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# y4xP, an Open Reading Frame Located in a Type III Protein Secretion System Locus of *Sinorhizobium fredii* USDA257 and USDA191, Encodes Cysteine Synthase

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***Sinorhizobium fredii* USDA257, a soybean symbiont, exports several nodulation outer proteins (Nops) into the rhizosphere. These proteins, which are exported by a type III secretion system (TTSS), have a pivotal role in host-specific nodulation. The entire TTSS of *S. fredii* lies within a 31-kb region that includes conserved genes that code for secretion machinery proteins, Nops, and several open reading frames (ORF) of unknown function. Identifying the functions of these ORF is essential to understand fully the role of TTSS in nodulation. Here, we report the characterization of y4xP, an ORF of previously unknown function. Southern blot analysis revealed that USDA257 contains two copies of y4xP, while a sibling, USDA191, contains a single copy. The amino acid sequence of Y4XP is homologous to both eukaryotic and prokaryotic cysteine synthase, a key enzyme in sulfur assimilation. The coding region of USDA257 y4xP under control of T7 promoter was expressed in *Escherichia coli*, and the recombinant protein was purified by nickel-affinity chromatography. Antibodies generated against soybean cysteine synthase cross-reacted with the recombinant protein. A nonpolar mutant of y4xP of USDA191 showed a marked reduction in cysteine synthase activity. Enzyme activity was completely restored when the mutant was complemented with a plasmid containing the y4xP sequence. Cysteine synthase activity was confined to the cell cytosol. Extracellular protein fraction from genistein-induced USDA191 showed no cysteine synthase activity. This observation indicates that cysteine synthase, which is located in the TTSS locus, is not a type III secreted protein. A nonpolar cysteine synthase mutant was able to export all the Nops to the rhizosphere, albeit in reduced amounts compared with the wild-type USDA191. Interestingly, USDA191 cysteine synthase mutant was able to initiate nodules on 'McCall' soybean more efficiently than the wild-type. Our results demonstrate that y4xP encodes a cysteine synthase and inactivation of this gene enhances the ability of USDA191 to form nodules on 'McCall' soybean by regulating Nops production.**

J. C. Lorio and D. Chronis contributed equally to this work.

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Soil bacteria belonging to the genera *Allorhizobium*, *Azorhizobium*, *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, and *Sinorhizobium*, collectively known as rhizobia, have the unique ability to form specialized structures called nodules on compatible legume roots or stems. Nodulation is a highly selective process in which symbiotic partners recognize each other through exchange of molecular signals (Broughton et al. 2000; Dénarié et al. 1993). Legume roots secrete a variety of chemical compounds, mostly flavonoids, that interact with NodD protein, a member of the LysR family of transcriptional regulators (Schlaman et al. 1992). NodD acts as a transcriptional activator of both "common" and "host-specific" *nod* genes that direct the synthesis and secretion of lipochitooligosaccharide signal molecules called Nod factors (Dénarié et al. 1996; Long 1996). These are oligomers of β-1,4-linked *N*-acetylglucosamine residues carrying specific modifications at the reducing and nonreducing ends (Dénarié et al. 1996; Long 1996). Nod factors initiate several initial events of symbiosis, including root-hair deformation, entry of the bacteria into the root hairs, and cortical cell division (Dénarié et al. 1996). Bacteria are delivered to the cortical cells by the infection threads and cause cell division and enlargement of the nodule primordia. Once inside the nodules, rhizobia differentiate into bacterioids, structures that are capable of reducing diatomic atmospheric nitrogen to ammonia (Lodwig et al. 2003; Patriarca et al. 2002).

*Sinorhizobium fredii* USDA257, primarily known as a soybean symbiont, forms nitrogen-fixing nodules on diverse legumes (Pueppke and Broughton 1999). In spite of its broad host-specificity, this strain exhibits cultivar-specific nodulation with respect to soybean (Keyser et al. 1982). USDA257 initiates nitrogen-fixing nodules on primitive soybean cultivars but fails to nodulate agronomically improved North American cultivars such as 'McCall' (Annapurna and Krishnan 2003; Heron and Pueppke 1984; Heron et al. 1989). The inability of USDA257 to nodulate 'McCall' soybean is regulated by the *nolWXBTUV* locus which is located on a *sym* plasmid (Meinhardt et al. 1993). Mutations in any of these genes enable *S. fredii* USDA257 to nodulate 'McCall'. Subsequent characterization of this locus has revealed it to be an integral part of a type III secretion system (TTSS). TTSS, which have been well characterized in animal and plant pathogenic bacteria, are utilized to deliver effector proteins to eukaryotic cells. The TTSS of USDA257 is contained in a 31-kb *sym* plasmid DNA region and includes *rhc* (rhizobia-conserved) genes that encode components of secretion machin-

ery and nodulation outer proteins (Nops). The genomic organization of the TTSS of USDA257 is remarkably similar to another broad-host range soil bacterium, *Rhizobium* sp. strain NGR234 (Krishnan et al. 2003; Marie et al. 2001). In both USDA257 and NGR234, inactivation of the different components of the TTSS significantly influences the ability of these two rhizobia to nodulate legumes in a host-dependent manner (Krishnan et al. 2003; Marie et al. 2001). In addition to Nops, the TTSS of USDA257 contains several open reading frames (ORF) of unknown function (Krishnan et al. 2003). To fully understand the role of TTSS in symbiosis, it is essential to identify these ORF and elucidate their functions. In the present investigation, we have focused our attention on y4xP, an ORF located between two Nops-encoding genes, *nopX* and *nopP*. Our results demonstrate y4xP encodes cysteine synthase, a pivotal enzyme in sulfur assimilation. USDA257 contains two copies of y4xP, while USDA191, a sibling of USDA257 has a single copy. Inactivation of USDA191, y4xP drastically reduced the cysteine synthase activity, reduced Nops production, and enhanced the ability of this mutant to nodulate 'McCall' soybean.

## RESULTS

### *S. fredii* USDA257 contains two copies of y4xP.

Sequence analysis of the soybean cultivar specificity locus of USDA257 has revealed the presence of y4yB, y4yA, y4xP, y4xO, y4xN, and y4xM located in the midst of a functional type III protein secretion system (Krishnan et al. 2003). These ORF are located between *nopX* and *nopL*, two genes that encode proteins secreted by TTSS (Fig. 1). Transcriptional analysis revealed that *nopX*, y4yB, y4yA, y4xP, y4xO, y4xN, and y4xM may form a single transcriptional unit (Perret et al. 1999). Earlier, we demonstrated that USDA257 contains two copies of y4yB and y4yA, one located on the *sym* plasmid and the other on the chromosome (Jiang and Krishnan 2000). The chromosomal copies are identified by a superscript c (y4yB$^c$ and y4yA$^c$). However, USDA191, a sibling of USDA257 carries a single copy of y4yB and y4yA on the *sym* plasmid. The USDA257 chromosomal copy of the y4yB$^c$ and y4yA$^c$ was located in a 4.0-kB *Eco*RI fragment (Jiang and Krishnan 2000). We now have sequenced the DNA region immediately following y4yA$^c$ and have identified the presence of y4xP$^c$ and

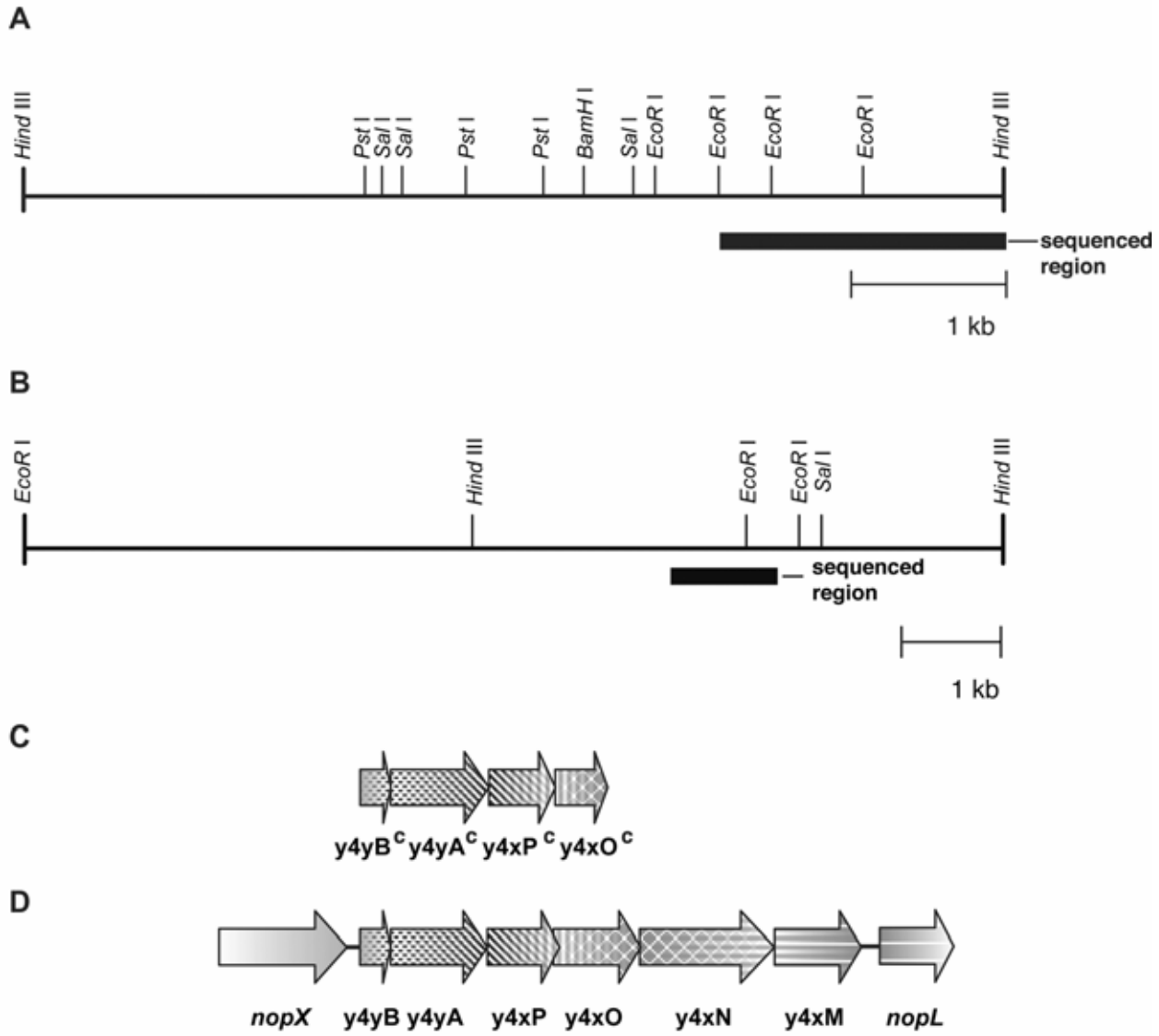


**Fig. 1.** Physical map of restriction endonuclease sites in the DNA region containing the y4xP$^c$ of **A,** *Sinorhizobium fredii* USDA257 and **B,** USDA191. The superscript 'c" refers to the chromosomal copy. The thick lines indicate the sequenced regions. **C,** Physical organization of y4xP located in the chromosome and on **D,** *sym* plasmid. Note the plasmid copy of y4xP is located in the type III secretion system locus of USDA257 and USDA191.

y4xO$^c$, which have the same orientation as the plasmid copy (Fig. 1). Southern hybridization analysis with *Hin*dIII and *Sal*I-digested genomic DNA from USDA257 and USDA191 showed strong hybridization signals when probed with the polymerase chain reaction (PCR)-amplified $^{32}$P-labeled coding region of y4xP. In the case of USDA257, two hybridization bands were detected, while only a single band was found in USDA191 (Fig. 2).

**y4xP shows sequence homology to both prokaryotic and eukaryotic cysteine synthase.**

A comparison of y4xP-deduced amino acid sequences of chromosomal (y4xP$^c$) and plasmid copies revealed that they are 94% identical. Similarly, the USDA191 y4xP$^c$ sequence was found to be highly homologous to the USDA257 sequences. Each of these genes encodes a 35-kDa protein with an isoelectric point of 5.9. A BLAST search against the SWISSPROT database showed that Y4XP shared significant homology to cysteine synthases from prokaryotes including, *Escherichia coli*, *Rhizobium* sp. strain NGR234, *Mesorhizobium loti*, *Erwinia carotovora*, *Synechocystis* spp., and *Nostoc* spp., and eukaryotes, *Glycine max*, *Arabidopsis thaliana*, and *Solanum tuberosum*. Cysteine synthase is a pyridoxal phosphate-dependent enzyme and contains a conserved lysine residue at the active site (Fig. 3). Both USDA257 and USDA191 Y4XP have this conserved lysine residue near the N-terminal region (Fig. 3). A comparison of the amino acid sequence shows that the residues that interact with pyridoxal phosphate and the substrate are conserved in both prokaryotes and eukaryotes (Fig. 3).

**Recombinant Y4XP shows antigenic homology to soybean cysteine synthase.**

To demonstrate that Y4XP has cysteine synthase activity, we first purified this protein. An ample supply of the protein was obtained by cloning the coding region of the USDA257 y4xP plasmid copy into *E. coli* protein expression vector pET28a. The bacterial culture grown overnight in the presence of isopropyl-beta-D-thiogalactoside (IPTG) produced a recombinant 45-kDa protein that was absent in noninduced cultures (Fig. 4A). The size of the recombinant protein is slightly larger than the size of the protein deduced from the y4xP nucleotide sequence. The reason for this aberrant migration of the recombinant Y4XP on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is not clear. Since the recombinant protein contains six-histidine residues at the C-terminus of Y4XP, we were able to purify this protein using nickel-affinity column chromatography under denaturing conditions (Fig. 4). However, our repeated attempts to purify the recombinant Y4XP under nondenaturing conditions were not successful because we were unable to solubilize Y4XP, which was found exclusively in inclusion bodies. Expression of the USDA257 chromosomal copy of y4xP$^c$ also resulted in insoluble recombinant protein. Earlier, we had raised antibodies against soybean cysteine synthase (Chronis and Krishnan 2003). Western blot analysis using soybean cysteine synthase antibodies revealed strong cross-reactivity against the recombinant Y4XP (Fig. 4B) indicating that this protein has antigenic homology to the soybean cysteine synthase.

**A nonpolar Δy4xP deletion mutant has drastically reduced cysteine synthase activity.**

To obtain biochemical evidence that y4xP encodes for cysteine synthase, we created nonpolar y4xP deletion mutants. The coding region of y4xP in both USDA257 and USDA191 plasmid copies were precisely excised and replaced by the *npt*II cassette. A comparison of cysteine synthase activity between these mutants and wild-type parents are shown in Figure 5. Cysteine synthase was barely detected in the USDA191 Δy4xP mutant, while the USDA257 Δy4xP mutant showed only a minimal reduction in enzyme activity. The difference in cysteine synthase activity between these two rhizobia could be explained by the fact that USDA191 has a single copy of y4xP while USDA257 has two copies (Fig. 1). Even though USDA257 contains two copies of y4xP, the cysteine synthase activity in USDA191 was three times greater than that of USDA257. When a plasmid copy of y4xP was introduced into the Δy4xP deletion mutants, cysteine synthase activity was completely restored and the levels were higher than in the wild type (Fig. 5).

**Genistein induces the transcription of y4xP.**

Earlier, we demonstrated that the transcription of *nop*X is inducible by flavonoids (Bellato et al. 1997; Meinhardt et al. 1993). A 185-bp intergenic region separates *nopX* and y4xP. We cloned this region in the promoter probe vector pMP220 and found no evidence for promoter activity in this region. This suggests that the y4xP could be a part of a single transcriptional unit encompassing *nopX*, y4yB, y4yA, y4xP, y4xO, y4xN, and y4xM (Fig. 1). To determine if y4xP can be induced by flavonoids, we performed semiquantitative reverse transcriptase (RT)-PCR analysis using RNA isolated from rhizobia that were grown in the presence or absence of genistein, a potent inducer of *nopX* and other *nod* genes of *S. fredii* (Pueppke et al. 1998). For these experiments, we utilized USDA191, since it has only a single copy of y4xP. When primers designed to amplify the coding region of the y4xP were utilized in the PCR reaction, a 1-kB RT-PCR product was obtained from wild-type USDA191. Genistein

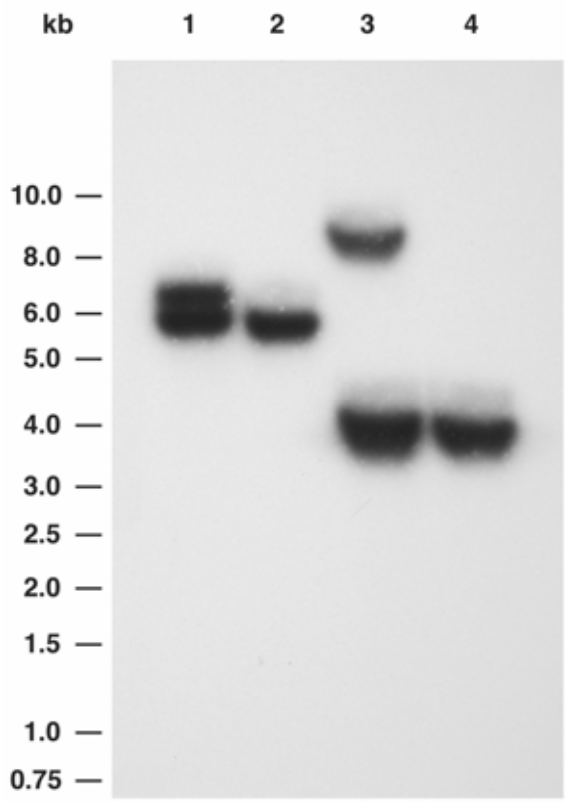


**Fig. 2.** Southern blot analysis of y4xP in *Sinorhizobium fredii* USDA257 and *Sinorhizobium fredii* USDA191. Genomic DNA from USDA257 (lanes 1 and 3) and USDA191 (lanes 2 and 4) were digested with *Hin*dIII (lanes 1 and 2) and *Sal*I (lanes 3 and 4), respectively, and were separated on a 0.7% agarose gel. The gel was blotted onto nitrocellulose and probed with $^{32}$P-labeled y4xP coding region. The numbers on the left indicate the positions and sizes of molecular weight marker.

addition to the growth media clearly enhanced the y4xP transcript levels (Fig. 6). No RT-PCR products were amplified from RNA isolated from the Δy4xP deletion mutant. However, the production of the RT-PCR product was restored when the mutant was complemented with a plasmid copy of y4xP. As in the wild type, the accumulation of y4xP transcript was severalfold greater in cells cultured in the presence of genistein (Fig. 6). To determine if deletion of y4yP had any effect on TTSS, we monitored the transcription of flavonoid-inducible genes *nopA, nopX, and nopL* (Deakin et al. 2005; Krause et al. 2002; Krishnan et al. 2003). RT-PCR analysis revealed the accumulation of *nopA*, *nopX*, and *nopL* transcripts in the wild type, the Δy4xP deletion mutant, and the complemented strain (Fig. 6). As in the case of y4xP, addition of genistein clearly enhanced the transcription of all these genes.

Since y4xP transcription is inducible by flavonoids, we wanted to ascertain if cysteine synthase activity is also elevated in rhizobia grown in the presence of the *nod*-gene inducer. In our initial experiments, cysteine synthase activity was measured in USDA257 and USDA191 cells that were grown for 38 h in the absence or presence of genistein. Under such conditions, we were unable to detect any significant difference in the cysteine synthase activity between induced and noninduced cultures. Therefore, we monitored cysteine synthase activity at different timepoints after the addition of genistein. Clearly, genistein elevated cysteine synthase activity during the first 3 h of incubation after its addition. This difference was not seen when genistein was present in the culture longer than 6 h (Fig. 7).

**Symbiotic properties of nonpolar Δy4xP mutants.**

We have earlier shown that mutations in the genes encoding the Nops and TTSS machinery extend the ability of USDA257 to nodulate 'McCall' soybean. To determine if USDA257 Δy4xP mutant has a similar phenotype, we tested the ability of this mutant to form nodules on 'McCall' soybean. The Δy4xP mutant failed to form nodules on 'McCall', indicating that y4xP may not be involved in regulating soybean cultivar specificity. Interestingly, USDA191, a strain that does not exhibit

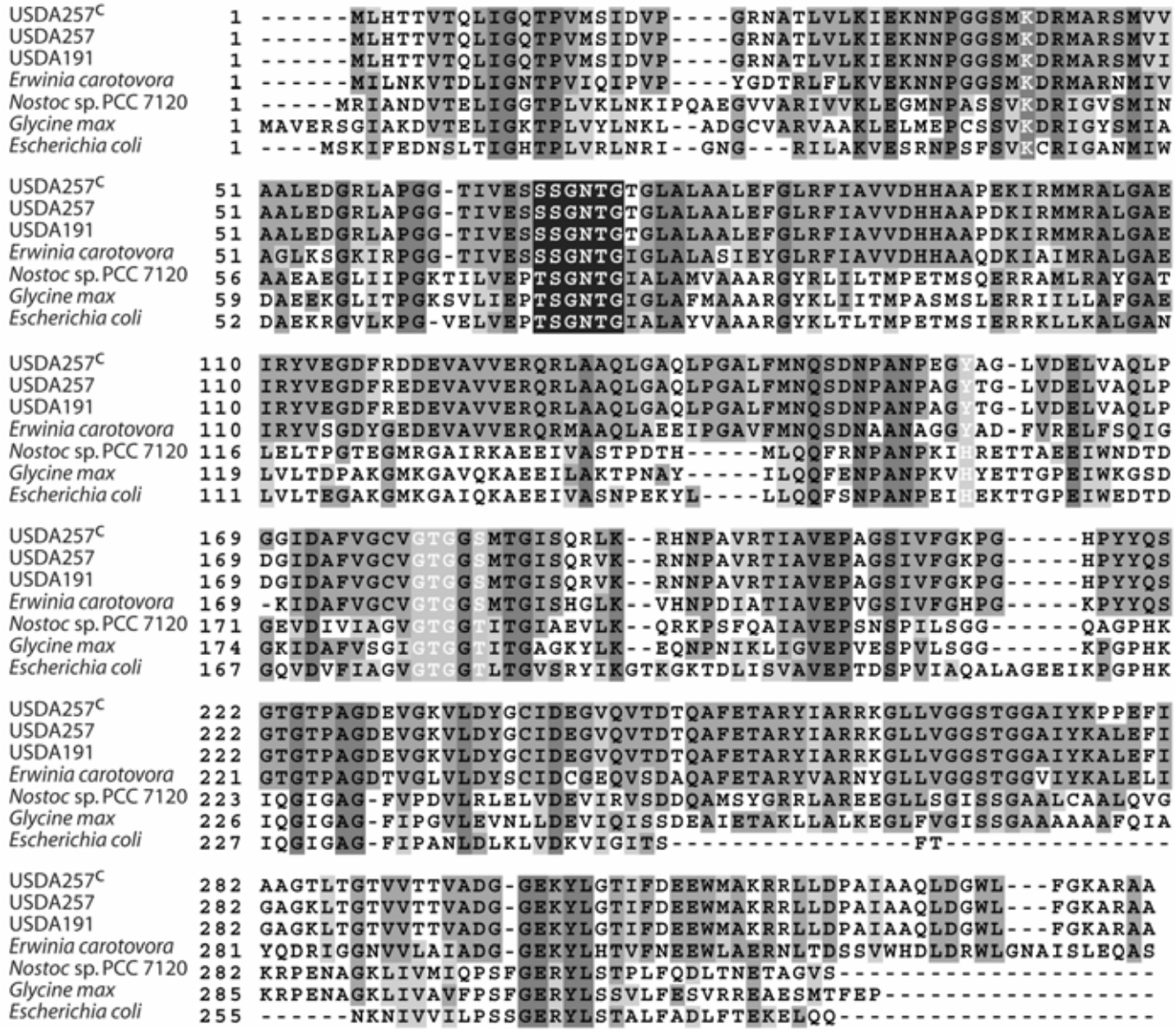


**Fig. 3.** Multiple alignment of the amino acid sequences of cysteine synthase. Dashes indicate gaps to facilitate best alignment. Dark gray shading indicates conserved residues; mid gray shading indicates residues showing more than 60% identity; light gray shading indicates those residues showing more than 60% similarity. The active site lysine (white on dark gray), the substrate loop (white on black), and residues that interact with pyridoxal phosphate (white on light gray) are indicated.

soybean cultivar specificity was able to form more nodules on 'McCall' soybean. An examination of the kinetics of nodule formation revealed that the USDA191 Δy4xP mutant derivative was more efficient in initiating nodules (Fig. 8). Twelve days after inoculation, the y4xP mutant had twice the number of nodules than did the wild type. This difference in the ability to efficiently initiate nodules appears to be controlled by y4xP, since the complemented strain exhibited similar nodulation kinetics as the wild-type USDA191 (Fig. 8).

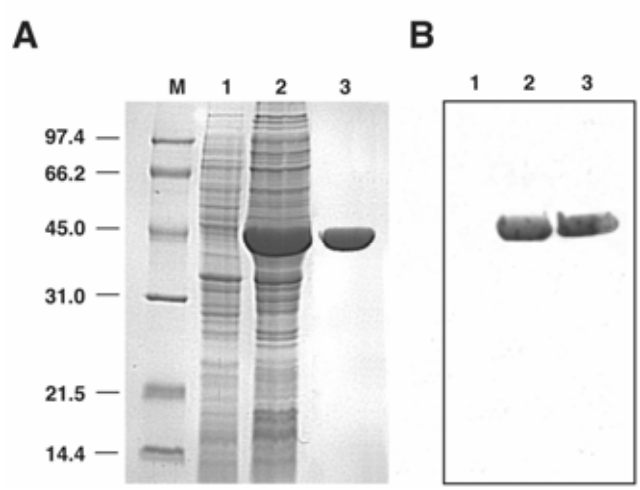


**Fig. 4.** Expression and purification of recombinant USDA257 Y4XP. The coding region of y4xP was cloned in an expression vector pET28a (+) and introduced into *Escherichia coli* ER2566. Recombinant protein expression was induced by the addition of 1 mM isopropyl-beta-D-thiogalactoside (IPTG) to the culture media. Following overnight growth at 37°C, the cells were harvested and the recombinant protein was purified on a nickel-affinity column. The proteins were fractioned in duplicate gels by 12.5% sodium dodecylsulphate polyacrylamide gel electrophoresis and were either **A,** stained with Coomassie brilliant blue or **B,** transferred to a nitrocellulose membrane Western blot analysis. The blot was probed with antibodies raised against soybean cysteine synthase. Lane M: molecular weight markers; lane 1, protein from noninduced cultures; lane 2, protein from IPTG-induced cultures; and lane 3. purified recombinant Y4XP. The sizes of the molecular weight markers in kilodaltons are shown on the left margins of each figure.

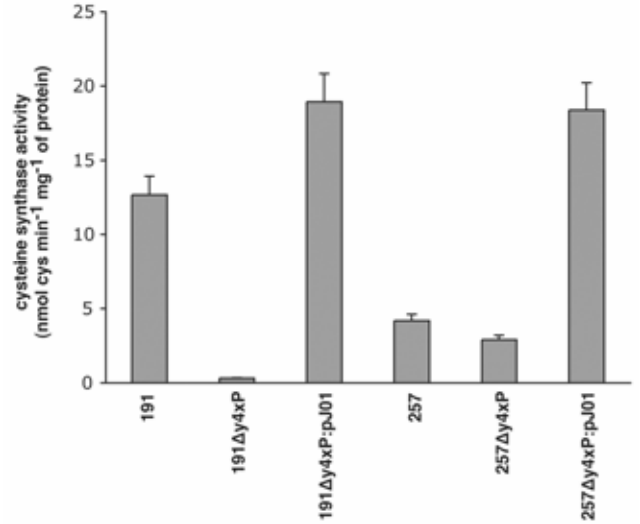


**Fig. 5.** Cysteine synthase activity in *Sinorhizobium fredii*. Crude cell-free protein extracts obtained from Δy4xP deletion mutants, complemented strains, and the wild-type USDA257 and USDA191 were used to measure cysteine synthase activity. Cysteine synthase activity was measured by the conversion of *O*-acetylserine into cysteine following spectrophotometric absorbance at 550 nm.

**USDA191 Δy4xP mutant shows subtle differences in Nops production.**

Previous studies have demonstrated that Nops are involved in either positively or negatively influencing nodulation in a host-dependent manner (Marie et al. 2001). Since the USDA191 Δy4xP mutant forms a greater number of nodules on 'McCall' soybean than does the wild-type parent, we wanted to determine

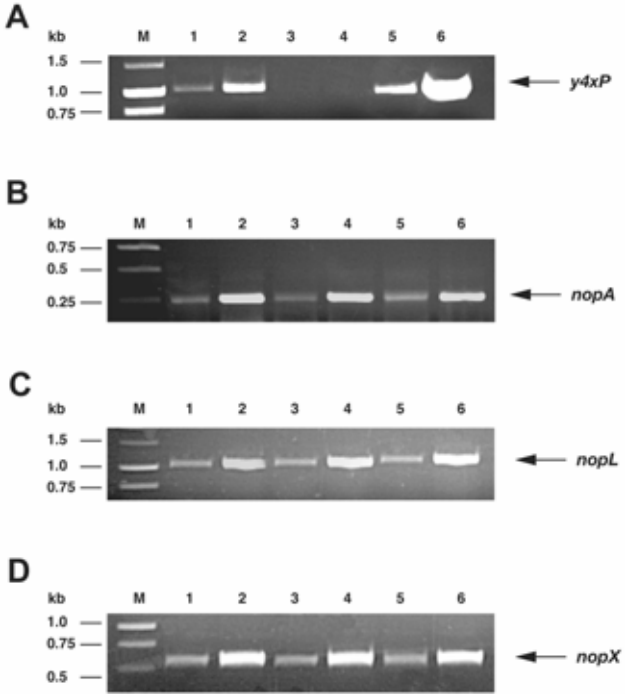


**Fig. 6.** Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of cysteine synthase mRNA in *Sinorhizobium fredii* USDA191. Total RNA isolated from USDA191 grown in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of 1 μM genistein was used as a template for RT-PCR. The products obtained utilizing primers designed to amplify either the complete coding regions of **A,** y4xP, **B,** *nopA*, and **C,** *nopL*, or **D,** a segment of the coding region of *nopX* are shown. Sizes of the molecular weight markers are indicated on the left side of the figure. Lanes 1 and 2, USDA191; lanes 3 and 4, USDA191 Δy4xP; lanes 5 and 6, USDA191 Δy4xP (pJO1).

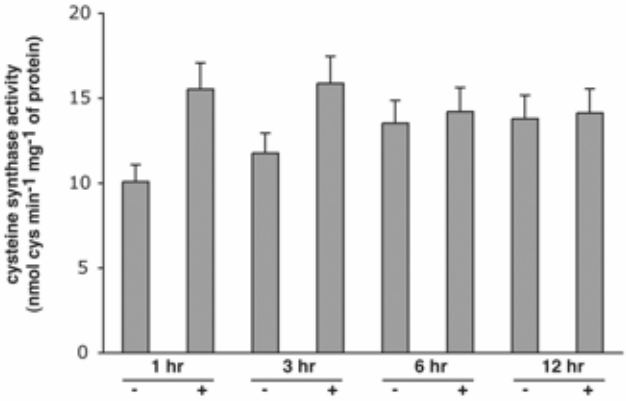


**Fig. 7.** Flavonoid induction transitionally increases cysteine synthase activity. USDA191 cells were harvested at different times after the addition of genistein, and cysteine synthase activity was measured from the crude cell-free extracts. Genistein was added when the culture reached an optical density of 0.5 (absorbance at 600 nm), and samples were taken at 1, 3, 6, and 12 h after induction. Cysteine synthase activity was measured by the conversion of *O*-acetylserine into cysteine following spectrophotometric absorbance at 550 nm.

if this phenotype is mediated through changes in Nops production. We isolated extracellular proteins from equal volumes of bacterial cultures that were grown in the presence of genistein. A comparison of proteins isolated from the wild type and the USDA191 Δy4xP mutant revealed similar protein profiles (Fig. 9A). However, the y4xP mutant contained a lesser amount of protein than the wild type. When equal amounts of protein were analyzed by SDS-PAGE, both USDA191 and the y4xP mutant exhibited the same protein profiles (Fig. 9B). Western blot analysis using antibodies raised against NopX, NopB, and NopA confirmed that these proteins were present in lesser amounts in the y4xP mutant (Fig. 9C).

**y4xP does not encode a protein of the TTSS nor is its product secreted.**

Since y4xP is located in the midst of the TTSS, we performed experiments to ascertain whether it encodes a component of the TTSS and whether the y4xP-encoded protein is secreted. Since the USDA191 y4xP mutant produces the same profile of TTSS proteins as the wild type (Fig. 9), it appears that y4xP is not an integral part of the TTSS. The TTSS is highly conserved among symbionts, plant, and animal bacterial pathogens. In these bacteria, cysteine synthase has not been reported to be a component of the TTSS. To verify whether Y4XP is secreted, we purified extracellular proteins from genistein-induced cultures of USDA191 and assayed for the presence of cysteine synthase activity. We were unable to detect cysteine synthase activity in the extracellular protein fraction, while the activity was clearly seen in the protein extract from the cell pellet. Our observation indicates that Y4XP is not an extracellular protein.

## DISCUSSION

In this study, we have shown that y4xP codes for cysteine synthase. Several lines of evidence support our contention that Y4XP is a cysteine synthase. A nonpolar Δy4xP mutant revealed drastically reduced cysteine synthase activity. This reduction in cysteine synthase activity was completely restored when a plasmid containing y4xP was introduced into this mutant. When the deduced amino acid sequence of y4xP was compared with cysteine synthase primary structures representing both eukaryotic and prokaryotic sources, a significant homology was noted. Additionally, the active site lysine residue and other amino acids that putatively bind the pyridoxal phosphate cofactor are all conserved in Y4XP. Our attempts to demonstrate cysteine synthase activity with a recombinant Y4XP expressed in *E. coli* were not successful. The *E. coli*-expressed heterologous protein formed inclusion bodies, making isolation of nondenatured protein for enzymatic analysis difficult. However, we were able to demonstrate immunological relatedness of Y4XP to that of soybean cysteine synthase.

An examination of the genetic organization of the USDA257 TTSS locus revealed that six ORF (y4yB, y4yA, y4xP, y4xO, y4xN, and y4xM), some of which share overlapping start and stop codons, are found between two Nops-encoding genes, *nopX* and *nopL*. We have demonstrated that inactivation of NopX abolishes the transcription of y4yB and y4yA, indicating that NopX and the two ORF may constitute a single transcriptional unit (Jiang and Krishnan 2000). A similar situation has been reported in NGR234 (Perret et al. 1999). In both USDA257 and NGR234, this operon was induced by flavonoids indicating that it may have a role in symbiosis. Inactivation of y4yB and y4yA, however, had no observable effect on the ability of USDA257 to nodulate soybean cultivar Peking (Jiang and Krishnan 2000). In this present study, we observed that inactivation of y4xP in USDA191 slightly enhanced the ability of the bacteria to nodulate soybean cultivar McCall. It is not clear how cysteine synthase, a key enzyme in sulfur assimilation could lead to enhanced nodulation. One possibility is that the inactivation of y4xP could have affected metabolism, resulting in alterations in symbiotic signals such as lipopolysaccharides and Nod factors. Since USDA257 and USDA191 do not contain sulfated Nod factors (Bec-Ferte et al. 1994, 1996), it is unlikely that the symbiotic phenotype of y4xP mutants is mediated by altered Nod factors. Although the y4xP mutant was created by the insertion of an *npt*II cassette lacking the *rho*-independent transcriptional terminator, it is possible that this insertion had an unexpected effect on the transcription of genes located downstream of y4xP. However, RT-PCR analysis indicates that transcription of *nopA*, *nopX*, and *nopL*, a gene located downstream of y4xP, are not affected by inactivation of y4xP. Reduced Nops production by the Δy4xP mutant is a more plausible explanation for the enhanced nodulation phenotype exhibited by this mutant. Altered Nops have been shown to either enhance, decrease, or have no effect on nodulation, in a host-dependent manner. We have demonstrated that NopX mutant of USDA257 can initiate nodules

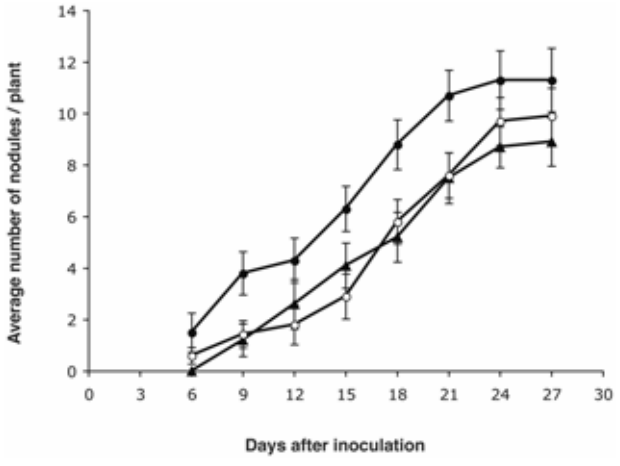


**Fig. 8.** Symbiotic properties of the Δy4xP deletion mutant of USDA191. Seedlings of *Glycine max* cv. McCall were inoculated with either USDA191 (○-----○), USDA191Δy4xP (●-----●), or USDA191Δy4xP(pJO1) (▲-------▲). Nodules were counted on day 6 after inoculation and on every third day thereafter until day 27. Results are from three independent experiments, using 10 plants per treatment in each case.

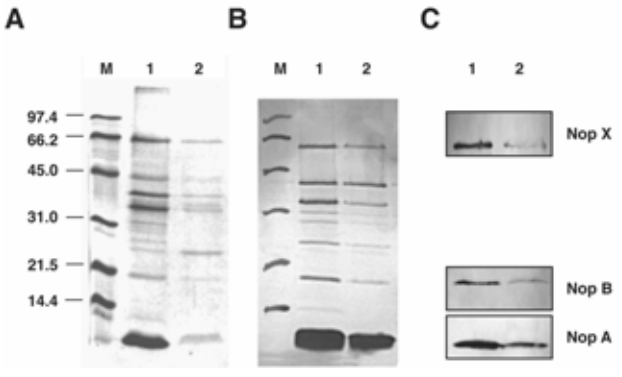


**Fig. 9.** Comparison of Nops production in USDA191 and USDA191 Δy4xP mutant. Extracellular proteins from genistein-induced cultures of USDA191 (lane 1) and USDA191 Δy4xP mutant (lane 2) were resolved by 15% sodium dodecylsulphate polyacrylamide gel electrophoresis and either **A** and **B,** stained with Coomassie brilliant blue or **C,** transferred to a nitrocellulose membrane for Western blot analysis. Blots of identical gels were immunostained with NopX, NopB, and NopA antibodies, respectively. Panel A shows the protein profile obtained from equal numbers of bacterial cells, while B reveals the profile obtained when equal amounts of protein (50 μg) was used. The sizes of the molecular weight markers in kilodaltons are indicated.

on 'McCall' soybean while the wild-type parent is unable to do so (Meinhardt et al. 1993). In contrast, USDA191, which elaborates the same set of Nops as USDA257, albeit in different amounts when grown in the presence of flavonoids, is still able to nodulate 'McCall' soybean (H. B. Krishnan, *unpublished observations*; Krishnan and Pueppke 1993; Krishnan et al. 1995). Thus, it appears that soybean cultivar–specific nodulation may be mediated by the relative concentration of different Nops secreted by USDA257. A quantitative comparison of the Nops production by USDA191 and USDA257 is necessary to determine if a difference in Nops concentration contributes to host specificity.

USDA257 contains two copies of y4xP, one is localized on the *sym* plasmid while the other is contained within the bacterial chromosome. In contrast, USDA191 contains one copy of the gene, which is located on the *sym* plasmid. Similarly, y4yB and y4yA are also duplicated in the USDA257 genome (Jiang and Krishnan 2000). Recently, the occurrence of duplicated copies of y4yB, y4yA, y4xp, y4xO, y4xN, and y4xM has been shown in NGR234 (Streit et al. 2004). One set of these genes is located on the *sym* plasmid (pNGR234*a*), which contains most of the symbiotic genes of this bacteria, and the second copy is on a megaplasmid (pNGR234*b*). In both USDA257 and NGR234, a copy of y4yB, y4yA, y4xP, y4xO, y4xN, and y4xM is located in a region that contains genes encoding a functional TTSS (Krishnan et al. 2003; Viprey et al. 1998). This cluster of ORF does not appear to be directly involved in type III protein secretion, since mutation in three of the ORF (y4yA, y4yB, and y4xP) does not abolish the production and secretion of Nops (Jiang and Krishnan 2000; this study). Their presence in the midst of a functional TTSS of USDA257 indicates acquisition by horizontal gene transfer from other microbes. This contention is supported by the recent finding that some gene clusters located on pNGR234*b* were similar to those in other soil- and plant-associated microbes (Streit et al. 2004).

## MATERIALS AND METHODS

### Bacterial strains and growth conditions.

Bacterial strains and plasmids used in this study are listed in Table 1. USDA257 and USDA191 were maintained on yeast extract mannitol medium (Vincent 1970) at 30°C. *E. coli* strains were grown in Luria-Bertani broth at 37°C. When necessary, antibiotics were added to the medium at the following concentrations: tetracycline, 10 μg/ml; kanamycin, 50 μg/ml; ampicillin, 100 μg/ml; spectinomycin, 50 μg/ml; and gentamicin, 10 μg/ml.

### Nucleic acid manipulation and analysis.

Recombinant DNA techniques were performed according to standard protocols (Ausubel et al. 1991). Genomic DNA and total RNA were isolated from *S. fredii* as described (Kovacs et al. 1995). DNA sequence was determined with an Applied Biosystems 3730 DNA analyzer using Applied Biosystems Prism BigDye Terminator cycle sequencing chemistry (Applied Biosystems, Foster City, CA, U.S.A.). Polymerase chain reaction (PCR) was performed with *Taq* DNA polymerase according to the manufacturer's protocol. Radioactive hybridization probes were prepared using the random primer labeling kit (Takara Mirus Bio, Madison, WI, U.S.A.). Southern blotting and hybridizations were performed according to established protocols (Sambrook et al. 1989).

### Construction of the nonpolar Δy4xP deletion mutant.

Two 1-kb DNA regions flanking start and stop codons of the y4xP ORF were amplified by PCR utilizing pHBK447 as a template. The primers for the upstream region, 5′-cttccgcggtcg atgacgagccccggcccttgtg-3′ and 5′-gcagatatctgaccatttgcggcgctg gccgcaccgg-3′, contained *Sac*II and *Eco*RV restriction sites, while those for the downstream region, 5′-ctggtccaggatatcaacct ctccaattgtgtcgg-3′ and 5′atcggtggactcgagggaagaactggagg-3′, included *Eco*RV and *Xho*I sites to facilitate cloning. A *npt*II cassette minus the *rho*-independent transcriptional terminator was isolated from pCPP2988 (Alfano et al. 1996) and was inserted between the two PCR fragments, thus eliminating the y4xP coding region. This DNA fragment was subsequently cloned into the *Sma*I site of pJQ200 mp18 (Quandt and Hynes 1993), resulting in pJO2. Utilizing triparental mating with helper plasmid pRK2013, this construct was mobilized into USDA257 and USDA191 (Figurski and Helinski 1979). Double recombination events were selected by plating bacteria on yeast extract medium containing 5% (wt/vol) sucrose. Putative USDA257Δy4xP and USDA191Δy4xP mutants were confirmed by Southern blot analysis.

### Expression of y4xP in *E. coli.*

The coding region of USDA257 y4xP was amplified by PCR, using DNA from pHBK447 as a template. The N and C

**Table 1.** Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant characteristics | Source or reference |
|---|---|---|
| Strains | | |
| *Sinorhizobium fredii* USDA257 | Broad-host range, $Nod^+$ on soybean | Heron and Pueppke 1984 |
| *Sinorhizobium fredii* USDA191 | Broad-host range, $Nod^+$ on soybean | Heron and Pueppke 1984 |
| USDA257Δy4xP | USDA257 derivative in which y4xP was replaced with *npt*II, $Km^r$ | This study |
| USDA257Δy4xP::pJO1 | USDA257 Δy4xP mutant carrying pJO1, $Km^r$, $Tc^r$ | This study |
| USDA257Δy4xP::pRF447 | USDA257 Δy4xP mutant carrying pRF447, $Km^r$, $Tc^r$ | This study |
| USDA191Δy4xP | USDA191 derivative in which y4xP was replaced with *npt*II, $Km^r$ | This study |
| USDA191 Δy4xP::pJO1 | USDA191 Δy4xP mutant carrying pJO1, $Km^r$, $Tc^r$ | This study |
| USDA191 Δy4xP::pRF447 | USDA191 Δy4xP mutant carrying pRF447, $Km^r$, $Tc^r$ | This study |
| DH5α | ϕ80*lacZ*ΔM15Δ(*lacZYA-argF*) U169 *hsdR*17 *recA*1 *endA*1 *thi*-1 | Gibco BRL |
| Plasmids | | |
| pGEM-T Easy | PCR cloning vector, $Ap^r$ | Promega |
| pBSKSII | Cloning vector, $Ap^r$ | Stratagene |
| pHBK447 | pLAFR1 cosmid carrying TTSS genes from USDA257, $Tc^r$ | Krishnan et al. 2003 |
| pRK2013 | *incP1* helper plasmid, $Km^r$ | Figurski and Helinski 1979 |
| pCPP2988 | pBluescript II KS vector carrying 1.5-kb *Hin*dIII and *Sal*I fragment from pRZ102 with *npt*II lacking terminator | Alfano et al. 1996 |
| pJQ200mp18 | Suicide vector for gene replacement, $Gm^r$ | Quandt and Hynes 1993 |
| pRK415 | Broad-host-range vector, $Tc^r$ | Keen et al. 1988 |
| pJ01 | y4xP coding region cloned in pRK415, $Tc^r$ | This study |
| pJ02 | pJQ200mp18 carrying 3.49-kb insert containing Δy4xP::*npt*II, $Km^r$, $Gm^r$ | This study |

terminal–specific primers were 5′-CCAACATATGATGCTG CACACGACCGTAAC-3′ and 5′-GGTTCTCGAGTCATGCT GCCCGCGCCTTTC-3′, which contained a *Nde*I and *Xho*I restriction site, respectively, to facilitate subsequent cloning into *E. coli* expression vector pET28(a)+ (Calbiochem-Novabiochem, San Diego, CA. U.S.A.). The resultant recombinant plasmid was introduced into *E. coli* ER2566 (New England Biolabs, Beverly, MA, U.S.A.). Bacterial cells transformed with the recombinant plasmid were grown at 37°C in Luria broth medium. When the culture reached optical density at 600 nm ($OD_{600}$) of 0.9 (absorbance at 600 nm), IPTG was added to a final concentration of 1 mM and growth was allowed to continue overnight. Recombinant protein was purified by affinity chromatography under denaturing conditions using a Ni-NTA agarose column following the manufacture's protocol (Qiagen, Valencia, CA, U.S.A.).

**Electrophoresis and Western blot analysis.**

Extracelluar proteins were isolated from *S. fredii* grown in the presence or absence of 1 μM genistein as described by Krishnan and associates (2003). Bacterial proteins were resolved by SDS-PAGE, using the discontinuous buffer system of Laemmli (1970). Protein samples in SDS sample buffer (2% SDS, 60 mM Tris-HCl (pH 6.8), 5% β-mercaptoethanol) were boiled for 5 min at 100°C before commencement of electrophoresis. The resolving gels (10 × 8 × 0.75 cm) contained 15% acrylamide and the stacking gel 5% acrylamide. Electrophoresis was conducted at a constant current of 20 mA per slab for 1 h or until the tracking dye reached the bottom of the gel. Proteins were visualized by staining with Coomassie brilliant blue (Sigma-Aldrich, St. Louis). For Western blot analysis, proteins separated by SDS-PAGE were electroblotted onto nitrocellulose as described (Burnett 1981). NopX, NopB, and NopA antibodies were diluted 1:3,000 in TBS (Tris-HCl 10 mM, pH 7.5, NaCl 500 mM) containing 5% (wt/vol) nonfat dried milk. Immunoreactive polypeptides were detected employing the horseradish peroxidase color-development protocol provided by the manufacturer (Bio-Rad Laboratories, Inc., Richmond, CA, U.S.A.).

**RT-PCR analysis.**

Duplicate sets of *S. fredii* USDA191, Δy4xP deletion mutants, and complemented derivatives were incubated on a rotary shaker at 30°C in liquid yeast mannitol extract (YEM) medium. When the cultures reached an $OD_{600}$ of 0.5, genistein at a final concentration of 1 μm was added to one of the sets and were grown for an additional hour. Cells were harvested and RNA was prepared following the general procedure (Sambrook et al. 1989). The RNA was treated with DNaseI (Invitrogen, Carlsbad, CA, U.S.A.) to eliminate any contaminating DNA. The primer set combination used for amplification are as follows: y4xP (forward primer: 5′-CCAACATATGATGCTGCACACGACCGTAAC-3′; reverse primer: 5′-GGTTCTCGAGTCATGCTGCCCGCGCCTTTC-3′), *nopA* (forward primer: 5′-ATGTCTAAAATAGGTACTGTCACGAG-3′; reverse primer: 5′-CTGTACGCGTTCATCCGCCGCCTTC-3′), *nopL* (forward primer: 5′-ATGGATATCAATTCAACCCGCCCACTAAAC-3′; reverse primer: 5′-TCAAATGTCAAAATCCAGCGATGGCCTG-3′), and *nopX* (forward primer: 5′-ATGTCGGCCAGCAACCTTTTACCAATGATC-3′; reverse primer: 5′-GCCTCCACAGCGACCATCGCCCTGCGAGC-3′). RT-PCR was carried out in a final volume of 50 μl using the One Step RT-PCR kit (Qiagen, Valencia, CA, U.S.A.). The thermal cycler program was 50°C for 30 min, 95°C for 15 min, 26 cycles at 94°C (1 min), and 72°C (1 min), followed by a final cycle of 10 min at 72°C. After electrophoretic separation on a 0.8% (wt/vol) agarose gel, an image of the resolved RT-PCR products was recorded (LabRepco, Horsham, PA, U.S.A.).

**Cysteine synthase assays.**

USDA 257, USDA191, Δy4xP deletion mutants, and the complemented strains were grown in liquid YEM media in the presence or absence of 1 μM genistein. Cells were pelleted by centrifugation and were resuspended in extraction buffer (100 mM Tris-HCl, pH 8.0, 100 mM KCl, 20 mM $MgCl_2$, 1% Tween 80, and 10 mM dithiothreitol [DTT]). The slurry was sonicated twice for 90 s at 4°C, and the cell debris was removed by centrifugation. Cysteine synthase activity in the supernatant was measured in a total volume of 200 μl containing 5 mM *O*-acetylserine (OAS), 3 mM sodium sulphide, 10 mM DTT, and 100 mM sodium phosphate, pH 8.0 (Warrilow and Hawkesford 1998). The reaction was initiated by the addition of OAS and was incubated at 26°C for 10 min prior to spectrophotometric analysis at 550 nm. One unit of cysteine synthase activity is defined as the conversion of 1 mM of OAS into cysteine per minute. Protein concentration in the cell extracts was determined with a Bio-Rad protein assay kit using bovine serum albumin as the standard.

**Plant nodulation assays.**

Surface-sterilized soybean seeds were germinated on 1% water-agar at 30°C for three days. Seedlings were first inoculated by immersing the roots in bacterial cultures of known density and were placed into sterile pouches premoistened with nitrogen-free water. Plants were placed in a growth chamber at a constant temperature of 28°C, with light intensity of 500 μE $m^{-2}$ $s^{-1}$ under a 12-h day and night cycle. Nodulation responses were assessed visually, and nodules were counted every three days beginning six days after inoculation.

**Nucleotide sequence accession number.**

The nucleotide sequence of the chromosomal copy of USDA257 y4xP$^c$ and USDA191 y4xP are deposited in the GenBank database under accession number DQ008443 and DQ008444, respectively.

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