

Research Note

***Sinorhizobium fredii* USDA257, a Cultivar-Specific Soybean Symbiont, Carries Two Copies of y4yA and y4yB, Two Open Reading Frames That Are Located in a Region That Encodes the Type III Protein Secretion System**

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Sinorhizobium fredii USDA257 forms nitrogen-fixing nodules on primitive soybean (*Glycine max*) cultivar Peking but fails to nodulate the improved cultivar McCall. Cultivar specificity is governed by a plasmid-borne locus, *nolXBTUV*. By DNA sequence analysis, we have identified two open reading frames, y4yA and y4yB, immediately downstream of *nolX*. Northern (RNA) blot analysis indicated that the expression of both y4yA and y4yB is inducible by isoflavonoids, and an intact copy of *nolX* is required. Two copies each of y4yA and y4yB are present in *S. fredii* USDA257, one on the *sym* plasmid (y4yAsp and y4yBsp), and the other on the chromosome (y4yAc and y4yBc). The cultivar-nonspecific strain USDA191 lacks y4yAc and y4yBc. Introduction of y4yAc plus y4yBc from USDA257 into USDA191 did not influence the ability of the latter strain to nodulate McCall soybean plants. Unlike *nolX*, the inactivation of y4yAsp and y4yBsp of USDA257 did not extend the host range of this strain. A double mutant, in which both the plasmid and chromosomal copies of y4yA and y4yB were mutated, had no observable effect on symbiotic ability of USDA257. The y4yAsp and y4yBsp mutants did not influence flavonoid-dependent extracellular protein production. *Rhizobium* sp. strain NGR234 and *S. saheli* USDA4893 both contain sequences similar to *S. fredii* USDA257 y4yAsp and y4yBsp; however, *Bradyrhizobium* spp., the traditional soybean symbionts, lack these genes.

Two genera of rhizobia, *Sinorhizobium* and *Bradyrhizobium*, form nitrogen-fixing nodules on soybean (*Glycine max* (L.) Merr.) roots. Research in our laboratory is focused on the *Sinorhizobium fredii*-soybean symbiosis. *S. fredii* USDA257 forms nitrogen-fixing nodules on primitive soybean cultivars such as Peking, but fails to nodulate agronomically improved North American cultivars (Keyser et al. 1982; Heron and Pueppke 1984). A different strain of *S. fredii* (USDA191) forms nodules and fixes nitrogen with both primitive and advanced soybean cultivars (Heron and Pueppke 1984). By means of random Tn5 mutagenesis, several mutants of USDA257 were obtained that were capable of forming nodules on advanced soybean cultivars (Heron et al. 1989). Two such mutants, 257DH4 and 257DH5, have been examined in some detail (Krishnan and Pueppke 1991a; Meinhardt et al. 1993; Kovács et al. 1995; Bellato et al. 1996 1997). 257DH5 contains the transposon in a chromosomal region named *nolC*. Inactivation of *nolC* enables 257DH5 to form Fix⁻ nodules on McCall (Krishnan and Pueppke 1992). In contrast, mutant 257DH4 forms Fix⁺ nodules on McCall and the transposon is located in an 8.0-kb *EcoRI* fragment located on the symbiosis (*sym*) plasmid (Heron et al. 1989). DNA sequence analysis of this region has identified several open reading frames (ORFs; *nolXWBTUV*) and this region has been designated as the host-specificity locus (Meinhardt et al. 1993).

Insertional mutations within *nolB*, *nolT*, *nolU*, *nolV*, *nolW*, and *nolX* all enabled *S. fredii* USDA257 to form nitrogen-fixing nodules on McCall soybean. The results of transcriptional analyses indicate that the genes in the host-specificity locus are transcribed from three promoters (Kovács et al. 1995). The promoters of *nolW* and *nolBTUV* are divergently oriented and initiate transcription at sites that are 14 bp apart. Compounds (daidzein and genistein) that regulate the expression of *nodABC* (Krishnan and Pueppke 1991b) are the most powerful inducers of *nolX* and *nolBTU* (Meinhardt et al. 1993; Gu et al. 1997). Unlike the *nodABC* genes, the soybean cultivar-specificity genes are not preceded by a conventional *nod* box (Meinhardt et al. 1993).

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Nucleotide and/or amino acid sequence data can be found at the GenBank data base as accession nos. AF229440 and AF229441.

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Flavonoids from soybean roots stimulate *S. fredii* USDA257 to secrete several proteins into extracellular medium (Krishnan and Pueppke 1993). The extracellular proteins have been designated as signal-responsive (SR) proteins and their secretion is dependent on a functional regulatory gene, *nodD1* (Krishnan et al. 1995). In addition, interruption of the cultivar-specificity locus, *noIXWBTUV*, also prevents extracellular accumulation of the SR proteins. Recently, we have shown that some of the proteins encoded by the cultivar-specificity locus are extracellular. For example, NoIX is an extracellular protein that is exported without N-terminal processing, indicating that type III protein secretion machinery is involved in its transport (Pueppke et al. 1999).

A cluster of genes that encode components of a bacterial type III secretion system (TTSS) has been identified in *Rhizobium* sp. strain NGR234 (Freiberg et al. 1997; Viprey et al. 1998), a broad-host-range symbiont of numerous legumes (Pueppke and Broughton 1999). The TTSS cluster of NGR234 contains 27 ORFs and includes the soybean cultivar-specificity locus *noIXWBTUV* (Viprey et al. 1998). Earlier, we reported the nucleotide sequence of a 5,596-bp DNA fragment that encompasses the soybean cultivar-specificity locus (Meinhardt et al. 1993; Kovács et al. 1995). Some of genes encoded in this region have significant sequence similarity with components of type III protein secretion systems of gram-negative pathogens of plants and animals.

To identify all the components of the type III protein secretion system and to locate the boundaries of the cultivar-specificity locus of *S. fredii* USDA257, we initiated sequenc-

ing of DNA regions downstream of *noIX* (Fig. 1). By sequence analysis of 2,000 bp beyond *noIX*, we have identified two ORFs, one beginning 185 bp downstream of *noIX* followed immediately by the second ORF. Both ORFs were of the same polarity as *noIX*. The first ORF is 429 bp in length and the deduced protein has a molecular mass of 15,602 Da. The potential initiation codon for the next ORF overlaps with the previous ORF termination codon. The second ORF is 1,350 bp in length and the deduced 450 amino acid sequences contribute to a protein of 49,175 Da. A computer-assisted search of protein data bases indicated a striking similarity between the newly identified ORFs from *S. fredii* and y4yA and y4yB from *Rhizobium* sp. strain NGR234. Based on this extensive identity, the ORF immediately downstream of *noIX* was designated as y4yB and the second ORF as y4yA (Fig. 1).

Southern blot analysis with *EcoRI*-digested genomic DNAs from *S. fredii* USDA257, USDA191, and *Rhizobium* sp. strain NGR234, a broad-host-range strain related to *S. fredii* (Perret et al. 1994) and its *sym*-plasmid cured derivative, ANU 265, revealed strong hybridizing signals when probed with a 1.4-kb *EcoRI/PstI* fragment that contains most of y4yB and y4yA (Fig. 2). *S. fredii* USDA191 contained a single 8.0-kb *EcoRI* hybridizing band, while *S. fredii* USDA257 and *Rhizobium* sp. strain NGR234 revealed two hybridizing bands (Fig. 2). The sizes of hybridizing bands in *S. fredii* USDA257 are approximately 8.0 and 4.0 kb, while similar fragments in *Rhizobium* sp. strain NGR234 are 8.0 and 3.2 kb (Fig. 2). DNA from the plasmid-cured derivative of NGR234 hybridized only to the 3.2-kb fragment, indicating that this fragment was located on

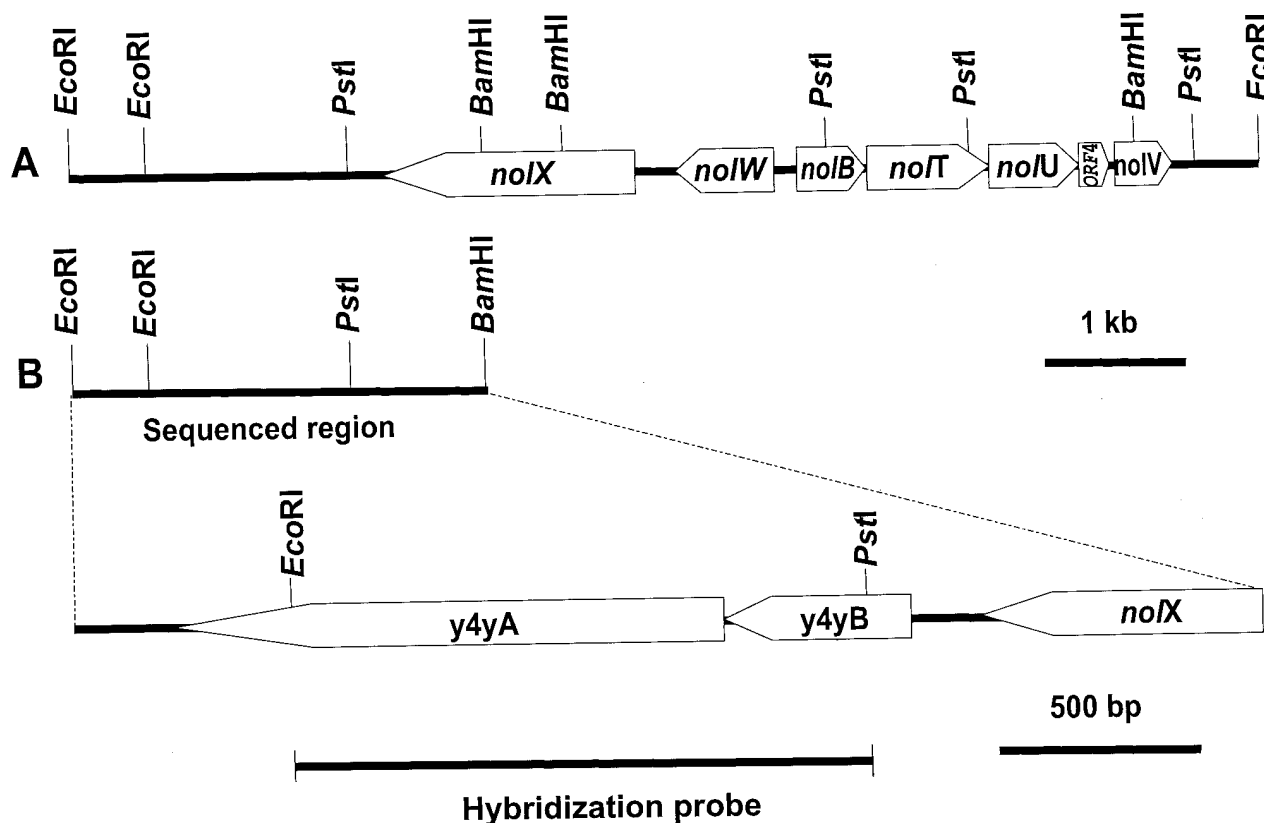


Fig. 1. A, Coordinated physical and genetic maps of cultivar-specificity region of *Sinorhizobium fredii* USDA257. B, DNA segment that was sequenced and orientation of open reading frames are shown. The 1.4-kb *PstI* and *EcoRI* fragment used as a y4yA- and y4yB-specific probe is highlighted.

the chromosome. To distinguish between the chromosomal and symbiotic plasmid copies of *y4yA* and *y4yB* of *S. fredii* USDA257, we have designated *y4yAsp*, *y4yBsp* to represent the symbiotic plasmid copies and *y4yAc*, *y4yBc* to represent the chromosomal copies.

To isolate *y4yAc* and *y4yBc*, we screened a genomic library of *S. fredii* USDA257 with the 1.4-kb *PstI-EcoRI* fragment (Fig. 1) as a hybridization probe. Four cosmid clones were isolated and a 4.0-kb *EcoRI* hybridizing fragment was subcloned in pGEM-7Zf(+) and pRK415 as pHBK39 and pHBK40, respectively. Subsequently, adjacent DNA fragments were also cloned and a total of 2,580 bp was sequenced. Analysis of the sequenced region revealed two ORFs that were very similar to *y4yBsp* and *y4yAsp*. The *y4yAc* and *y4yBc* gene products encode proteins of 49,526 and 15,609 Da, respectively. The 600-bp sequences upstream of *y4yBc* had no similarity to the sequences present in the plasmid copy, indicating that *nolX* was not reiterated in the chromosome. A comparison of the deduced amino acid sequence of *S. fredii* USDA257 Y4YAsp and Y4YBsp to that of *Rhizobium* sp. strain NGR234 homologues revealed greater than 90% identity (Fig. 3). *S. fredii* USDA257 Y4YAsp and Y4YAc share 85% amino acid identity, and Y4YBsp and Y4YBc exhibit 78% identity (Fig. 3). *Rhizobium* sp. strain NGR234 *y4yA* and *S. fredii* USDA257 *y4yAc* encode a protein of 457 amino acids while *S. fredii* USDA257 *y4yAsp* encoded a protein of 450 amino acids (Fig. 3).

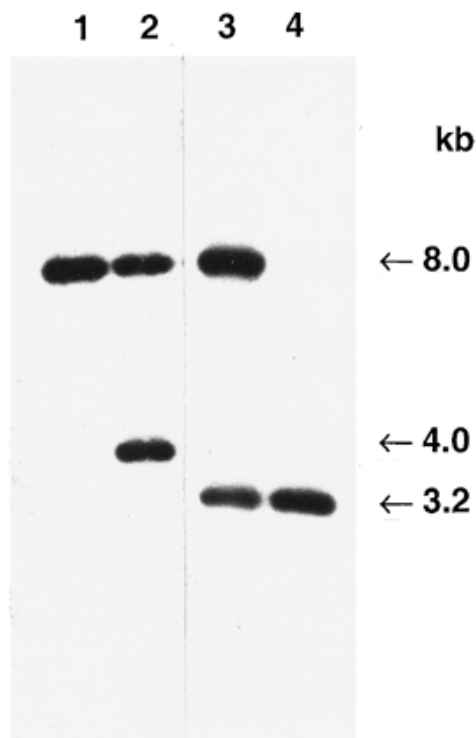


Fig. 2. Southern blot analysis of *y4yA* and *y4yB* in *Sinorhizobium fredii* and *Rhizobium* sp. strain NGR234. Genomic DNA from USDA191 (lane 1), USDA257 (lane 2), *Rhizobium* sp. strain NGR234 (lane 3), and its plasmid-cured derivative *Rhizobium* sp. strain ANU265 (lane 4) was digested with *EcoRI* and the blot probed with the 1.4-kb *EcoRI/PstI* fragment (see Figure 1). Arrows indicate sizes of hybridizing fragments.

Nucleotide sequence analysis revealed that *nolX*, *y4yBsp*, and *y4yAsp* are closely spaced and transcribed in the same direction, suggesting that these genes could be a part of an operon. To test this, we isolated RNA (Wang and Stacey 1991) from USDA191, USDA257, and USDA257 *nolX* mutant grown in the presence and absence of 1 μ M genistein. Ten micrograms of RNA was resolved on a 1.5% agarose gel containing formaldehyde, then transferred to a Nylon membrane by capillary blotting. After transfer, the membrane was baked at 80°C for 1 h and hybridized to a ³²P-labeled, 1.4-kb *PstI/EcoRI* fragment that contains most of *y4yAsp* and *y4yBsp*. Northern (RNA) blot analysis indicated that in both *S. fredii* USDA191 and USDA 257, *y4yAsp* and *y4yBsp* probe hybridized to a 2.5-kb RNA transcript only when the cells were grown in the presence of genistein (Fig. 4). However, inactivation of *nolX* resulted in no detectable RNA transcript. Our Northern blot analysis clearly shows that expression of *y4yAsp* and *y4yBsp* is dependent on *nolX*. Thus, *nolX*, *y4yBsp*, and *y4yAsp* appear to constitute an operon. Similar transcriptional organization of these genes is also found in *Rhizobium* sp. strain NGR234 and the expression of this gene cluster is induced within 1 h after induction with flavonoids (Perret et al. 1999).

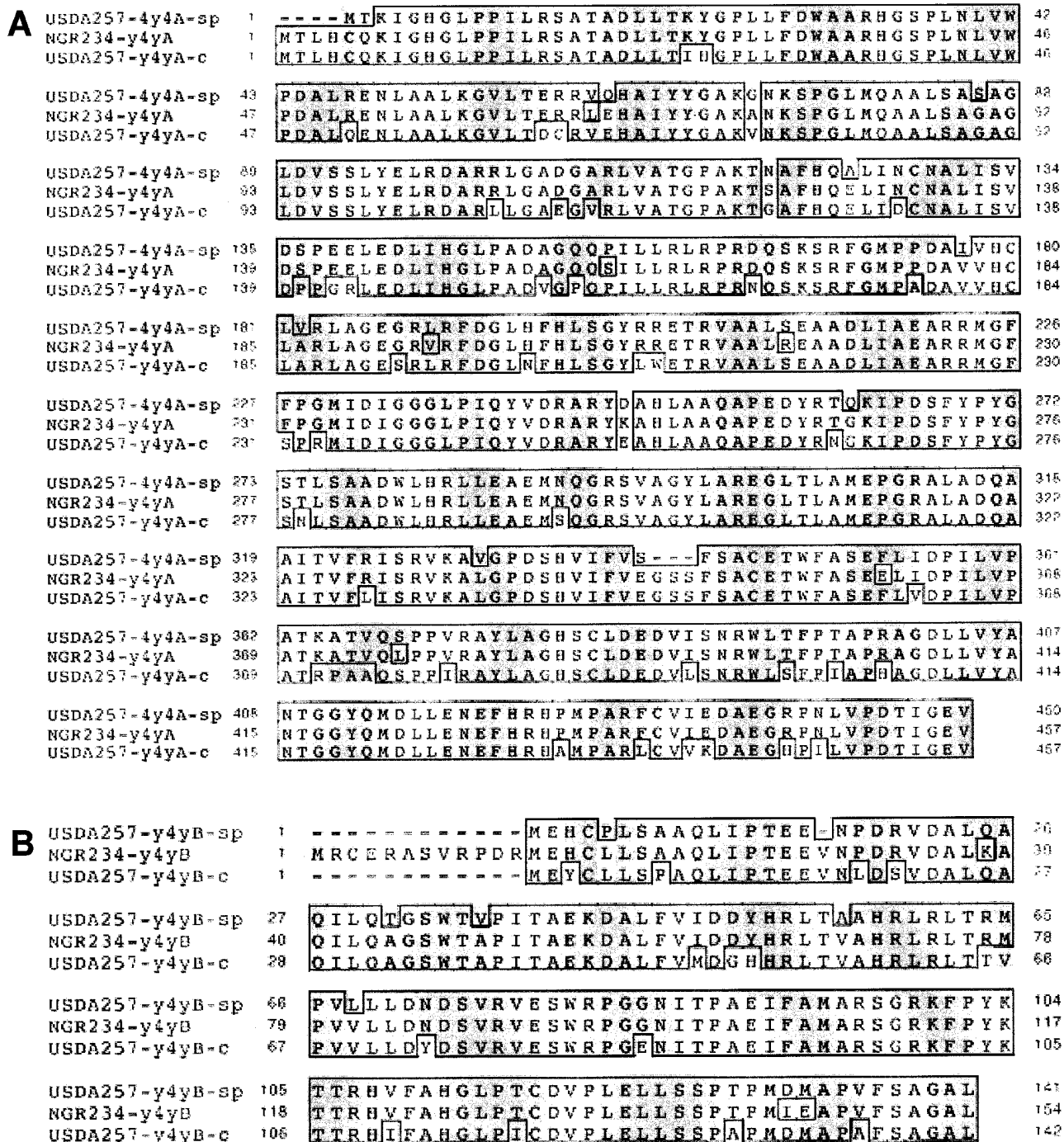
Since *S. fredii* USDA191 contains only the plasmid copy and, unlike USDA257, lacks the chromosomal copy, we tested the role of the chromosomal copy in cultivar-specific nodulation of soybeans. We first introduced the pHBK40 into *S. fredii* USDA191 by triparental mating. *S. fredii* USDA191 transconjugants carrying both the plasmid and chromosomal copies nodulated both Peking and McCall soybean cultivars. To create mutants of *y4yAsp* and *y4yBsp*, a 2.4-kb *EcoRI-BamHI* fragment from pRfDH412 was subcloned into the *SmaI* site of the suicide plasmid pJQ200uc1 (Quandt and Hynes 1993) to produce pJQ200uc1-24. A 1.3-kb *PstI* kanamycin cassette from pUC-4K (Vieira and Messing 1982) was inserted into the *PstI* site, inactivating the *y4yBsp* and *y4yAsp* genes and producing the plasmid pJQ200uc1-24km. The *y4yBc* and *y4yAc* genes were mutated as follows. A 6.3-kb *HindIII* fragment was excised from pHBK366 and subcloned in the *SmaI* site of pJQ200uc1 to produce plasmid pJQ200uc1-63. Subsequently this plasmid was digested with *PstI*, resulting in the loss of 508-bp and 648-bp regions within *y4yBc* and *y4yAc*, respectively. A 1-kb chloramphenicol cassette from pCAT19 was excised and cloned into the *PstI*-digested plasmid pJQ200uc1-63 and the resulting plasmid was named pJQ200uc1-63CAT. The mutant constructs were mobilized into *S. fredii* USDA257 by triparental mating with the helper plasmid pRK2013 (Figurski and Helinski 1979). Marker exchange was achieved by selection on YEM (yeast extract-mannitol) plates (Vincent 1970) containing 5% (wt/vol) sucrose. Mutants were confirmed by Southern blot hybridization (Maniatis et al. 1982). Inactivation of either the plasmid or chromosomal copy, or the inactivation of both copies, had no effect on the inability of USDA257 to nodulate McCall. However, USDA257 *y4yAsp* and *y4yBsp* mutants retained their ability to form nitrogen-fixing nodules on Peking soybean.

We have earlier shown that inactivation of *nolX* abolishes the SR extracellular protein production by *S. fredii* USDA257 (Krishnan et al. 1995). To examine whether inactivation of *y4yAsp* and *y4yBsp*, whose expression is dependent on *nolX*, had any effect on SR extracellular protein production, we iso-

lated the extracellular proteins from these mutants grown in the presence and absence of genistein, a potent inducer of *nod* genes of *S. fredii* (Krishnan and Pueppke 1991b). Induction and detection of SR extracellular proteins were performed as described earlier (Krishnan and Pueppke 1993). Inactivation of either the chromosomal or plasmid-borne copies of *y4yA* and *y4yB* did not prevent these mutants from elaborating the SR extracellular proteins. A double mutant, in which both the

chromosomal and plasmid copies of *y4yA* and *y4yB* were inactivated, also was not impaired in its ability to produce SR extracellular proteins (data not shown).

We tested the occurrence of *y4yAsp* and *y4yBsp* sequences in 23 different *Rhizobium* spp. obtained from the USDA collection by Southern blot analysis with a 1.4-kb *PstI-EcoRI* fragment (Fig. 1) as a hybridization probe. We observed strong hybridization to two *EcoRI* fragments in USDA257,



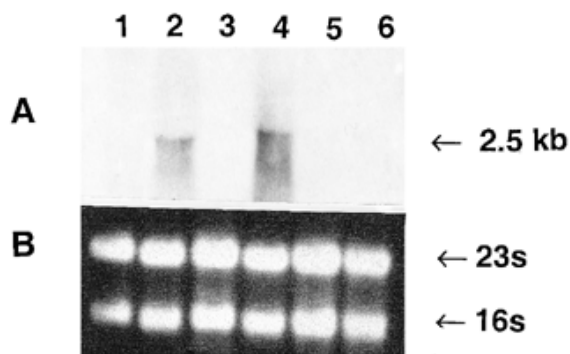


Fig. 4. Northern (RNA) blot analysis of flavonoid induction of y4yA and y4yB of *Sinorhizobium fredii*. RNA was isolated from bacteria grown in the absence (lanes 1, 3, 5) or presence (lanes 2, 4, 6) of 1 μ M genistein. Ten micrograms of RNA was resolved on a formaldehyde agarose gel and transferred to a Nylon membrane. **A**, Blots were hybridized with a 1.4-kb *PstI/EcoRI* fragment that contains most of y4yA and y4yB. **B**, Ethidium-bromide-stained gel picture showing uniform loading and integrity of RNA samples. RNA sources: lanes 1 and 2, USDA191; lanes 3 and 4, USDA257; lanes 5 and 6, USDA257 *nolX* mutant.

Rhizobium sp. strain NGR234, and *S. saheli* USDA4893. Weakly hybridizing, single *EcoRI* fragments were also detected in *Mesorhizobium tianshanense* USDA3592, *M. amorphae* USDA10001, *R. tropici* USDA9030, *R. mongolense* USDA1844, *R. etli* USDA9032, *R. galegae* USDA4128, and *R. hainanense* USDA3588. No homologous sequences to USDA257 y4yA_{sp} and y4yB_{sp} were detected in *Azorhizobium caulinodans* USDA4892, *Bradyrhizobium japonicum* USDA6, *B. liaoningense* USDA3622, *B. elkanii* USDA76, *R. huautlaense* USDA4900, *R. leguminosarum* USDA2370, *Mesorhizobium ciceri* USDA3383, *M. huakuii* USDA4779, *M. loti* USDA3471, *M. mediterraneum* USDA 3392, and *S. medicae* USDA 1037. The occurrence of *nolX*, y4yB_{sp}, and y4yA_{sp} sequences in *Rhizobium* sp. strain NGR234, and their absence in *Bradyrhizobium* spp., indicates that these genes may be involved in cellular processes other than soybean cultivar specificity. In fact, USDA257 y4yB_{sp} and y4yA_{sp} are significantly similar to diaminopimelate decarboxylase, the last enzyme of the lysine biosynthetic pathway in bacteria.

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