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A Functional *myo*-Inositol Dehydrogenase Gene Is Required for Efficient Nitrogen Fixation and Competitiveness of *Sinorhizobium fredii* USDA191 To Nodulate Soybean (*Glycine max* [L.] Merr.)

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Inositol derivative compounds provide a nutrient source for soil bacteria that possess the ability to degrade such compounds. *Rhizobium* strains that are capable of utilizing certain inositol derivatives are better colonizers of their host plants. We have cloned and determined the nucleotide sequence of the *myo*-inositol dehydrogenase gene (*idhA*) of *Sinorhizobium fredii* USDA191, the first enzyme responsible for inositol catabolism. The deduced IdhA protein has a molecular mass of 34,648 Da and shows significant sequence similarity with protein sequences of *Sinorhizobium meliloti* IdhA and MocA; *Bacillus subtilis* IolG, YrbE, and YucG; and *Streptomyces griseus* StrI. *S. fredii* USDA191 *idhA* mutants revealed no detectable *myo*-inositol dehydrogenase activity and failed to grow on *myo*-inositol as a sole carbon source. Northern blot analysis and *idhA-lacZ* fusion expression studies indicate that *idhA* is inducible by *myo*-inositol. *S. fredii* USDA191 *idhA* mutant was drastically affected in its ability to reduce nitrogen and revealed deteriorating bacteroids inside the nodules. The number of bacteria recovered from such nodules was about threefold lower than the number of bacteria isolated from nodules initiated by *S. fredii* USDA191. In addition, the *idhA* mutant was also severely affected in its ability to compete with the wild-type strain in nodulating soybean. Under competitive conditions, nodules induced on soybean roots were predominantly occupied by the parent strain, even when the *idhA* mutant was applied at a 10-fold numerical advantage. Thus, we conclude that a functional *idhA* gene is required for efficient nitrogen fixation and for competitive nodulation of soybeans by *S. fredii* USDA191.

Sinorhizobium fredii and *Bradyrhizobium* spp. form nitrogen-fixing nodules on soybeans. On Midwestern soils, soybeans are predominantly nodulated by *Bradyrhizobium japonicum* serogroup 123 (34). However, serogroup 123 is a poor nitrogen fixer; hence, attempts have been made to overcome this problem. Commercial inoculants with better nitrogen-fixing ability than serogroup 123 are often utilized to enhance biological nitrogen fixation. However, serogroup 123 is extremely competitive on Midwestern soils and is able to exclude the introduced strains (17). Therefore, it becomes imperative that competitiveness of the commercial strains be improved to obtain beneficial effects from a commercial inoculum. We are interested in improving the nitrogen-fixing ability of *S. fredii* USDA191. This strain is a fast-growing Chinese isolate that is able to form nitrogen-fixing nodules on soybean and several other legumes (18, 21). *S. fredii* USDA191 is better suited for commercial inoculation production because it is a fast grower and produces fewer extracellular polysaccharides than the traditional symbiont *B. japonicum*. We are currently exploring strategies that will enhance USDA191 competitiveness relative to the indigenous rhizobia.

Symbiotic nitrogen fixation has been shown to be elevated in *Rhizobium* strains that have an increased acid tolerance, an uptake of hydrogenase, reduced levels of cytochrome *o*, increased expression of the nitrogen fixation gene (*nifA*) and C_4-dicarboxylic acid transport genes (*dctABD*), and a flavonoid-independent hybrid NodD (8, 9, 10, 37, 39, 48). Ronson et al. (31) constructed rhizobial strains with additional copies of *nifA* and *dctABD* and demonstrated that such a genetically modified strain increased plant biomass under controlled environmental conditions. Bosworth and others (7) extended this study to field conditions. One of the recombinant *Rhizobium meliloti* strains (RMBPC-2) increased the alfalfa biomass by 13% when compared to the wild-type strain RMBPC on sites where soil nitrogen and organic matter content were lowest. However, on locations where soil nitrogen concentrations were high and native rhizobial populations were large, the recombinant rhizobium did not affect the yield of alfalfa (*Medicago sativa* [L.]) (7). In this study, the extra copies of *nif*A and *dctABD* genes were inserted in two symbiotically silent sites. The first site was referred to as the P3 region (4, 5) and the second as the *ino* region (46). The *ino* region is involved in inositol catabolism. Interestingly, when *dctABD* genes were inserted in the *ino* region, an increase in plant biomass was observed. However, insertion of the same genes in the P3 region resulted in a yield decrease (36). Based on this observation, it was suggested that the *ino* region may not be symbiotically silent and that inactivation of this region may actually benefit the host plants (36).

Plants secrete a wide array of compounds, some of which can

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be used as a carbon and/or nitrogen source by the rhizosphere bacteria. *myo*-Inositol is abundant in soil, and several microorganisms, including *Rhizobium leguminosarum* bv. viciae and *Sinorhozobium meliloti,* can grow using inositol as the sole carbon source (1, 14, 28). *Rhizobium* strains containing catabolism genes for the degradation of inositol may have a competitive advantage since inositol is abundant in the rhizosphere (47). Recently, Galbraith et al. (14) isolated a *myo*-inositol dehydrogenase gene (*idhA*) from *S. meliloti* and demonstrated that the activity of this gene product is essential for inositol catabolism as well as rhizopine utilization. A previous study established that bacterial strains that were capable of utilizing rhizopine had a fitness advantage and were able to nodulate their host plants more efficiently (15). These studies, along with the speculation that the inositol locus may have a role in symbiosis (36), prompted us to investigate the role of *myo*-inositol dehydrogenase in soybean-*S. fredii* USDA191 symbiosis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this work are listed in Table 1. Rhizobia were grown on a reciprocal shaker at 30°C in yeast extract mannitol (YEM) medium (44), and *Escherichia coli* was cultured in Luria-Bertani broth at 37°C (33). When appropriate, antibiotics were added at the following concentrations: kanamycin, 75 μg/ml; tetracycline, 10 μg/ml; spectinomycin, 50 μg/ml; gentamicin, 10 μg/ml; ampicillin, 100 μg/ml; and trimethoprim, 10 μg/ml (for counterselection against *E. coli* donor strains).

Molecular techniques. Recombinant DNA techniques were performed according to standard protocols (33). Rhizobial genomic DNA was isolated according to the method described previously by Jagadish and Szalay (16), and DNA probes were labeled with [^{32}P]dCTP by using a Multiprime DNA Labeling System (Amersham Life Science, Cleveland, Ohio). The construction of the cosmid clone bank of *S. fredii* USDA191 used in this study was described previously (3). DNA sequencing was performed with a *Taq* Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, Calif.) at the University of Missouri.

Cloning and nucleotide sequence analysis of *idhA*. Based on the published sequence of *idhA* from *S. meliloti* (GenBank accession no. AF059313) we designed two primers: 5′-CAGTGATGACAGTGAGATTTG-3′ and 5′-TGCTGA TCTGGCGCTTTTCC-3′. These primers were used to amplify a 990-bp DNA fragment from genomic DNA of *S. fredii* USDA191 by PCR. The PCR product was cloned into pGEM-T Easy vector (Promega, Madison, Wis.) to produce pHBK191–1. We used this 990-bp PCR fragment as a hybridization probe to screen a cosmid library of *S. fredii* USDA191 constructed in the vector pLAFRI (12). Three positive colonies were identified that yielded positive hybridization signals. Cosmid DNA was isolated from these three clones and digested with the restriction endonuclease *Eco*RI and separated by agarose gel electrophoresis. The DNA was transferred to a nylon membrane and hybridized with ^{32}P-labeled 990-bp PCR product. All three positive cosmid clones revealed strong hybridization to a 2.1-kb *Eco*RI fragment. The 2.1-kb *Eco*RI fragment was subsequently cloned into pGEM-7zf(+) to produce pHBK191–2. A 1.3-kb region from pHBK191–2 was sequenced with appropriate primers synthesized by the DNA Core Facility at the University of Missouri.

Construction of strains and plasmids. A 2.4-kb *Xho*I fragment (Fig. 1) from one of the cosmid clones was subcloned into pGEM-7zf(+) and pHBK191–6 to produce pHBK191–3 and pHBK191–7. For the construction of the *idhA* mutant (HBK101), a 1.3-kb *Eco*RI kanamycin cassette from pUC-4K (40) was inserted into the *Eco*RI site of pHBK191–7 to produce pHBK191–8. A 3.7-kb *Xho*I fragment from pHBK191–8 was purified from the gel and cloned into the *Sma*I site of the suicide plasmid pJQ200uc1 (29) to yield pHBK191–9. A second *idhA* mutant (HBK106) was created by digesting pHBK192 DNA with *Sal*I, which resulted in a loss of a 120-bp fragment in the coding region of *idhA* (Fig. 1A). A 2.0-kb Ω fragment was inserted into the above *Sal*I site to yield pHBK191–10. A 4.2-kb *Eco*RI fragment from pHBK191–10 was cloned into the *Eco*RI site of pHBK191–7 to yield pHBK191–11. A 4.4-kb *Xho*I fragment was isolated from pHBK191–11 and cloned into the *Sma*I site of pJQ200uc1 to yield pHBK191–12. pHBK191–9 and pHBK191–1 were individually transferred into *S. fredii* USDA191 by triparental mating using the helper plasmid pRK2013 (11). Marker exchange was achieved by selection on YEM plates containing 5% (wt/vol) sucrose. Mutants were confirmed by Southern blotting and hybridization with the wild-type region.

Northern analysis. Five-milliliter starter cultures of *S. fredii* USDA191, HBK101, and HBK106 were grown at 30°C in YEM overnight. The cells were harvested by centrifugation and washed with minimal media. The cells were transferred to a fresh 20 ml of minimal media and grown for another 8 h either in the presence or absence of 0.2% *myo*-inositol. Total RNA was isolated by the hot-phenol method (45). Fifteen micrograms of RNA was resolved on a 1.5% agarose gel containing formaldehyde and transferred to a nylon membrane by capillary blotting. After transfer, the membrane was baked at 80°C for 2 h and hybridized with the *S. fredii* USDA191 *idhA* gene that had been labeled with [^{32}P]dCTP. Prehybridization (4 h) and hybridization (18 h) were at 68°C in a solution containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 10× Denhardt's solution, 0.1% (wt/vol) sodium dodecyl sulfate (SDS), and 100 μg of denatured salmon sperm DNA/ml. After hybridization, membranes were washed twice for 20 min at 68°C in 2× SSC–0.5% SDS and were then washed for 30 min at 68°C in 0.5× SSC–0.5% SDS.

Enzyme assays. Bacteria were grown in 250 ml of minimal medium with 0.2% succinate as the carbon source and 0.02% *myo*-inositol as the inducer. The specific *myo*-inositol dehydrogenase activity was measured according to the method described previously by Poole et al. (28) and was expressed as nanomoles of NADH reduced min^{-1}/milligram of protein. The values represent the mean of three determinations.

Nodulation and nitrogenase assays. Nodulation assays were performed as described previously by Balatti et al. (2). Acetylene reduction rates were determined by the method of Schwinghamer et al. (35).

Assessment of competition for nodulation. Soybean cv. McCall seeds were surface sterilized and germinated on 1% water agar at 30°C for 3 days (19). Rhizobial strains HBK102 and HBK103 (Table 1) were pelleted from log-phase cultures, washed in YEM, and resuspended in YEM to 10^8 cells/ml. Bacterial growth in liquid cultures was estimated turbidimetrically relative to a standard curve that had been validated by bacterial counts with a Petroff-Hauser counter. For assessing the competition for soybean nodulation, strains HBK102 and HBK103 were mixed to provide ratios of 1:0, 1:1; 1:5, 1:10, 0:1, 5:1, and 10:1. Three-day-old soybean seedlings were dipped into bacterial suspensions, and the seedlings were transferred to autoclaved, plastic growth pouches that had been premoistened with nitrogen-free nutrient solution. The pouches were incubated in a growth chamber at 400 μmol of photons/m^2/s with a 12-h photoperiod. Twenty-five days after inoculation, 100 nodules were randomly selected from a total of 30 plants per treatment and were histochemically stained for β-galactosidase activity.

β-Galactosidase assay. The β-galactosidase assays were performed as described previously by Miller (24). In planta expression of *idhA-lacZ* was measured histochemically as described previously by Krishnan and Pueppke (20). Briefly, soybean nodules were sliced in two, and halves were fixed in glutaraldehyde and treated with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), essentially as described by Boivin et al. (6). Each nodule was viewed by bright-field microscopy for the presence of blue, an indication of β-galactosidase activity driven by the *idhA* promoter. Photographs representative of these nodules were recorded on Kodak Gold 200 film (Eastman Kodak Company, Rochester, N.Y.) with the help of an Olympus SZH microscope (Olympus Optical Co., Ltd., Tokyo, Japan).

Ultrastructural analysis. Soybean nodules induced by *S. fredii* USDA191 and HBK101 were harvested at 15 and 25 days after inoculation. They were cut into small cubes with a razor blade and were immediately fixed in 2.5% glutaraldehyde buffered at pH 7.2 with 50 mM sodium phosphate for 4 h at room temperature. After the primary fixation, the samples were washed extensively with several changes of phosphate buffer. Tissue samples were then postfixed with 2% aqueous osmium tetroxide and embedded in Spurr's resin as described previously by Krishnan et al. (22). Thin sections were cut with a diamond knife on an ultramicrotome, mounted on uncoated 200-mesh nickel grids, and stained with uranyl acetate and lead citrate. Sections were examined with a JEOL JEM 100B (JEOL USA, Inc., Peabody, Mass.) electron microscope at 80 kV.

RESULTS

Cloning the *S. fredii* USDA191 *idhA* gene region. To isolate the *idhA* gene of *S. fredii* USDA191, we synthesized primers corresponding to the 5′ and 3′ of the published *S. meliloti idhA* gene sequence (13). These primers were utilized to amplify a

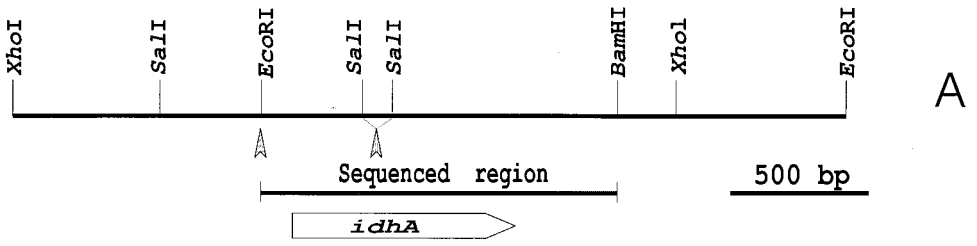
TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Strain		
S. fredii USDA191	Nod$^+$ on soybean	18
S. meliloti USDA1002	Nod$^+$ on alfalfa	USDA-ARS[a]
S. terangae USDA4894	Nod$^+$ on *Acacia* spp.	USDA-ARS
S. medicae USDA1037	Nod$^+$ on *Medicago* spp.	USDA-ARS
S. saheli USDA4893	Nod$^+$ on *Sesbania* spp.	USDA-ARS
B. japonicum USDA6	Nod$^+$ on soybean	USDA-ARS
B. liaoningense USDA3622	Nod$^+$ on *Arachis hypogaea*	USDA-ARS
B. elkanii USDA76	Nod$^+$ on soybean	USDA-ARS
Rizobium sp. NGR234	Broad host range	W. J. Broughton
R. etli USDA9032	Nod$^+$ on *Phaseolus vulgaris*	USDA-ARS
R. galegae USDA4128	Nod$^+$ on *Galega* spp.	USDA-ARS
R. mongolense USDA1844	Nod$^+$ on *Medicago ruthenica*	USDA-ARS
R. tropici USDA9030	Nod$^+$ on *P. vulgaris*	USDA-ARS
R. leguminosarum USDA2370	*Nod*$^+$ on peas	USDA-ARS
M. loti USDA3471	Nod$^+$ on *Lotus japonicus*	USDA-ARS
M. amorphae USDA10001	Nod$^+$ on *Amorpha fruticosa*	USDA-ARS
M. ciceri USDA3383	Nod$^+$ on *Astragalus cicer*	USDA-ARS
M. huakuii USDA4479	Nod$^+$ on *Astragalus sinicus*	USDA-ARS
M. mediterraneum USDA3392	Nod$^+$ on *Cicer arietinum*	USDA-ARS
M. plurifarium USDA3707	Nod$^+$ on *Acacia* spp.	USDA-ARS
M. tianshanense USDA3592	Arid saline desert soil isolate	USDA-ARS
HBK101	*idhA* mutant of USDA191, Kan^r	This work
HBK102	USDA191 containing pMP220	This work
HBK103	HBK101 carrying the *idhA* promoter-*lacZ* fusion	This work
HBK104	HBK101 carrying pLAFRI	This work
HBK105	HBK101 carrying pHBK191-4	This work
HBK106	*idhA* mutant of USDA191, Spc^r	This work
E. coli DH5α	ϕ80*lacZ*ΔM15Δ(*lacZYA-argF*) U169 *hsdR17 recA1 endA1 thi-1*	Gibco BRL
Plasmid		
pGEM-7zf(+)	Ap^r	Promega
pBluescript II SK(+)	Ap^r	Stratagene
pGEM-T Easy	Ap^r	Promega
pMP220	Tc^r	38
pJQ200uc1	Gm^r	29
pUC-4K	Km^r	43
pHBK191-1	Ap^r, 990-bp *idhA* PCR product in pGEM-T Easy	This work
pHBK191-2	Ap^r, 2.1-kb *Eco*RI *idhA*-containing fragment in pGEM-7zf(+)	This work
pHBK191-3	Ap^r, 2.4-kb *Xho*I *idhA*-containing fragment in pGEM-7zf(+)	This work
pHBK191-4	Tc^r, pLAFRI cosmid containing *idhA*	This work
pHBK191-5	Tc^r, 900-bp *idhA* promoter sequences in pMP220	This work
pHBK191-6	Ap^r, pBluescript II SK(+) modified to remove *Sma*I, *Eco*RI, and *Eco*RV restriction sites	This work
pHBK191-7	Ap^r, 2.4-kb *Xho*I *idhA*-containing fragment in pHBK191-6	This work
pHBK191-8	Ap^r and Km^r, 1.3-kb Km cassette into *Eco*RI site of pHBK191-7	This work
pHBK191-9	Gm^r and Km^r, 3.7-kb *Xho*I fragment from pHBK191-7 cloned in pJQ200uc1	This work
pHBK191-10	Ap^r and Spc^r, 2.0-kb Ω fragment cloned into *Sal*I site of pHBK191-7	This work
pHBK191-11	Spc^r, 4.2-kb *Eco*RI fragment from pHBK191-7 cloned into the *Eco*RI site of pHBK191-7	This work
pHBK191-12	Gm^r and Spc^r, 4.4-kb *Xho*I fragment from pHBK191-11 cloned into the *Sma*I site of pJQ200uc1	This work

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

990-bp fragment from *S. fredii* USDA191 genomic DNA. Using this PCR product as a hybridization probe, we screened a genomic cosmid library of *S. fredii* USDA191. Three positive cosmids were identified by colony hybridization. Southern blot analysis revealed that all three cosmids contained a 2.1-kb *Eco*RI hybridization DNA fragment. This fragment was subcloned in pGEM-7zf(+) to produce the plasmid pHBK191–2. Subsequently, a 2.4-kb *Xho*I fragment that overlaps the 2.1-kb *Eco*RI fragment was also cloned. The physical map of this DNA region is shown in Fig. 1A.

Nucleotide sequence of *idhA*. In order to characterize the *idhA* gene, the DNA sequence of a 1,301-bp *Eco*RI and *Bam*HI fragment was determined (Fig. 1B). Analysis of the DNA sequence using the open reading frame (ORF) finder program identified a 990-bp-long ORF. Twenty base pairs downstream of the stop codon, a palindromic structure with the potential to form a stem-loop structure was identified (Fig. 1B). The predicted ORF encodes a protein of 329 amino acids with a molecular weight of 34,648. The theoretical isoelectric point of the protein was estimated to be 5.39. The amino acid

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   1 - GAATTCGAGAGAGACGAAGATGACAGTGAGATTTGGTCTTCTGGGCGCCGGACGCATCGG - 60
                       M  T  V  R  F  G  L  L  G  A  G  R  I  G
  61 - CAAGGTTCACGCGAAAGCCGTCAGCGGCAATCCGGACGCCGTGCTCGTGGCGGTTGCCGA - 120
       K  V  H  A  K  A  V  S  G  N  P  D  A  V  L  V  A  V  A  D
 121 - TGCCTTTCCGGCCGCCGCTGAAGCAATCGCCAAGGCCTATGGCTGCGAGGTTCGCAGCAT - 180
       A  F  P  A  A  A  E  A  I  A  K  A  Y  G  C  E  V  R  S  I
 181 - CGAGGCGATCGAGGCGGCCTCCGACATCGACGCCGTGGTCATCTGCACGCCGACCGACAC - 240
       E  A  I  E  A  A  S  D  I  D  A  V  V  I  C  T  P  T  D  T
 241 - GCATGCCGACCTGATCGAGCGTTTTGCCCGGGCCGGCAAGGCCATCTTCTGCGAAAAGCC - 300
       H  A  D  L  I  E  R  F  A  R  A  G  K  A  I  F  C  E  K  P
 301 - GATCGATCTCGACGTCGATCGCGTCAAGGCCTGCCTGAAGGTGGTCTCCGAAACCGGGGC - 360
       I  D  L  D  V  D  R  V  K  A  C  L  K  V  V  S  E  T  G  A
 361 - GAAGCTGATGGTCGGCTTCAACCGCGCTTCTACCCCATTTATGGCCGTGCGAAAGGCGAT - 420
       K  L  M  V  G  F  N  R  A  S  T  P  F  M  A  V  R  K  A  I
 421 - CGACGCCGGCACGATTGGCGACGTCGAGATGGTGACGATCACCTCGCGCGATCCGGGCGC - 480
       D  A  G  T  I  G  D  V  E  M  V  T  I  T  S  R  D  P  G  A
 481 - CCCGCCGGTCGACTATATCAAGCGGTCGGGCGGCATCTTCCGCGACATGACGATCCACGA - 540
       P  P  V  D  Y  I  K  R  S  G  G  I  F  R  D  M  T  I  H  D
 541 - TTTCGACATGGCGCGCTTCCTGCTCGGCGAGGAGCCGGTTTCAGTGACGGCCACCGCCGC - 600
       F  D  M  A  R  F  L  L  G  E  E  P  V  S  V  T  A  T  A  A
 601 - CGTATTGGTCGACAAGGCGATCGGCGCAGCGGGCGACTTCGACAGCGTCTCCGTGATCCT - 660
       V  L  V  D  K  A  I  G  A  A  G  D  F  D  S  V  S  V  I  L
 661 - GCAGACGGCATCCGGCAAGCAGGCGGTCATCTCCAACTCGCGTCGCGCCACCTACGGCTA - 720
       Q  T  A  S  G  K  Q  A  V  I  S  N  S  R  R  A  T  Y  G  Y
 721 - CGATCAGCGCATCGAGGTGCATGGCTCGAAGGGCGCCGTCGCGGCCGAGAACCAGCGCCC - 780
       D  Q  R  I  E  V  H  G  S  K  G  A  V  A  A  E  N  Q  R  P
 781 - CGTGTCGATCGAGATCGCCACCGGCGAGGGTTATACGCGCCCGCCGCTGCACGATTTCTT - 840
       V  S  I  E  I  A  T  G  E  G  Y  T  R  P  P  L  H  D  F  F
 841 - CATGACGCGCTACACCGAAGCCTATGCCAACGAGATCGAGAGCTTCATCGCCGCGATCGA - 900
       M  T  R  Y  T  E  A  Y  A  N  E  I  E  S  F  I  A  A  I  E
 901 - GAAGGGCGCGGAAATCACCCCGTCCGGCAAGGATGGCCTCGCGGCACTCGCCCTTGCCGA - 960
       K  G  A  E  I  T  P  S  G  K  D  G  L  A  A  L  A  L  A  D
 961 - CGCGGCCGTCCGCTCGGTCGCGGAAAAGCGCCAGATCAGCGTCGCCTGAGGTTTCAGCGA - 1020
       A  A  V  R  S  V  A  E  K  R  Q  I  S  V  A  *
1021 - TAGTGAATCACAAAGGCCGGGGCTTCCCCGGCCTTTGTCGTTTCCAAGACTGGCTACGCC - 1080
1081 - TCGGCGTGTTGAAGTGCCGGGGCGTCCGACAACGAGTTCGCTCCCGCACTCGGCGCAACA - 1140
1141 - CCCGGGCCGGGCCGGTCAGGAAGCCCTGAATCTGCCGGCAGCCCTCCCCGACGATGATCT - 1200
1201 - GCTTCTGCCGCTCGGTTTCGACGCCCTCGGTCACGACCGGCAGGTCGAGACTGTGGCCAA - 1260
1261 - GGGCGATGATTGCCCGGATGATCGCATGGGCGGCGGGATCC                     - 1301
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FIG. 1. (A) Physical map of a 4-kb DNA segment of *S. fredii* USDA191 containing the *idhA* gene. An empty arrow indicates the location and the direction of the *idhA* gene. The arrowheads point to the sites into which a kanamycin or spectinomycin cassette was inserted to inactivate the *idhA* gene. (B) Nucleotide sequence of the *idhA* gene of *S. fredii* USDA191. The sequenced region covers 1,301 nucleotides. The ORF for *idhA* begins at position 20 and ends at position 1009. The predicted amino acid sequence is given below the nucleotide sequence. Transcriptional termination sequences represented by an inverted repeat are underlined. These sequences appear in the GenBank database as accession no. AF274867.

sequences of *S. fredii* USDA191 that were submitted to a search of the SwissProt data bank revealed striking sequence similarity to the deduced protein sequences of *S. meliloti idhA* (79% identity, 82% similarity), *B. subtilis yrbE* (36% identity, 51% similarity), *B. subtilis yisS* (34% identity, 47% similarity), *Streptomyces griseus strI* (27% identity, 43% similarity), *S. meliloti mocA* (26% identity, 40% similarity), and *B. subtilis iolG* (22% identity, 40% similarity). The *idhA*, *iolG*, and *strI* genes encode *myo*-inositol dehydrogenase. The *mocA* gene product is involved in rhizopine catabolism. The *yrbE* and *yisS* genes encode hypothetical proteins of unknown function. Conserved among all these sequences is an N-terminal NADH-binding motif that is involved in the binding of the ADP moiety to NADH in dehydrogenases (41).

***S. fredii idhA* mutant is unable to utilize *myo*-inositol.** In order to verify if the cloned region contains the inositol catabolism region, we mutated the cloned region by inserting a kanamycin cassette into the *Eco*RI site or a spectinomycin cassette into the *Sal*I site, respectively (Fig. 1A). These cassettes were recombined into the genome to create the *idhA* mutants. When inositol mutants were grown on minimal media containing *myo*-inositol as the sole carbon source, no noticeable growth was observed. However, when a cosmid containing the wild-type gene was introduced into the inositol mutants, it

restored the ability of these mutants to utilize *myo*-inositol (data not shown). In order to demonstrate that the cloned region encodes an enzyme with *myo*-inositol dehydrogenase activity, we performed an enzyme assay utilizing cell extracts from *S. fredii* USDA191 grown in minimal media with and without 0.02% *myo*-inositol. *S. fredii* USDA191 grown in the absence of *myo*-inositol had a specific activity of *myo*-inositol dehydrogenase of 5 nmol of NADH reduced min^{-1}/mg of protein. When the medium was supplemented with 0.02% *myo*-inositol, the enzyme activity was elevated to 70 nmol of NADH reduced min^{-1}/mg of protein, indicating that the *myo*-inositol dehydrogenase activity is inducible. We were also able to detect *myo*-inositol dehydrogenase activity from soybean nodule extracts, although at considerably lower levels than those observed from *Rhizobium* grown in culture media. The inositol mutants, HBK101 and HBK106, did not display any detectable *myo*-inositol dehydrogenase activity either in the presence or absence of *myo*-inositol. HBK105, which harbors the cloned *idhA* gene, displayed specific *myo*-inositol dehydrogenase activity of 4 nmol of NADH reduced min^{-1}/mg of protein and 209 nmol of NADH reduced min^{-1}/mg of protein when grown in the absence and presence of *myo*-inositol, respectively.

***S. fredii idhA* is inducible by *myo*-inositol.** In order to determine if the *idhA* gene is inducible by *myo*-inositol, we cloned a 900-bp upstream sequence (*Xho*I-*Eco*RI fragment) (Fig. 1) into pMP220, a promoter probe vector (36). This construct was mobilized into *S. fredii* USDA191 by triparental mating to produce the strain HBK191–5. The promoter activity was measured by assaying β-galactosidase activity. HBK191–5 grown in minimal media exhibited β-galactosidase activity of 579 Miller units. The activity was elevated about fourfold (2,743 Miller units) when the cultures were grown in the presence of 0.2% *myo*-inositol, indicating that the *idhA* promoter is inducible by *myo*-inositol. The relatively high levels of β-galactosidase activity observed in HBK191–5 even in the absence of *myo*-inositol could be related to the copy number of the promoter probe vector. The induction of *idhA* by *myo*-inositol was further verified by Northern blot analysis. No RNA transcripts were detected using *idhA*-specific probe from USDA191 and HBK101 grown in the absence of *myo*-inositol. Supplementation of the culture medium with 0.2% *myo*-inositol clearly induced the transcription of USDA191 *idh*A (Fig. 2). The probe hybridized to a 1.0-kb RNA transcript (Fig. 2). RNA from the inositol mutant grown either in the presence or absence of *myo*-inositol revealed no hybridizing signal (Fig. 2).

Nodulation and nitrogen fixation by *idhA* mutant of *S. fredii*. The symbiotic phenotypes of parental strain USDA191 and the *idhA* mutant (HBK101) were examined on McCall soybean. Both strains formed nodules on soybean tap and lateral roots. The nitrogen fixation levels were estimated by measuring acetylene reduction to assay nitrogenase activity. A comparison of nitrogen-fixing ability of these strains at 15 days after inoculation showed that the nitrogenase activity of the *idhA* mutant was several times lower than that of the wild type. This difference was particularly pronounced 25 days after inoculation, at which point the acetylene reduction rates of the USDA191 and the *idhA* mutant were 7.9 and 0.06 μmol/mg/h, respectively. Plants inoculated with HBK101 were stunted with yellow leaves. In order to rule out the possibility that the *idhA* mutant

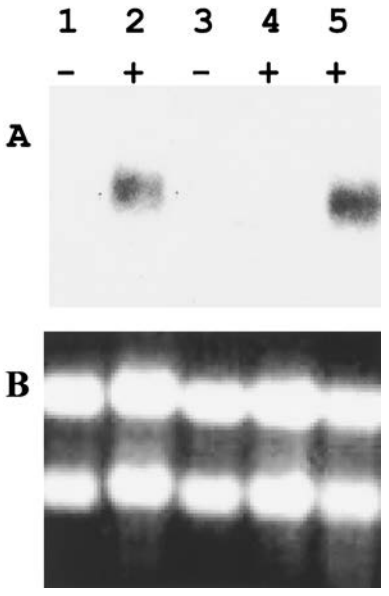

FIG. 2. (A) Effect of *myo*-inositol on the transcription of *idhA* of *S. fredii* USDA191. The bacteria initially grown in YEM media were harvested, washed, and grown in minimal slat medium in the presence or absence of 0.2% *myo*-inositol to an A_{660} of 1.0. Total RNA was isolated and used for Northern blot analysis. The RNA (15 μg) was resolved on a 1.5% formaldehyde gel and probed with a 1-kb *Eco*RI fragment containing the *idhA* gene. Lanes 1 and 2, USDA191; lanes 3 and 4, HBK101; lane 5, HBK104. The + and − signs indicate the presence and absence of *myo*-inositol in the culture media. (B) Ethidium bromide-stained gel picture showing uniform loading and integrity of RNA samples.

was delayed in its ability to fix nitrogen, we measured the nitrogenase activity in 35- and 50-day-old soybean nodules induced by the *idhA* mutant. The acetylene reduction was barely detectable (less than 0.02 μmol/mg/h) in 35- and 50-day-old nodules, suggesting that the *idhA* mutant was defective in its ability to fix nitrogen. Furthermore, we isolated bacteria from the nodules and found that about threefold fewer bacteria were present in the nodules initiated by the *idhA* mutant than in the wild-type nodules.

Soybean nodules initiated by *S. fredii idhA* mutant have aberrant ultrastructure. Since the inositol mutant had reduced nitrogen-fixing capacity when compared with the parent strain, we examined whether the mutant-induced nodules were altered in their ultrastructure. Figure 3 compares the ultrastructure of 15-day-old McCall nodules induced by the wild-type *S. fredii* USDA257 with the ultrastructure of those initiated by the inositol mutant. Nodules produced by the parental strain exhibited the typical internal structure of soybean nodules (Fig. 3A). Cells that contained numerous bacteria and a few noninfected cells predominantly occupied the central region of the nodules. The noninfected cells contained prominent vacuoles, and the cytoplasm of these cells contained several peroxisomes and starch granules. In contrast, the infected cells contained bacteroids that were enclosed by peribacteroid membranes. Most of the symbiosome contained more than one bacteroid (Fig. 3A). In contrast the nodules initiated by the inositol mutant revealed a symbiosome that contained only one bacteriod (compare Fig. 3A and B). A closer examination of the

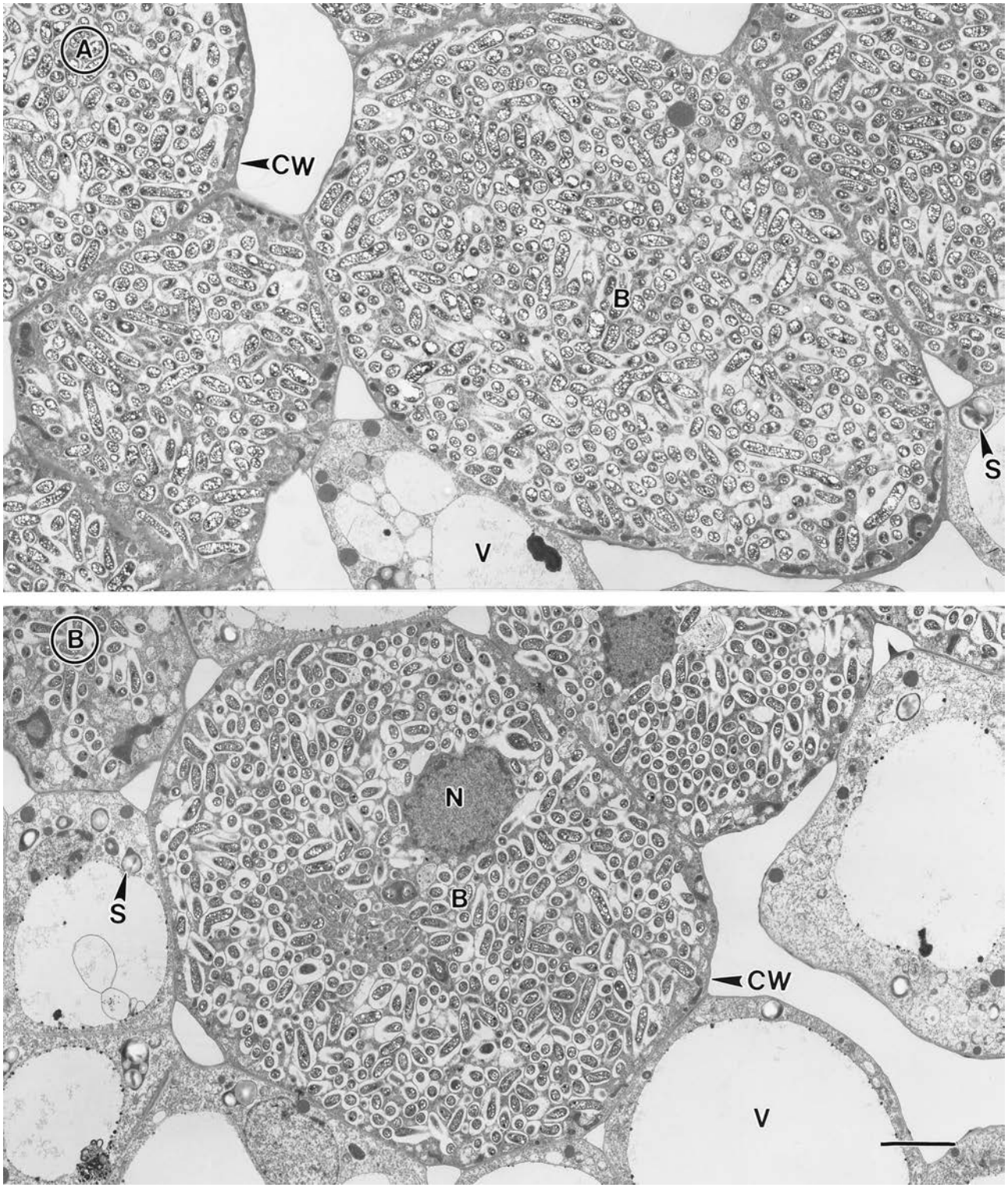

FIG. 3. Low-magnification transmission electron micrographs of 15-day-old soybean nodules initiated by *S. fredii* USDA191 (A) and the *idhA* mutant (B). The infected cells contain numerous bacteria that are surrounded by peribacteroid membranes. Note the presence of densely packed poly-β-hydroxybutyrate granules within the bacteroids. Bar, 5 μm; CW, cell wall; B, bacteroids; S, starch; V, vacuole; and N, nucleus.

nodules at higher magnification revealed abnormal structural features, including the occurrence of whorls of loosely arranged membranes and bacteroids showing signs of senescence (data not shown).

***S. fredii idhA* mutant is impaired in its ability to compete for soybean nodulation.** We also compared the competitiveness of the *S. fredii* USDA257 inositol mutant with that of its parent strain. Since the *idhA-lacZ* fusions were strongly expressed in

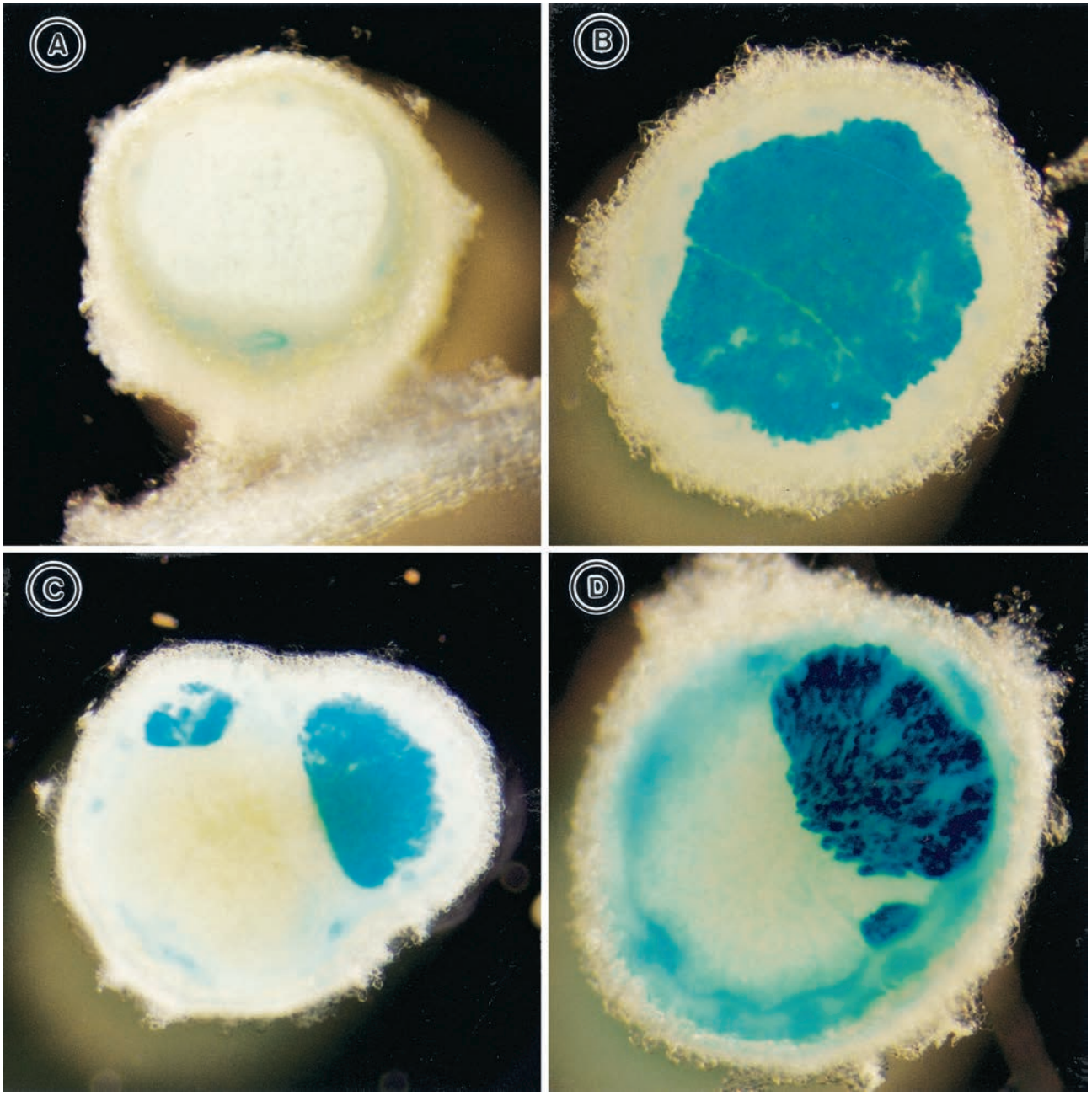

FIG. 4. Histochemical staining of nodules produced by *S. fredii* HBK102 and HBK103. HBK102 carries the cloning vector pMP220, and HBK103 is an *idhA* mutant carrying the *idhA* promoter cloned in pMP220. Twenty-five-day-old nodules were sectioned into two halves and stained with X-Gal as described in Materials and Methods. Inocula are as follows: HBK102 (A), HBK103 (B), and a mixture of HBK102 and HBK103 (C and D).

culture, even in the absence of induction, we introduced the *idhA-lacZ* constructs into the USDA191 inositol mutant. Our preliminary experiments indicated that the *idhA-lacZ* fusion was expressed strongly in nodules when visualized by histochemical staining with X-Gal, a chromogenic substrate for the *lacZ*-encoded β-galactosidase. Nodules occupied by USDA191 containing the vector pMP220 (HBK102) were unstained, while the nodules occupied by USDA191 carrying the *idhA* promoter-*lacZ* fusion (HBK103) stained blue (Fig. 4A and B). Nodules occupied by both the competitors contained blue and white sectors (Fig. 4C and D). Table 2 gives the results of competition experiments in which strain HBK102 was paired with HBK103 at various ratios. Controls inoculated with HBK102 or HBK103 yielded nodules that were 100% unstained or 100% blue, respectively (Table 2). Under competitive conditions, USDA191 always dominated nodule occupancy, even when the competitor strain had been applied at a greater numerical advantage (Table 2). At a 1:1 ratio in the inoculum, the inositol mutant occupied only 11% of the nodules, while USDA191 was found in 59% of the nodules. Inter-

TABLE 2. Nodule occupancy in dually inoculated McCall soybean[a]

USDA191/USDA191 *idhA* mutant ratio	No. of nodules examined	% Nodules occupied by:		
		USDA191	USDA191 *idhA* mutant	Both
1:0	108	100	0	0
0:1	101	0	100	0
1:1	112	59	11	30
5:1	102	80	4	16
10:1	112	85	0	15
1:5	108	29	33	38
1:10	122	28	33	39

[a] Nodules were harvested 25 days after inoculation and were stained histochemically with X-Gal. Results are from replicated experiments.

estingly, both the competitors occupied 30% of the nodules. A fivefold increase in inoculum concentration of USDA191 in comparison with the *idhA* mutant almost completely prevented the latter strain from forming nodules (Table 2). In contrast, increasing the *idhA* mutant concentration by 5- to 10-fold over that of USDA191 only marginally improved its ability to compete for soybean nodulation. In spite of increased numbers, the *idhA* mutant was found only in 29% of the nodules, while the USDA191, in spite of a 10-fold-lower concentration, was found in 33% of the nodules (Table 2). These results clearly indicate that the inositol mutant is severely affected in competitiveness.

Sequences similar to *S. fredii idhA* are widely distributed in rhizobia. Earlier studies have shown that the occurrence of rhizopine synthesis (*mos*) and catabolism (*moc*) genes is restricted in their distribution (32). Since the *idhA* gene of *S. fredii* USDA191 has significant sequence similarity to the *S. meliloti mocA* gene, we examined the occurrence of the *idhA* gene among 24 different *Rhizobium* species. Unlike the *moc* genes, *idhA* is widely distributed in different *Rhizobium* species from diverse geographical locations. Under stringent hybridization conditions, we were able to detect sequences similar to *idhA* in *Rhizobium* sp. strain NGR234, *Rhizobium etli*, *Rhizobium galegae*, *Rhizobium mongolense*, *Rhizobium tropici*, *R. leguminosarum*, *Mesorhizobium amorphae*, *Mesorhizobium ciceri*, *Mesorhizobium huakuii*, *Mesorhizobium loti*, *Mesorhizobium mediterraneum*, *Mesorhizobium plurifarium*, *Mesorhizobium tianshanense*, *S. meliloti*, *Sinorhizobium terangae*, *Sinorhizobium medicae,* and *Sinorhizobium saheli*. However, under similar hybridization conditions we were unable to detect any sequences similar to *idhA* in *Rhizobium huautlaense*, *Azorhizobium caulinodans*, *B. japonicum*, *Bradyrhizobium liaoningense,* and *Bradyrhizobium elkanii*. We also examined the occurrence of the *idhA* gene in 10 different *S. fredii* strains obtained from the U.S. Department of Agriculture collection. All *S. fredii* strains revealed strong hybridization to a 2.1-kb *Eco*RI DNA fragment.

DISCUSSION

myo-Inositol, which is abundantly present in soil, is widely used by soil bacteria as a carbon source. *myo*-Inositol dehydrogenase is the first enzyme responsible for the catabolism of *myo*-inositol in microorganisms such as *Cryptococcus melibiosum* (42), *B. subtilis* (30), *R. leguminosarum* bv. viciae (28), and *S. meliloti* (14). We have demonstrated that *S. fredii* USDA191, a soybean symbiont, also contains a *myo*-inositol dehydrogenase that is required for growth on inositol as the sole carbon source. The nucleotide sequence of the *S. fredii* USDA191 *idhA* gene, which consists of 329 amino acids and has a molecular weight of 34,648, is very similar to those for the *S. meliloti idhA* (14) and *B. subtilis yrbE* (23) and *iolG* (13) genes. The *B. subtilis iolG* gene has been identified as the *myo*-inositol 2-dehydrogenase gene (*idh*) and is a part of an operon consisting of 10 *iol* genes (26, 47). Mutation analysis has shown that the *iol* operon is probably transcribed as an 11.5-kb mRNA that encodes all the 10 *iol* genes (47). In case of *S. fredii* USDA191, the *idhA* does not appear to be a part of an operon. Our Northern blot analysis indicates that the *idhA* of *S. fredii* USDA191 is transcribed as a 1-kb mRNA. Our results are consistent with the findings from *S. meliloti*, where the entire inositol degradation gene was localized within a 2-kb *Bam*HI fragment and no other adjacent DNA region was required for growth on *myo*-inositol as the sole carbon source (14).

The IdhA of *S. fredii* USDA191 also reveals amino acid similarity to MocA of *S. meliloti* (32). Both IdhA and MocA contain, in their N-terminal region, a NADH-binding motif, which has been suggested to bind to the ADP moiety of NADH in dehydrogenases (41). The MocA is required for rhizopine utilization (32). Rhizopine (L-3-*O*-methyl-scyllo-inosamine), which occurs in alfalfa nodules induced by specific *S. meliloti* strains, can serve as a nutrient source for bacterial growth. Soybean nodules also accumulate several inositol derivatives. *myo*-Inositol, D-*chiro*-inositol, 3-*O*-methyl-D-*chiro*-inositol, and 4-*O*-methyl-*myo*-inositol are the most abundant water-soluble forms of carbon in the soybean nodules (40). Even though these compounds have been proposed to function as osmotic protectants, their precise role in symbiosis still needs to be elucidated. Our ultrastructural studies of soybean nodules initiated by *idhA* mutants indicate that the bacteroids undergo structural alterations. In the absence of *myo*-inositol dehdrogenase activity, the bacteroids within soybean nodules will be unable to catabolize *myo*-inositol. The accumulation of *myo*-inositol may result in toxicity, leading to a reduction in the number of viable bacteroids within the nodules. The occurrence of nonfunctional bacteroids within soybean nodules will lead to lower nitrogen-fixing capacity, as in the case of the *idhA* mutant of *S. fredii* USDA191. The other possibility is that inositol may be essential for the growth and maturation of the bacterioids prior to nitrogen fixation. If the maturation is blocked, as in the case of the *idhA* mutant, then senescence may follow rapidly, resulting in the loss of nitrogen fixation. This possibility appears to be more likely since dicarboxylic acids rather than sugars are needed for nitrogen fixation.

Inactivating *idhA* in *S. meliloti* and *S. fredii* produced different symbiotic effects. *myo*-Inositol dehydrogenase mutants of *S. meliloti* were reported to have no observable symbiotic phenotype (14). These mutants were able to form nitrogen-fixing nodules on alfalfa that were indistinguishable from those induced by the parental strain (14). Based on this observation, it was concluded that the *idhA* gene of *S. meliloti* is not essential for the establishment of nitrogen-fixing symbiosis (14). Another study showed that inactivation of the inositol site by insertion of *dctABD* genes resulted in increased plant biomass (36). In our study, we found that the *idhA* mutant of *S. fredii* USDA191, even though it formed pink, nitrogen-fixing nodules on soybean roots, exhibited a considerably lower acetylene

reduction rate than that of the parental strain. In addition, the *idhA* mutant also revealed developmental abnormalities in the nodule ultrastructure. Our results indicate that the *idhA* gene may have an important function in soybean symbiosis.

It has been suggested that bacterial strains that can synthesize and degrade rhizopine have a competitive advantage in the rhizosphere (15). Since rhizopine is an inositol derivative, it was suggested that genes involved in inositol catabolism might be involved in rhizopine degradation. It is now known that the *idhA* gene of *S. meliloti* is essential for rhizopine utilization (14). However, the competitive ability of *S. meliloti idhA* mutants has not been examined. Our study clearly shows the importance of an intact *idhA* gene for *S. fredii* USDA191's ability to successfully compete for soybean nodulation. Presumably *idhA* plays an important role in utilizing *myo*-inositol and inositol derivatives that are abundantly found in the rhizosphere. Soil bacteria containing an active *idhA* gene will have a competitive advantage in the rhizosphere. Attempts are currently being made to establish "biased rhizospheres" by creating transgenic plants expressing *mos* genes and beneficial soil bacteria expressing *moc* genes (32). A similar approach can also be taken to create "biased rhizospheres" by expressing *myo*-inositol synthesis genes in transgenic plants and expressing the *idhA* gene in desirable soil bacteria.

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