar sequences in diverse rhizobia. Measurement of β -galactosidase activity of a USDA257 strain containing a transcriptional fusion of *gcvT* promoter sequences to the *lacZ* gene revealed that the USDA257 *gcvTHP* operon was inducible by glycine. Inactivation of either *gcvT* or *gcvP* of USDA257 enabled the mutant to nodulate several agronomically improved North American soybean cultivars. These nodules revealed anatomical features typical of determinate nodules, with numerous bacteroids within the infected cells. Unlike for the previously characterized soybean cultivar specificity locus *nolBTUVW*, inactivation of the *gcv* locus had no discernible effect on the secretion of nodulation outer proteins of USDA257.

Sinorhizobium fredii USDA257 is a fast-growing rhizobium that forms nitrogen-fixing nodules on *Glycine max*, *Glycine soja*, *Neonotonia wightii*, *Phaseolus vulgaris*, and several other legumes (24, 36). The symbiotic relationship between *S. fredii* USDA257 (here called USDA257) and soybean is of particular scientific and economic interest because this bacterium nodulates soybean in a cultivar-specific manner (13, 19). USDA257 effectively nodulates the primitive soybean cultivar Beijing but fails to nodulate agronomically improved cultivars such as McCall (2, 13). Histological studies have shown that USDA257 is able to infect McCall root hairs and induce cortical cell divisions but fails to form infection threads (5).

Previously, we have demonstrated the presence of negatively acting nodulation genes by creating Tn5 mutants (14). In one of the mutants (DH4) the Tn5 insertion was located in *sym* plasmid, while in the second mutant (DH5), the mutation was located in the bacterial chromosome. In DH4, the Tn5 insertion was located in an 8.0-kb EcoRI fragment which harbors the *nolXWBTUV* locus (34). Disruption of any of these genes expands the host range of *S. fredii* USDA257. Subsequent studies have demonstrated that this locus is part of a USDA257 type III secretion system (T3SS) (27, 34). Both DH4 and DH5 were able to form nodules on agronomically improved soybean

cultivars. In contrast to DH4, which formed nitrogen-fixing nodules, the DH5 formed only ineffective nodules (14). The gene *nolC*, which is inactivated by Tn5 insertion in DH5, shows homology to *dnaJ* of *Escherichia coli*, a heat-shock gene (22).

In the present work, we report the identification and characterization of another genetic locus in USDA257 that regulates soybean cultivar specificity. This chromosomal locus was identified by random mutagenesis with a Tn5 transposon, followed by nodulation screening on different soybean hosts. Sequence analysis showed that a gene that codes for the aminomethyltransferase precursor (glycine cleavage system T protein) was disrupted at the N terminus by the Tn5 transposon. The *gcv* mutants of USDA257, unlike the wild type, were able to form nitrogen-fixing nodules on several agronomically improved North American soybean cultivars.

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 (48), and *E. coli* was cultured in Luria-Bertani broth at 37°C (42). When appropriate, antibiotics were added at the following concentrations: tetracycline, 10 μ g/ml; kanamycin, 50 μ g/ml; spectinomycin, 50 μ g/ml; ampicillin, 100 μ g/ml; gentamicin, 10 μ g/ml; and trimethoprim, 10 μ g/ml (for counter-selection against *E. coli* donor strains).

Molecular techniques. Recombinant DNA techniques were performed by using standard methods (42). Rhizobial genomic DNA was isolated by the method of Jagadish and Szalay (15), and DNA probes were labeled with [³²P]dCTP by using a Multiprime DNA labeling system (Amersham LifeScience, Cleveland, OH). Restriction mapping, cloning of restriction fragments, and Southern blot analysis were performed by using standard protocols (42).

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristic(s)	Source or reference
Strains		
<i>S. fredii</i> USDA257	Nod ⁺ on soybean cultivar Beijing	USDA-ARS
<i>S. fredii</i> USDA205	Nod ⁺ on soybean cultivar Beijing	USDA-ARS
<i>S. meliloti</i> USDA1002	Nod ⁺ on alfalfa	USDA-ARS
<i>S. teranga</i> USDA4894	Nod ⁺ on acacia	USDA-ARS
<i>B. liaoningense</i> USDA3622	Nod ⁺ on soybean	USDA-ARS
<i>R. galegae</i> USDA4128	Nod ⁺ on <i>Galega</i> spp.	USDA-ARS
<i>R. hainanense</i> USDA3588	Nod ⁺ on <i>Desmodium</i> spp.	USDA-ARS
<i>R. huautlaense</i> USDA4900	Nod ⁺ on <i>Sesbania herbacea</i>	USDA-ARS
<i>R. tropici</i> USDA9030	Nod ⁺ on <i>Phaseolus vulgaris</i>	USDA-ARS
<i>R. leguminosarum</i> USDA2370	Nod ⁺ on pea	USDA-ARS
<i>M. amorphae</i> USDA1001	Nod ⁺ on <i>Amorpha fruticosa</i>	USDA-ARS
<i>S. fredii</i> USDA191	Nod ⁺ on soybean	USDA-ARS
<i>S. fredii</i> HBK257-15A	USDA257 derivative containing a Tn5 insertion in <i>gcvT</i> ; Km ^r	This work
<i>S. fredii</i> HBK <i>gcvT</i> -Ω	USDA257 derivative containing an Ω insertion in <i>gcvT</i> ; Spec ^r	This work
<i>S. fredii</i> HBK <i>gcvP</i> -Ω	USDA257 derivative containing an Ω insertion in <i>gcvP</i> ; Spec ^r	This work
<i>E. coli</i> HB101	Restriction-minus, <i>recA</i> background	Takara Bio Inc.
<i>E. coli</i> DH5α	λ ⁻ φ80 <i>dlacZ</i> DM15 D(<i>lacZYA-argF</i>) <i>U169 recA1 endA hsdR17</i> (r _K ⁻ m _K ⁻) <i>supE44 thi-1 gyrA relA1</i>	Gibco BRL
Plasmids		
pGEM-7zf(+)	Ap ^r	Promega
pBluescript II SK(+)	Ap ^r	Stratagene
pGEM-T Easy	Ap ^r	Promega
pMP220	Tc ^r	45
pJQ200sk	Gm ^r	38
pGCV-1	Km ^r Tc ^r ; USDA257 <i>gcv</i> -Tn5 in pLAFR1	This work
pGCV-2	Tc ^r ; USDA257 <i>gcv</i> in pLAFR1	This work
pGCV-3	Ap ^r ; 1.5-kb USDA257 <i>gcvT</i> EcoRI fragment in pGEM-7zf(+)	This work
pGCV-4	Ap ^r ; 5.7-kb EcoRII fragment containing part of <i>gcvT</i> and <i>gcvHP</i> in pBluescript II SK(+)	This work
pGCV-5	Tc ^r ; 1.5-kb EcoRI fragment from pGVC-3 cloned in pMP220	This work
pGCV-6	Ap ^r ; 3.4-kb PstI fragment from pHKCS-4 in pBluescript II SK(+)	This work
pGCV-6Ω	Ap ^r Spe ^r ; 2.0-kb Ω fragment cloned into EcoRI site of pHKCS-6	This work
pGCV-7Ω	Gm ^r Spe ^r ; 5.4-kb PstI fragment from pHKCS-6Ω cloned in pJQ200sk	This work

Tn5 mutagenesis and screening. USDA257 Tn5 mutants were created with the help of suicide vector pSUP1011 as described by Heron et al. (14). Mutants capable of nodulating McCall soybean, which is not nodulated by parental strain USDA257, were assayed by pooling kanamycin-resistant colonies that were adjusted turbidimetrically to 1×10^8 cells/ml. Roots of 3-day-old McCall seedlings were inoculated with 200 μl of bacterial suspension and transferred to aseptically prepared Leonard jars that had been filled with vermiculite (21). The jars were kept in a growth chamber at a light intensity of 400 μmol of photons/m²/s with a 12-h photoperiod. Nodulation was evaluated 20 days after inoculation. USDA257 Tn5 mutants that produced nodules on McCall soybean were recovered by squeezing the nodule contents on a YEM agar plate containing kanamycin.

Cloning and nucleotide sequence analysis of the genes encoding the glycine cleavage system of USDA257. A cosmid library of the parental strain USDA257 and the Tn5 mutant strain USDA257-15A in pLAFR1 (8) was constructed as described previously (14). To identify cosmid clones containing the Tn5 insertion, the library was streaked on YEM plates containing tetracycline and kanamycin. DNA from four cosmid clones that were resistant to both of the antibiotics were digested individually with EcoRI and BamHI and subjected to Southern blot analysis. An internal HindIII fragment of Tn5 was used as a probe to identify DNA fragments harboring the Tn5. A 7.5-kb EcoRI fragment, which contained USDA257 DNA sequences flanking the Tn5 insertion, was used to probe a cosmid library of the parental strain USDA257. Three cosmid clones that yielded positive hybridization signals were identified. Cosmid DNA was isolated from these clones, restricted individually with EcoRI, and fractionated by agarose gel electrophoresis. The DNA was transferred to a nitrocellulose membrane and hybridized with the ³²P-labeled 7.5-kb EcoRI fragment containing DNA sequences flanking the Tn5 insertion. All the positive cosmid clones exhibited strong hybridization with a 1.5-kb EcoRI fragment. This EcoRI fragment from one of the cosmid clones (pGCV-2) was cloned into pGEM 7zf(+) to obtain pGCV-3 (Table 1). DNA sequencing was performed at the University of Mis-

souri DNA Core Facility by utilizing appropriate primers synthesized by Integrated DNA Technologies (Coralville, IA).

Construction of *gcvT-lacZ* fusion constructs. A 1.5-kb EcoRI DNA fragment which includes the putative promoter sequences of the *gcvT* was cloned in both orientations in the promoter probe plasmid pMP220 (45). Each of these constructs was introduced into *S. fredii* USDA257 by triparental mating using the helper plasmid pRK2013 (7). Transconjugants were grown for 2 days in YEM medium, followed by centrifugation to pellet the cells and suspension in minimal medium. Afterwards, cells were grown in the absence and in the presence of 200 mM glycine for 4 to 6 h. β-Galactosidase activity (35) was measured in each of the induced and noninduced samples.

Mutagenesis and marker exchange of USDA257 *gcvT* and *gcvP* genes. A 3.4-kb PstI fragment (Fig. 1) from pGCV-4 was subcloned into modified pBluescript II SK(+) in which the EcoRI site was removed to produce pGCV-6. For construction of the *gcvT* mutant, a 2.0-kb Ω fragment was cloned into an EcoRI site of pGCV-6 to produce pGCV-6Ω. The 5.4-kb PstI fragment from pGCV-6Ω was cloned into the suicide plasmid pJQ200sk to produce pGCV-7Ω. For construction of a *gcvP* mutant, a similar strategy was used, resulting in the insertion of the Ω fragment into an EcoRV site (Fig. 1). The *gcvT*Ω and *gcvP*Ω constructs were mobilized into USDA257 by triparental mating with helper plasmid pRK2013 (7). 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.

Isolation of extracellular proteins and Western blot analysis. USDA257 and the *gcvT* mutant were grown in YEM medium in either the presence or absence of 1 μM apigenin for 48 h at 30°C. Bacterial cells were pelleted by centrifugation at 12,000 × g for 15 min. To the resulting supernatant 3 volumes of ice-cold acetone was added and left at -20°C overnight. Precipitated proteins were recovered by centrifugation as before and resuspended in SDS sample buffer. Protein samples were boiled for 3 min, and aliquots were resolved by 15% SDS-PAGE (28) using a Hoefer SE260 minigel electrophoresis apparatus (GE

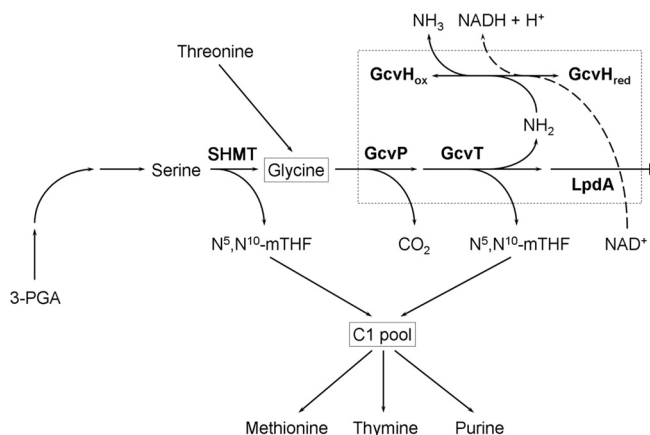


FIG. 1. Metabolic pathway of the glycine cleavage enzyme system. This system is a multimeric assembly of four loosely associated proteins known as P protein (GcvP pyridoxal phosphate-containing glycine decarboxylase), H protein (GcvH, lipoic acid-containing carrier), T protein (GcvT, tetrahydrofolate requiring aminomethyltransferase or glycine synthase), and L protein (GcvL, lipoamide dehydrogenase). 3-PGA, 3-phosphoglycerate; N⁵,N¹⁰-mTHF, N⁵,N¹⁰-methylene tetrahydrofolate; C₁ pool, pool of compounds containing only one carbon.

Healthcare, Piscataway, NJ). Resolved proteins were visualized by silver staining. Western blot analysis was performed using antibodies raised against the entire Nop (for nodulation outer protein) protein SR (also known as signal response [SR] protein) at a final dilution of 1:10,000. Immunoreactive proteins were detected with an enhanced chemiluminescent substrate (Super Signal West Pico kit; Pierce Biotechnology, Rockford, IL) according to the manufacturer's recommendations.

Nodulation tests. Soybean seeds were surface sterilized and germinated on water agar plates as described previously (21). Cells of USDA257 and *gcv* mutants were harvested from liquid YEM medium cultures by centrifugation at 7,700 × g for 15 min. The cell concentrations were adjusted turbidimetrically to 1 × 10⁸ cells/ml. Three-day-old seedlings were dip inoculated with bacterial cells and transferred immediately to autoclaved Leonard jars containing vermiculite and Jensen's nitrogen-free solution (21). 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/m²/s with 12 h of light. Nodules were counted at 20 days postinoculation. Nodulation experiments were repeated two times with five plants per treatment in each experiment. Acetylene reduction rates were determined by the method of Schwinghamer et al. (44).

Light and transmission electron microscopy. Embedment of soybean nodules in paraffin was carried out as described earlier (26). Paraffin-embedded nodules were sectioned (10 μm thick) with a microtome and stained with hematoxylin and eosin. For ultrastructural studies, soybean nodules harvested at 20 days after inoculation were dissected into small pieces (2 to 4 mm) with a razor blade and fixed at room temperature for 4 h in buffered 2.5% glutaraldehyde (pH 7.2; 50 mM sodium phosphate). The tissue samples were washed four times at 15-min intervals with 50 mM phosphate buffer (pH 7.2) and postfixed with 2% aqueous osmium tetroxide for 1 h at room temperature. After extensive rinses in distilled water, the samples were dehydrated in a graded acetone series and infiltrated with Spurr's resin. Thin sections were cut with a diamond knife and collected on uncoated copper grids, stained with 0.5% aqueous uranyl acetate and 0.4% aqueous lead citrate, and viewed with a JEOL JEM 100B (Tokyo, Japan) electron microscope at 100 kV.

Nucleotide sequence accession number. USDA257 *gcvTHP* sequences have been deposited in the GenBank database under accession number GQ214396.

RESULTS AND DISCUSSION

Isolation of a Tn5 mutant regulating soybean cultivar specificity. Previously, two Tn5 mutants of USDA257, DH4 and DH5, that are involved in regulating soybean cultivar specificity were identified and characterized (4, 14, 20, 22, 34). The genes interrupted by the Tn5 insertion in DH4 was found to

code for a type III secretion system (27), while in the case of DH5 the Tn5 inactivated a gene having homology to *dnaI* of *E. coli* (22). In order to identify additional genes involved in soybean cultivar specificity, we extended our search by Tn5 transposon mutagenesis. About 12,000 presumptive Tn5-containing mutants of USDA257 were tested on McCall soybeans, which is not nodulated by the parental strain USDA257. A single mutant, HB15A, capable of nodulating McCall soybeans was selected. Rhizobia were isolated from the nodules and confirmed to be kanamycin resistant and a derivative of USDA257.

As a first step in identifying and characterizing the gene inactivated by Tn5 insertion, total DNA from the mutant was isolated, digested with either EcoRI or BamHI, and subjected to Southern blot analysis utilizing the internal HindIII fragment of Tn5 as a probe. The mutant revealed a single hybridizing EcoRI fragment and two BamHI fragments (data not shown). Since Tn5 has no EcoRI site and contains a single BamHI site, the results of our Southern blot analysis indicate that the mutant contains a single copy of the transposon. To isolate the gene inactivated by Tn5, we constructed a cosmid library of the mutant in pLAFR1 and screened the genomic library with a radiolabeled internal HindIII fragment of Tn5 as a probe. Cosmid DNA from several positive clones was isolated, digested with EcoRI, and subjected to Southern hybridization. All of these cosmid clones showed a single 7.5-kb hybridizing EcoRI fragment. One such cosmid clone (pGCV-1) was selected for further characterization. The Tn5-containing 7.5-kb EcoRI fragment was subcloned into the vector pBSKS. In order to isolate the wild-type fragment, we constructed a genomic DNA library of the parental strain *S. fredii* USDA257. This library was screened with a ³²P-labeled Tn5-containing 7.5-kb EcoRI fragment; six positive clones were identified, and one was studied in detail. Southern blot analysis indicated that the Tn5 transposon was located within a 1.5-kb EcoRI fragment. We also performed Southern blot analysis using genomic DNA isolated from *S. fredii* USDA192 and its *sym* plasmid-cured derivative. In both cases strong hybridization was observed to a 5.7-kb EcoRI fragment, suggesting that this newly identified gene is not localized in the *sym* plasmid.

Since the Tn5 insertion was identified to a 1.5-kb EcoRI DNA fragment, we subcloned this fragment and sequenced it in its entirety. Nucleotide sequence analysis identified an incomplete open reading frame (ORF) at the 3' region of the 1.5-kb DNA fragment. This partial ORF showed extensive homology to the glycine cleavage (*gcv*) system T protein of *E. coli* and several other organisms. In *E. coli* and in other organisms, the T protein is a subunit of the glycine decarboxylase complex (47). This Gcv complex is composed of four proteins: GcvP, GcvH, GcvT, and GcvL (Fig. 1). The GcvP protein (glycine dehydrogenase) catalyzes the pyridoxal-phosphate (pyridoxal-P)-dependent decarboxylation of glycine and transfer of the remaining aminomethyl moiety to the lipoyl prosthetic group of the GcvH protein. The GcvT protein (aminomethyltransferase) catalyzes the release of ammonia from the intermediate attached to the H protein and the synthesis of methylenetetrahydrofolate in the presence of tetrahydrofolate (Fig. 1). To ascertain if a similar operon structure is also present in USDA257, we subcloned the adjacent 5.7-kb EcoRI

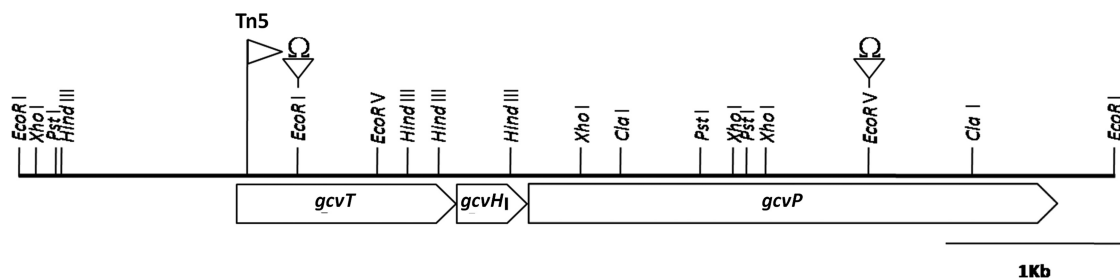


FIG. 2. Coordinated physical and genetic maps of *gcv* region of *S. fredii* USDA257. The orientation of the three ORFs and the location of the Tn5 and omega cassette insertions are also shown.

fragment and determined its nucleotide sequence. The DNA sequence was subjected to computer analysis, and three potential ORFs were identified. All three ORFs were of the same polarity, and they appeared to be part of an operon (Fig. 2). The first ORF was comprised of 387 amino acids encoding a protein with a molecular mass of 41 kDa. The second ORF, which initiated 15 bp after the termination of the first ORF, contained 120 amino acids and encoded a protein with a molecular mass of 12.7 kDa. The third ORF was the largest among the three ORFs, initiated immediately after the second ORF, and was made up of 954 amino acids with a molecular mass of 104 kDa.

Sequence homology among the glycine cleavage system from different organisms. The deduced amino acid sequences of the three open reading frames were subjected to BLAST analysis, which revealed high sequence identities with proteins encoding the glycine cleavage system from other rhizobia. On the basis of extensive sequence homology, we have named the three ORFs *gcvT*, *gcvH* and *gcvP*. The amino acid sequence of USDA257 *gcvT* shows 80 to 87% overall sequence similarity with *gcvT* proteins from *Sinorhizobium medicae*, *Sinorhizobium meliloti*, *Rhizobium leguminosarum* bv. trifolii, and *Rhizobium etli*. Similarly, a high degree of sequence similarity with *gcvH* (85 to 93%) and *gcvP* (84 to 91%) proteins among these rhizobia were also detected. The glycine cleavage system is widely distributed in both prokaryotes and eukaryotes. In order to determine the percent sequence similarity among the GcvT proteins from different organisms, the deduced amino acid sequence of the GcvT protein from *S. fredii* USDA257 was aligned with the corresponding protein sequences from *S. meliloti*, *Agrobacterium*, *E. coli*, *Arabidopsis*, and human and bovine GcvT proteins (Fig. 3). USDA257 revealed 84.5% and 72.3% sequence similarity to the GcvT protein from *S. meliloti* and *Agrobacterium*, respectively. USDA257 also showed 35 to 38% amino acid sequence similarity to GcvT from eukaryotic organisms like *Arabidopsis* and bovine and human proteins (Fig. 3). In contrast, USDA257 and *E. coli* showed only 23.8% amino acid sequence similarity.

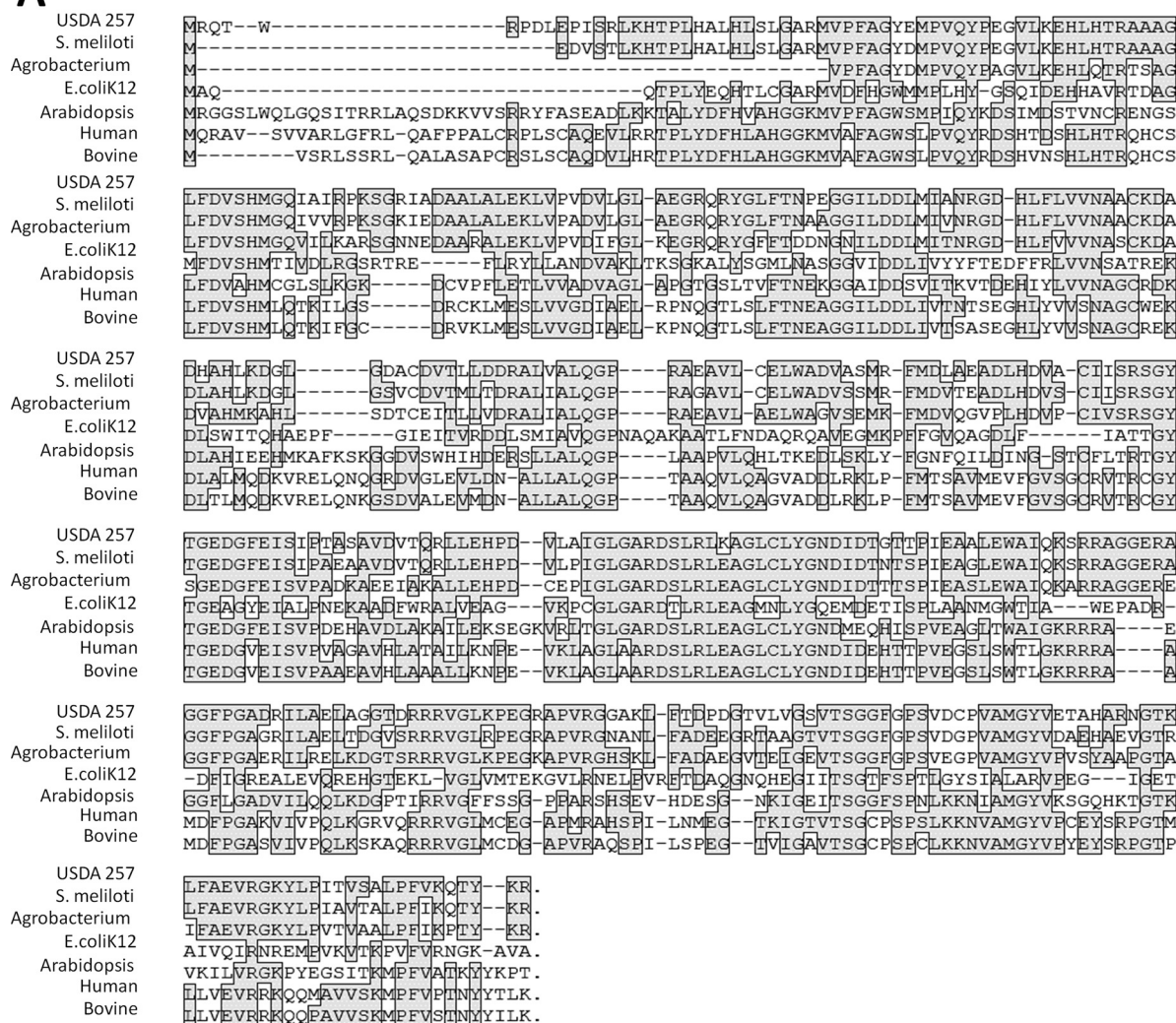
Computerized comparisons of the USDA257 *gcv* operon revealed the presence of similar sequences in *S. medicae*, *S. meliloti*, *R. leguminosarum* bv. trifolii, and *R. etli*. This prompted us to search for homologous sequences in several other rhizobia. The results of Southern blotting in which EcoRI-digested genomic DNAs of 11 rhizobial strains were probed with a 430-bp EcoRI and EcoRV DNA fragment corresponding to *gcvT* demonstrated that a single strong hybrid-

izing fragment was detected in *Bradyrhizobium liaoningense* USDA3622, *Mesorhizobium amorphae* USDA1001, *Rhizobium galegae* USDA4128, *Rhizobium hainanense* USDA3588, *Rhizobium huautlaense* USDA4900, *Rhizobium tropici* USDA9030, *R. leguminosarum* USDA2370, *S. meliloti* USDA1002, and *Sinorhizobium teranga* USDA4894 (Fig. 4). The sizes of the hybridizing fragments showed marked differences. *S. fredii* USDA205 and *Sinorhizobium saheli* USDA4894, however, revealed two strongly hybridizing EcoRI fragments (Fig. 4), indicating either the presence of two copies of *gcvT* or an additional EcoRI site in the *gcvT* coding region.

Recently, a number of genome projects for rhizobia have been completed, facilitating insights into the genome architecture of these symbiotic bacteria (31). An examination of the available complete rhizobial genome sequences revealed the presence of a *gcv* locus in *Rhizobium* sp. NGR234 (43), *S. meliloti* 1021 (9), *R. leguminosarum* bv. viciae (50), *R. leguminosarum* bv. trifolii (39), *R. etli* (11), *Bradyrhizobium japonicum* USDA110 (17), *Bradyrhizobium* sp. ORS278 (10), *Azorhizobium caulinodans* ORS571 (29), and *Mesorhizobium loti* MAFF303099 (16). Additionally, a *gcv* locus was also identified in the genome of the betaproteobacteria *Burkholderia phymatum* STM815 and *Cupriavidus taiwanensis* LMG19424 (1), both of which form root nodules on legumes. The organization of the *gcv* operon in these symbiotic nitrogen-fixing bacteria was similar to that of *S. fredii* USDA257 with the exception of *R. leguminosarum* bv. viciae and *A. caulinodans* ORS 571. In the case of *R. leguminosarum* bv. viciae, the *gcvH* was not identified in between *gcvT* and *gcvP* (Fig. 2). In contrast, the *gcvP* was not a part of the *gcv* operon in *A. caulinodans* ORS571. Proteins encoded by the *gcv* loci from these diverse symbiotic bacteria had similar molecular weights and exhibited significant amino acid sequence similarities between them. The amino acid sequence similarities of GcvT, GcvH, and GcvP among USDA257, *Rhizobium* sp. NGR234, and *S. meliloti* ranged from 85 to 95%. A similar comparison of the amino acid sequences between these proteins among USDA257 and the two betaproteobacteria *B. phymatum* STM815 and *C. taiwanensis* LMG19424 revealed similarities ranging from 40 to 58%, indicating a distinct phylogenetic relationship among these diverse symbiotic bacteria.

USDA257 *gcvTHP* operon is inducible by glycine. In *E. coli*, the expression of the glycine cleavage enzyme system is induced by glycine (33, 46), and the transcriptional regulation of the *gcvTHP* operon has been studied extensively. To test if the USDA257 *gcvTHP* operon is also inducible by glycine, we

A



B

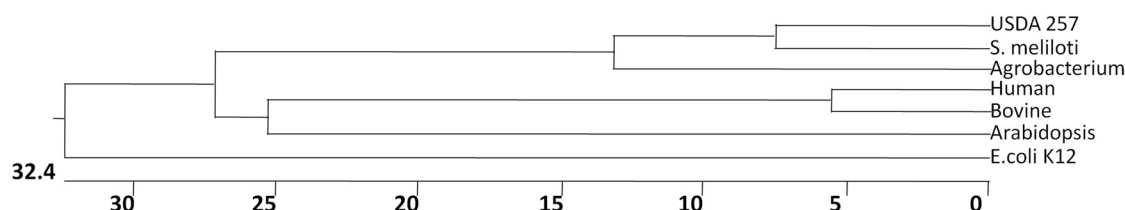


FIG. 3. Multiple amino acid sequence alignment of GcvT proteins from different organisms. (A) The accession numbers of the sequences are as follows: *S. meliloti*, AL591688; *Agrobacterium*, AE007869; *E. coli*, AP009048.1; *Arabidopsis*, AT1G11860; human, NM_000481; bovine, NM_177485. Sequences are aligned with the USDA257 GcvT sequence (GQ214396). Positions of amino acid identity are boxed. Multiple sequence alignment analysis was performed using the CLUSTAL W program from UniProt Knowledgebase (<http://www.uniprot.org/>). (B) Phylogenetic tree of GcvT. The scale shown in the phylogenetic tree represents the branch distance as the number of changes in character state between GcvT proteins from different organisms.

cloned a 1.5-kb EcoRI fragment (Fig. 2), which contains about 1,173 bp of DNA sequence upstream of the *gcvT* start codon, in both orientations into the promoter probe plasmid pMP220. Each of these constructs was then introduced into *S. fredii* USDA257, and their responsiveness to the addition of 200 mM glycine was evaluated by measuring the β -galactosidase activity of the transconjugants. USDA257 carrying the pMP220 or 1.5-kb EcoRI fragment cloned in the wrong orientation in

either the presence or absence of glycine revealed a basal level of β -galactosidase activity (less than 200 Miller units). USDA257 carrying the 1.5-kb EcoRI fragment cloned in the correct orientation and grown in the absence of glycine showed 224 ± 22 Miller units of β -galactosidase activity. However, when this transconjugant was grown in the presence of glycine, the activity of β -galactosidase was about seven times greater ($1,646 \pm 90$ Miller units) than the values obtained from the

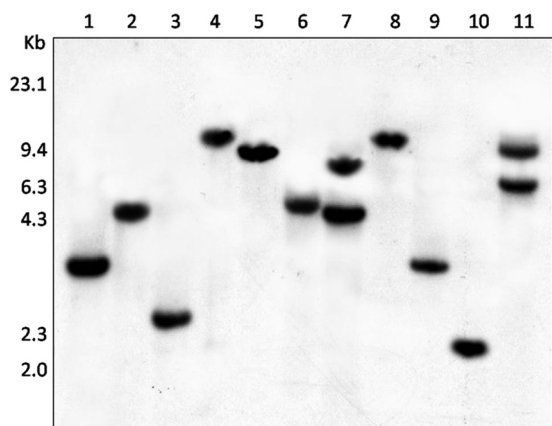


FIG. 4. Southern blot analysis of *gcvT* in rhizobia. Genomic DNAs from *B. liaoningense* USDA3622 (lane 1), *R. galegae* USDA4128 (lane 2), *R. hainanense* USDA3588 (lane 3), *R. huautlaense* USDA4900 (lane 4), *R. tropici* USDA9030 (lane 5), *M. amorphae* USDA1001 (lane 6), *S. fredii* USDA205 (lane 7), *S. meliloti* USDA1002 (lane 8), *S. teranga* USDA4894 (lane 9), *R. leguminosarum* USDA2370 (lane 10), and *S. saheli* USDA4893 (lane 11) were restriction enzyme digested with EcoRI and separated electrophoretically in 0.8% agarose. The gel was blotted onto nitrocellulose and probed with the ^{32}P -labeled *S. fredii* USDA257 *gcvT* gene. Molecular weight markers in kilobases are shown on the left side of the figure.

uninduced cultures, indicating that the USDA257 *gcvTHP* operon is inducible by glycine.

Inactivation of *gcvTHP* operon of USDA257 alters symbiotic behavior. The effect of inactivation of the glycine cleavage system was examined on several agronomically improved North American soybean cultivars (Table 2). Both USDA257 and USDA257-15A were able to form nitrogen-fixing nodules on Beijing soybean. In contrast, when agronomically improved soybean cultivars were inoculated with USDA257, no nodules were produced while the *gcvT* mutant (USDA257-15A) formed large nodules whose interiors were pink, presumably due to the accumulation of leghemoglobin. Nodules harvested from agronomically improved cultivars 20 days after inoculation with USDA257-15A showed significant acetylene reduction, ranging from 18.8 to 32.6 $\mu\text{mol/h/g}$ of fresh nodule weight. We also complemented the *gcvT* mutant by introducing a cosmid clone carrying the wild-type *gcv* operon of USDA257 and tested its nodulation phenotype on McCall soybean. Interestingly, even the complemented strain produced nodules on McCall soybean. However, the rhizobia recovered from those nodules were resistant to kanamycin but not to tetracycline. This indicates that the bacteria forming these nodules had lost the cosmid containing the wild-type *gcv* operon. This result is to be expected because selection generally favors the Nod^+ phenotype (14). To obtain further evidence that the glycine cleavage system regulates the host range of USDA257, we created two independent mutants (*gcvT Ω and *gcvP Ω) and tested their ability to form nodules on several agronomically improved North American soybean cultivars (Table 2). Both of these mutants were able to form nitrogen-fixing nodules with all the soybean hosts. Thus, inactivation of the glycine cleavage system clearly enables USDA257 to initiate nitrogen-fixing nodules on agronomically improved North American soybean cultivars.**

S. fredii USDA191, a close relative of USDA257, forms nitrogen-fixing nodules on agronomically improved soybean cultivars (2, 4, 13). To investigate if the nodules initiated by USDA257 *gcv* mutants were similar to those of USDA191, we examined the anatomical structure of the nodules with both light and transmission electron microscopy. Light microscopic examination of nodules initiated by *gcv* mutants on agronomically improved soybean cultivars exhibited a structure that was typical of determinate nodules (Fig. 5A). The nodule, which was differentiated into a central bacteria-filled region, was separated from the outer cortex region by a layer of sclerenchyma cells. In addition, several vascular bundles were seen in the outer cortex. Transmission electron microscopy of thin sections of the central region revealed the presence of cells that were filled with bacteria that were enclosed by symbiosomes (Fig. 5B). These bacteroids contained numerous prominent polyhydroxybutyrate inclusions. The anatomical features of nodules induced by *gcv* mutants and those from USDA191 were indistinguishable.

Inactivation of the *gcvTHP* operon does not affect the secretion of nodulation outer proteins. Previously, we have demonstrated that USDA257, when grown in the presence of the *nod* gene inducing flavonoids, can secrete *nod* factors and several extracellular proteins into the rhizosphere (3, 23). The secreted proteins were termed nodulation outer proteins (Nops), and their secretion was regulated by both *nodD1* and *nodD2* and was dependent on the soybean cultivar specificity locus *nodBTUVW* (25). This locus was found to code for components of the type III secretion system (T3SS) (20, 27). Mutants in this locus are defective in T3SS and consequently secrete only some or none of the nodulation outer proteins that have been demonstrated to have profound effects on nodulation (6, 12, 32, 49). For example, USDA257DH4, a Tn5 mutant of USDA257, is unable to secrete several Nops. Consequently, this mutant, unlike the wild-type strain, was able to nodulate agronomically improved soybean cultivars, suggesting an important role for the Nops in regulating soybean cultivar specificity (27, 30). To determine if the mutation in the *gcv* operon of USDA257 has any effect on Nops, we isolated extracellular proteins from USDA257 and the *gcv* mutant that were grown in either the presence or absence of apigenin, a potent inducer of the *nod* genes of USDA257 (21, 37). SDS-PAGE analysis followed by silver staining of the gels revealed no major

TABLE 2. Symbiotic phenotypes of USDA257 and *gcv* mutants

Legume species	Nodulation response with ^a :			
	USDA257	HBK257-15A	HBK <i>gcvT</i> - Ω	HBK <i>gcvP</i> - Ω
<i>Glycine max</i> cultivar or strain				
Beijing	+	+	+	+
McCall	-	+	+	+
Jack	-	+	+	+
Clark	-	+	+	+
Maverick	-	+	+	+
Pio94Y20	-	+	+	+
MFA4249	-	+	+	+
<i>Vigna unguiculata</i>	+	+	+	+

^a +, nitrogen-fixing nodules; -, no nodules.

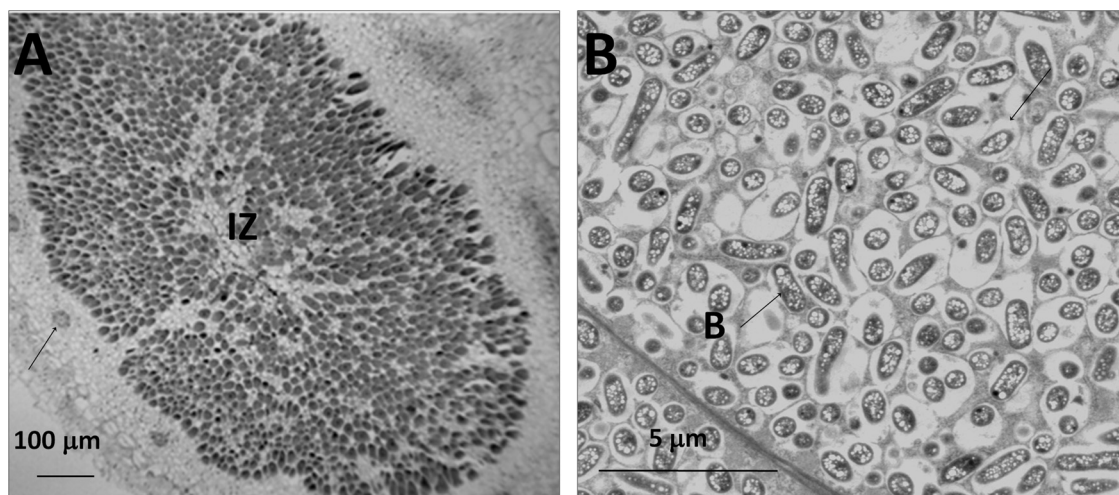


FIG. 5. Anatomy of soybean (*G. max* cv. McCall) nodules formed by the USDA257 *gcvT* mutant. (A) Light micrograph of a paraffin section of soybean nodule revealing a central infected zone (IZ). The arrows point to vascular bundles in the cortex. (B) Transmission electron micrograph of soybean nodule showing the presence of numerous bacteroids (B) which are surrounded by symbiosomes. Note the presence of numerous poly- β -hydroxybutyrate inclusions inside the bacteroids.

changes in the protein profiles between the parental strain and the *gcv* mutant (Fig. 6A). Western blot analysis utilizing antibodies raised against flavonoid-induced extracellular proteins of USDA257 clearly demonstrates that USDA257 secretes several proteins into the extracellular milieu when it is grown in the presence of flavonoids (Fig. 6B). This includes NopA, NopB, NopL, NopP, and NopX. The same set of Nops was also elaborated by the *gcv* mutant (Fig. 6). It is clear from this observation that *gcv* mutation has no obvious effect on the Nop production of USDA257. This observation is in drastic contrast

with USDA257DH4, another Tn5 mutant extensively studied in our laboratory (3, 4, 14, 20, 25, 27). Thus, it is evident that in addition to Nops, there are other yet unidentified factors that could influence soybean cultivar specificity.

The glycine cleavage system is the most important pathway in serine and glycine catabolism in various vertebrates including humans. Nonketotic hyperglycinemia, a genetic disorder characterized by abnormally high levels of glycine in human infants, results from defective glycine cleavage activity (18). It is not clear why inactivation of the glycine cleavage system of USDA257 enables this strain to nodulate soybean cultivar McCall. Previously, a Tn5 mutant of *B. japonicum* was reported to elicit pseudonodule-like structures on soybean (41). Subsequent studies have shown that the Tn5 mutation was located in the *glyA* gene which encodes serine hydroxymethyltransferase (SHMT) (Fig. 1) (40). SHMT catalyzes the biosynthesis of glycine from serine and the transfer of a one-carbon unit to tetrahydrofolate (40). Even though the specific role of SHMT and its reaction products in causing defects in early nodule and bacteroid development is not fully understood, it is speculated that an adequate supply of glycine and/or a functioning C₁ metabolism are essential for effective nodulation of soybean by *B. japonicum* (40). In contrast to *B. japonicum*, where inactivation of *glyA* leads to ineffective nodulation, the inactivation of the *gcv* locus of USDA257 leads to effective nodulation of soybean cultivars that are not originally nodulated by the parental strain. It is believed that both the *gly* and *gcv* loci are involved in the generation of C₁ units, respectively (33). C₁ units are used in a variety of biochemical reactions, including the synthesis of purines, histidine, thymine, and methionine and the formylation of aminoacylated initiator tRNA (46). We speculate that inactivation of the *gcv* locus could have altered some essential function of the cell, resulting in modification or synthesis of signal molecule(s) that may regulate soybean cultivar specificity. Further studies are re-

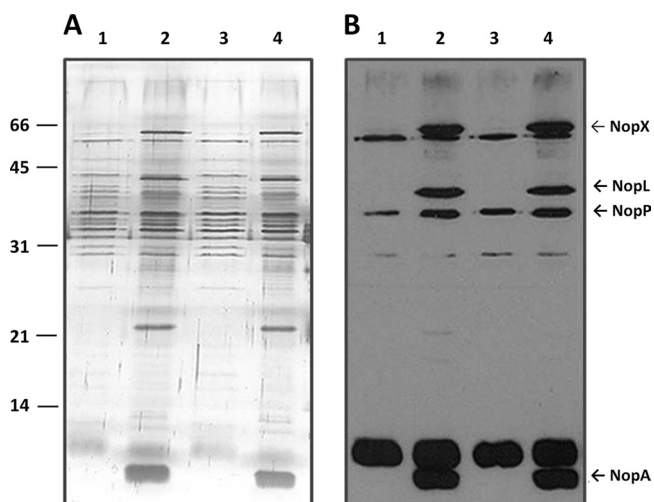


FIG. 6. Extracellular protein profile and immunoblot analysis of USDA257 and the USDA257 *gcvT* mutant. Extracellular proteins were prepared from cells grown in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 1 μ M apigenin. The proteins were resolved by 15% SDS-PAGE and silver stained (A) or transferred to a nitrocellulose membrane for immunological analysis with antibodies raised against Nops (B). Lanes 1 and 2, extracellular proteins from USDA257; lanes 3 and 4, extracellular proteins from the USDA257 *gcvT* mutant. The immunoreactive proteins are identified on the right side of the figure.

quired to elucidate the precise role of the *gcv* locus in regulating soybean cultivar specificity.

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The use of product names is necessary to report factually on available data; however, neither the University of Missouri nor USDA either guarantees or warrants any product mentioned, and the use of the name by the University of Missouri or USDA implies no approval of the product to the exclusion of others that may be suitable.

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