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Two Host-Inducible Genes of *Rhizobium fredii* and Characterization of the Inducing Compound

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Random transcription fusions with Mu d1(Kan *lac*) generated three mutants in *Rhizobium fredii* (strain USDA 201) which showed induction of β-galactosidase when grown in root exudate of the host plants *Glycine max*, *Phaseolus vulgaris*, and *Vigna ungliculata*. Two genes were isolated from a library of total plasmid DNA of one of the mutants, 3F1. These genes, present in tandem on a 4.2-kilobase *Hin*dIII fragment, appear in one copy each on the symbiotic plasmid and do not hybridize to the *Rhizobium meliloti* common nodulation region. They comprise two separate transcriptional units coding for about 450 and 950 nucleotides, both of which are transcribed in the same direction. The two open reading frames are separated by 586 base pairs, and the 5′ regions of the two genes show a common sequence. No similarity was found with the promoter areas of *Rhizobium trifolii*, *R. meliloti*, or *Bradyrhizobium japonicum nif* genes and with any known nodulation genes. Regions homologous to both sequences were detected in *Eco*RI digests of genomic DNAs from *B. japonicum* USDA 110, USDA 122, and 61A76, but not in genomic DNA from *R. trifolii*, *Rhizobium leguminosarum*, or *Rhizobium phaseoli*. Mass spectrometry and nuclear magnetic resonance analysis indicated that the inducing compound has properties of 4′,7-dihydroxyisoflavone, daidzein. These results suggest that, in addition to common nodulation genes, several other genes appear to be specifically induced by compounds in the root exudate of the host plants.

The establishment of the *Rhizobium*-legume symbiosis requires that complex biochemical, physiological, and molecular changes occur in both partners. The most pronounced changes which occur during the formation of nitrogen-fixing nodules are the production of nodulins (10, 24), and the differentiation of rhizobia into nitrogen-fixing bacteroids (45). However, many subtle changes also occur during the early stages of the symbiotic process and likely involve the induction and repression of a large number of bacterial and plant genes (29; see references 32 and 46).

While the early periods of the symbiosis have been shown to be important for nodulation and competition (23), most of our knowledge about plant-bacterium interactions has been limited to postinfection events (3). Recently, evidence has accumulated from several laboratories indicating that some form of bacterium-plant communication is important for the early symbiotic steps (3, 4, 7, 8, 13, 14, 17, 18, 29, 37; R. M. Kosslak, R. Bookland, J. Barkei, H. E. Paaren, and E. R. Applebaum, Proc. Natl. Acad. Sci. USA, in press). Legume plants excrete substances which allow *Rhizobium* species to nodulate their respective hosts effectively. Although the exact natures and modes of action of the excreted substances are unknown, crude soybean root exudates have been shown to stimulate chemotaxis in *Bradyrhizobium* sp. (14), influence lectin binding in *Bradyrhizobium japonicum* strains (8), elicit a faster nodulation response in *Bradyrhizobium* spp. strain 32H1 (7), cause a phenotypic reversion in slow-to-nodulate *B. japonicum* HS111 (18), induce symbiosis-associated genes in *Rhizobium fredii* (29), and increase the competitiveness of some *B. japonicum* strains (3). Both positive and negative interactions of root- or seed-derived compounds (flavanones, flavanols, flavones, and isoflavones) have been shown with the common nodulation genes of *Rhizobium meliloti* (28, 30), *Rhizobium trifolii* (22), *Rhizobium leguminosarum* (49), and *B. japonicum* (Kosslak et al., in press). Using Mu d1(Kan *lac*) transcription fusions, we have identified three *R. fredii* USDA 201 insertion mutants which have symbiosis-related genes specifically induced by soybean root exudate and extract (29). Here we report on the isolation, cloning, and molecular analysis of two host plant-inducible operons in one of these mutants and the characterization of the inducing substance.

MATERIALS AND METHODS

Bacteria, plasmids, and growth conditions. *R. fredii* USDA 201 and the Mu d1(Kan *lac*) insertion mutants (3F1, 6A6, and 3B2) inducible by host plant substances have been described previously (29). Plasmid pACYC184 (11) was used for cloning mutant (pAC3F1) and wild-type (pAC2A4) inducible genes, and *Escherichia coli* strains containing these plasmids were grown on LB medium (26) supplemented with 10 μg of tetracycline per ml and 30 μg of chloramphenicol per ml, respectively. The *R. fredii* USDA 201 cultures were grown at 28°C on YEM (47) or on PPM (35) agar slants. Insertion mutants were grown on PPM with 50 μg of kanamycin per ml and 10 μg of naladixic acid per ml. *lacZ* activity in *E. coli* (JM101) was determined on LB medium containing 40 μg of 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal) per ml.

DNA and RNA isolation and hybridization. *Rhizobium* total DNAs and plasmids were isolated as described previously (20). Large-scale *E. coli* plasmid DNA was prepared by the method of Holmes and Quigley (21), and plasmid mini-preps

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were prepared by the method of Birnboim and Doly (9). ^{32}P-labeled probes were prepared and hybridized to Southern blots as previously described (29).

Total RNA from root extract-induced and uninduced *R. fredii* USDA 201 and mutant 3F1 (29) was isolated by a modification of the hot phenol-lysozyme procedure (12). *Rhizobium* cultures were induced as described previously (29). Glyoxylated RNAs (10 μg) were separated by horizontal electrophoresis in 1.0% agarose gels in circulating 10 mM sodium phosphate buffer (26). RNA was transferred to GeneScreen (New England Nuclear Corp., Boston, Mass.) and hybridized in 50% (vol/vol) formamide–0.02% Ficoll–0.02% polyvinylpyrrolidone–0.02% bovine serum albumin–5× SSC (1× SSC is 8 g of NaCl per liter and 4.4 g of sodium citrate per liter)–1% sodium dodecyl sulfate–100 μg of denatured salmon sperm DNA per ml. The ^{32}P-labeled DNA probes were hybridized for 19 to 24 h at 42°C, and filters were washed at 65° in 2× SSC–1% sodium dodecyl sulfate.

Exonuclease VII mapping. Mapping of the transcription start site with exonuclease VII (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was done by hybridizing approximately 40,000 cpm of the 5′-^{32}P-end-labeled 651-base-pair (bp) *Hin*dIII-*Xho*I fragment of pAC2A4 to 20 μg of RNA from root exudate-induced USDA 201. Hybridizations were done at 51°C for 18 h in 10 μl of 0.04 M PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.4)–0.4 M NaCl–2 μg of denatured calf thymus DNA–1 mM EDTA in 80% formamide (5, 26). The mixture was diluted in 100 μl of ice-cold buffer (0.3 M KCl, 10 mM Tris hydrochloride [pH 7.6], 10 mM EDTA, 10 mM β-mercaptoethanol, 0.139 U of exonuclease VII) and incubated at 37°C for 40 min. The reaction was stopped by adding 12 μl of 7.5 M ammonium acetate and 10 μg of tRNA, and the nucleic acids were precipitated by ethanol. After washes with 70% ethanol, the pellet was dissolved in formamide containing 20 mM EDTA and 0.03% each of xylene cyanol and bromophenol blue and electrophoresed on a 4% polyacrylamide gel with Tris-borate-EDTA buffer containing 8 M urea.

β-Galactosidase assays. β-Galactosidase induction assays in microtiter plates were done as described previously (29). Assays for induction by commercially available isoflavones, flavones, flavanones, and flavanols were done in test tubes in TM medium (22) at final concentrations of 0.1 and 1.0 μM with mutant 3F1. The compounds were prepared as 1 mM stocks in 100% ethanol, and logarithmic-phase cultures were induced for 24 h and assayed by the chloroform-sodium dodecyl sulfate procedure of Miller (27). Three replicate cultures were assayed for each compound tested, and the values presented are in β-galactosidase units. Luteolin, genistein, and 4′,7-dihydroxyisoflavone (daidzein) were obtained from ICN Biomedicals; apigenin, naringenin, and biochanin A were obtained from Aldrich Chemical Co., Inc. (Milwaukee, Wis.); chrysin and quercetin were from Sigma Chemical Co., (St. Louis, Mo.); and formononetin was obtained from Spectrum Medical Industries (Los Angeles, Calif.). The 4′,7-dihydroxyflavone was kindly provided by Agrigenetics Advanced Science Co. (Madison, Wis.).

Preparation and purification of inducing substance. Plant extracts were prepared and assayed as described previously (29) from *Glycine max* cv. Peking roots, seeds, seed coats, leaves, epicotyls, hypocotyls, and cotyledons. Although the inducing substance is present in root exudates and root extract, concentrated seed extracts were used to obtain larger quantities of the inducing compound. *G. max* cv. Prize seeds (1 kg) were soaked in water overnight and ground to a fine slurry in a Waring blender. The mixture was filtered through several layers of cheesecloth, adjusted to a final ethanol concentration of 70% (vol/vol), and allowed to precipitate for 2 to 3 h at −20°C. The suspension was centrifuged at 7,000 × *g* for 10 min at 4°C, and the resulting supernatant was concentrated 10-fold by rotary evaporation at 60°C. The concentrate was dialyzed overnight against 2 liters of 70% (vol/vol) ethanol, using a 1,000-molecular-weight-cutoff Spectropor dialysis membrane (Spectrum). The dialysate was concentrated fivefold, adjusted to pH 1.0 with concentrated H_2SO_4, and extracted with an equal volume of anhydrous diethyl ether. The ether phase was extracted with 0.5 volume of 10 mM NH_4OH and adjusted to pH 10.0 with concentrated NH_4OH. The aqueous phase was then concentrated approximately fivefold by evaporation and filtered through a nylon filter (0.45-μm pore size; Fisher Scientific Co., Springfield, N.J.). The concentrate was fractionated by high-pressure liquid chromatography (HPLC) with a reverse-phase C-18 column with a flow rate of 0.3 ml/min and a 30 to 70% methanol-water gradient. Absorbance was measured at 254 nm, and fractions were assayed in microtiter plates for inducing activity with the Mu dl(Kan *lac*) insertion mutant 3F1 (29). A peak having inducing activity was converted to the trimethylsilyl (TMS) derivatives by heating at 50°C for 10 min with 50 μl of Tri-Sil–bovine serum albumin (Pierce Chemical Co., Rockford, Ill.). A 1-μl sample was analyzed in electron impact mode with a LKB 9000 gas chromatograph-mass spectrometer, using the gas chromatography inlet equipped with a 30-meter DB-1 Megabore column and temperature programmed from 150 to 290°C at 5°C/min. The active peak was also analyzed by nuclear magnetic resonance spectroscopy.

DNA-sequencing analysis. The 2.86-kilobase (kb) *Hin*dIII-*Sal*I fragment from pAC2A4 was treated with T4 DNA polymerase to produce overlapping fragments of convenient length (15) and sequenced by the dideoxy method of Sanger (36). DNA sequences in the putative promoter regions of two root exudate-inducible genes were compared with each other and with the known symbiotic promoters of *B. japonicum*, *R. meliloti*, *R. trifolii*, and *Klebsiella pneumoniae nif* genes (see references 6 and 38) and symbiotic nodulation genes (34, 39, 41, 42) by using Rapid Similarity Search computer programs (48).

RESULTS

Isolation of host plant-inducible genes from a random transcription fusion mutant, 3F1. Random Mu dl(Kan *lac*) transpositions in *R. fredii* USDA 201 resulted in a mutant (3F1) which exhibited β-galactosidase activity when grown in the presence of root exudate from its hosts (29). Southern hybridization analysis revealed that the insertion in mutant 3F1 had occurred in the 190-megadalton indigenous symbiotic plasmid (data not shown). *Eco*RI-digested total plasmid DNA of this mutant was cloned into the vector pACYC184, and tetracycline-resistant (Tc^r) transformants were screened for *lacZ* activity. One of the positive transformants had a plasmid, pAC3F1, which contained a 5.3-kb insert (Fig. 1A). When this plasmid was used as a probe to Southern blots of *Eco*RI-digested plasmid DNA from USDA 201 and mutant 3F1, it hybridized to a 5.3-kb fragment from mutant 3F1 and a 6.0-kb fragment from USDA 201 (Fig. 2A). In addition, pAC3F1 also hybridized to a *lacZ* probe (data not shown), indicating that the insert in pAC3F1 contains the Mu dl(Kan *lac*)-*Rhizobium* junction fragment from mutant 3F1.

To obtain the wild-type gene corresponding to this locus, USDA 201 plasmid DNA was digested with *Hin*dIII and

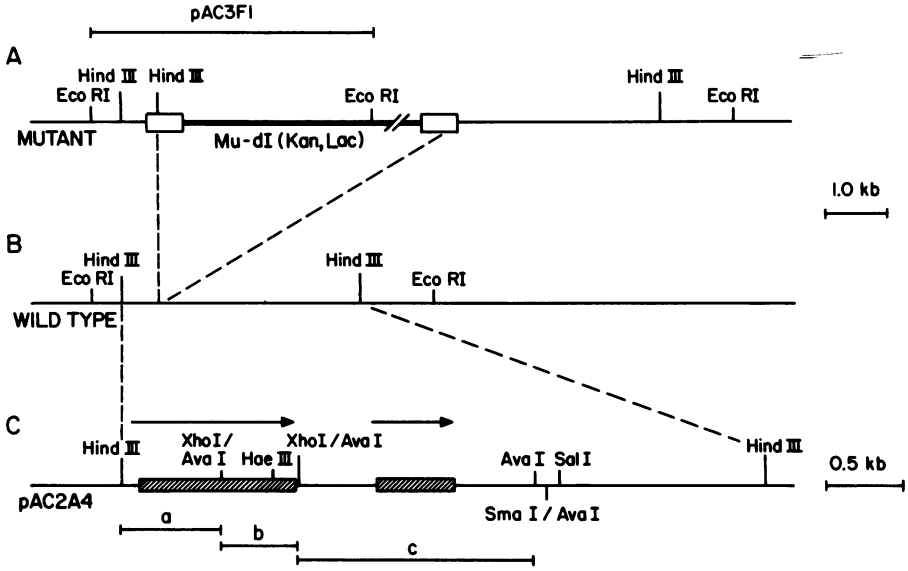

FIG. 1. Restriction maps and physical relationship of pAC3F1 and pAC2A4. The *Eco*RI insert from a USDA 201::Mu d1 mutant (3F1) plasmid library, pAC3F1 (A), is shown with respect to the wild-type *Hin*dIII insert (B) isolated from pAC2A4. The site of Mu d1(Kan *lac*) insertion in mutant 3F1 is indicated by dotted lines. The hatched boxes in panel C indicate the positions of the two root exudate-inducible genes, and the arrows indicate the direction of transcription. Fragments used as probes for Northern hybridizations (C) (see Fig. 3) are listed (a to c).

cloned into plasmid pACYC184. A plasmid (pAC2A4) from one of the chloramphenicol-resistant (Cm^r) and Tc^s transformants was found to have a 4.2-kb fragment which hybridized to the pAC3F1 *Eco*RI insert. Plasmid pAC2A4 hybridized to two bands in mutant 3F1, one of which was the same 5.3-kb fragment hybridizing to the pAC3F1 probe (Fig. 1B and 2B). The large *Eco*RI fragment hybridizing in mutant 3F1 (Fig. 2B) corresponds to the other Mu d1(Kan *lac*) junction fragment. When pAC2A4 was used as to probe

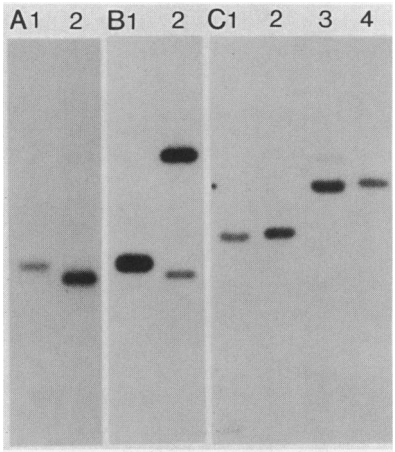

FIG. 2. (A and B) Southern hybridization of plasmid DNA from USDA 201 and Mu d1 mutant 3F1. DNAs were digested with *Eco*RI, electrophoresed through a 0.8% agarose gel, blotted to GeneScreen, and sequentially hybridized to ^{32}P-labeled pAC3F1 (A) and pAC2A4 (B) probes. Lanes: 1, USDA 201; 2, 3F1. (C) Hybridization of USDA 201 plasmid and genomic DNA with pAC2A4. Plasmid and genomic DNA from USDA 201 was digested with *Hin*dIII (lanes 1 and 2, respectively) and with *Eco*RI (lanes 3 and 4, respectively) and electrophoresed on a 1.0% agarose gel. The gel was blotted to GeneScreen and hybridized with ^{32}P-labeled pAC2A4 plasmid DNA.

*Eco*RI- and *Hin*dIII-digested USDA 201 genomic and plasmid DNAs, a 4.2-kb *Hin*dIII fragment and a 6-kb *Eco*RI fragment hybridized (Fig. 2C). This indicates that these sequences are not reiterated in the *R. fredii* genome and that they are present in one copy on the symbiotic plasmid. Similarly, in two other inducible mutants (3B2 and 6A6 [29]), Mu d1(Kan *lac*) was also found to be present on the 190-megadalton symbiotic plasmid, but in different locations (data not shown).

Transcription analysis. RNA from USDA 201 and mutant 3F1 grown in the presence of the inducer hybridized strongly to the pAC2A4 probe (Fig. 3A). In addition to an RNA species common to both strains (approximately 450 nucleotides), USDA 201 also has a hybridizing RNA species of approximately 1,000 nucleotides in length. To determine what region of the 4.2-kb *Hin*dIII fragment codes for each of these transcripts, we hybridized RNA blots to different DNA fragments from pAC2A4. Results from these hybridizations (Fig. 3B; see also Fig. 1C) indicated that there are two separate transcripts coded for by the 4.2-kb region contained in pAC2A4. The larger transcript is the one that is interrupted by the Mu d1(Kan *lac*) insertion in the mutant 3F1 (Fig. 1). The second transcript originates and ends within the 1.4-kb *Ava*I-*Ava*I fragment, suggesting that it is a product of another promoter. The RNA analysis also demonstrated that both transcripts are induced to about the same extent in response to root exudate or root extract (Fig. 3A).

Localization of inducible promoter regions. To localize the promoter region upstream from the Mu d1(Kan *lac*) insertion, we hybridized the small *Eco*RI-*Hin*dIII and *Hin*dIII fragments from pAC3F1 (Fig. 1) to Northern blots of RNA isolated from root extract-induced USDA 201. Only the *Hin*dIII fragment hybridized to the larger transcript (data not shown). To localize the promoter region more precisely, the 651-bp *Hin*dIII-*Xho*I fragment from pAC2A4 was 5′ end labeled with ^{32}P, hybridized to RNA from root exudate-induced USDA 201, and subjected to digestion with exonuclease VII. The size of the protected fragment (Fig. 4)

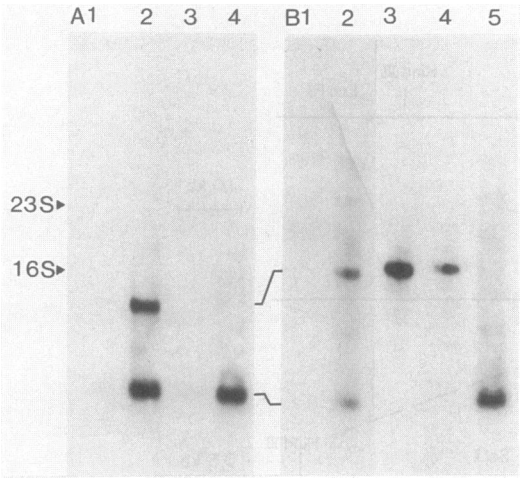

FIG. 3. (A) Northern hybridization analysis of root extract-induced RNA from USDA 201 and mutant 3F1. RNA was electrophoresed, blotted to GeneScreen, and hybridized to ^{32}P-labeled pAC2A4 DNA. Lanes contain RNA from USDA 201 grown in PPM medium only (lane 1), USDA 201 grown in PPM plus root extract (lane 2), mutant 3F1 grown in medium only (lane 3), and mutant 3F1 grown in medium plus root extract (lane 4). *E. coli* RNA was used as the reference for size. (B) Northern hybridization analysis of root extract-induced RNA from USDA 201. Northern blots were hybridized to different ^{32}P-labeled DNA probes from pAC2A4. Lane 1, Uninduced (control); lanes 2 to 5, induced RNA: lanes 1 and 2 hybridized with pAC2A4 probe, lane 3 with probe b, lane 4 with probe a, and lane 5 with probe c (probes as shown in Fig. 1).

localized the transcription start site in an area 180 bp from the *Hin*dIII site. The initiation position of the second transcript was not determined.

Structures of two host-inducible genes. To determine the sizes of the open reading frames and whether DNA sequences proximal to the start points of both transcripts share any common features, we sequenced the 2.86-kb *Hin*dIII-*Sal*I fragment from pAC2A4. Two open reading frames corresponding to the two inducible transcripts were found (Fig. 5). The first open reading frame corresponding to the larger RNA transcript is 296 codons and starts 43 bp from the transcription initiation site. The second open reading frame begins 1,517 bases from the first transcription initiation site and consists of 149 codons. Data from transcriptional mapping, orientation of the Mu d1 insertion, exonuclease VII, and sequence analysis indicate that both root exudate-induced messages are transcribed in the same orientation. Comparison of the nucleotide sequences upstream from both inducible genes indicated that they share a region of homology beginning at positions −128 and 1480, respectively, including a sequence showing significant homology (Fig. 5, underlined) having 82% consensus. A homology matrix performed on the entire 2.86-kb sequence indicated that no other areas had any appreciable homology to each other. A comparison of the nucleotide sequences upstream from both of the root exudate-inducible genes with promoter sequences from *K. pneumoniae* and *R. trifolii nifHDK* (38) and *nifLA* (16), *R. meliloti nifHDK* (6), *B. japonicum nifH* and *nifDK* (1), or any nodulation genes (34, 39, 41, 42) revealed no apparent sequence homology. In addition, there was no sequence homology with the coding regions of any of these genes.

Promoter-inducing host compound. To determine the nature of the plant-derived *Rhizobium* promoter-inducing substance, we attempted to characterize the active component(s). The promoter-inducing substance is heat stable and suffered no apparent loss in activity after treatment of 121°C for 15 min. In addition, the active component was acid and alkali stable and survived incubation at 50°C for 30 min at pH 1.0 or 14.0. The active component(s) did not appear to be a glycoprotein, since it did not bind to soybean agglutinin, wheat germ lectin, pea lectin, ulex lectin, or concanavalin A. Fractionation on DEAE suggested that the active component is relatively negatively charged as it was not eluted from the column until an NaCl concentration of 1 M at pH 7.5. The DEAE-eluted active component did not react with ninhydrin reagent and thus does not appear to contain any free amino groups. It was dialyzable and readily passed through a 1,000-molecular-weight-cutoff membrane. Chromatography on a Bio-Gel P2 column indicated that the promoter-inducing compound has an apparent molecular weight of approximately 300.

Results in Table 1 show that while the majority of induction activity was in soybean roots, the compound(s) was also present in extracts from seed coat and from seed devoid of its coat. While virtually no activity was found in the leaf fraction, a significant amount was found in hypocotyl, cotyledon, and epicotyl.

To purify the inducing compound, we fractionated a large-scale ethanolic-ether extract from *G. max* seeds by HPLC with a reverse-phase C-18 column with a water-methanol gradient. Two large peaks of activity were found in

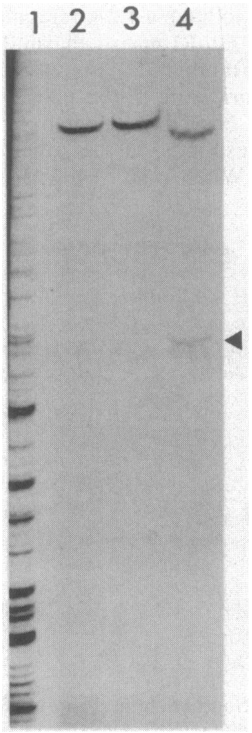

FIG. 4. Exonuclease VII mapping of the larger root exudate-inducible transcript from USDA 201. The 5′-[^{32}P]ATP-labeled 651-bp *Hin*dIII-*Xho*I fragment from pAC2A4 was hybridized to induced RNA and digested with exonuclease VII. Fragments were electrophoresed on a 4.0% polyacrylamide sequencing gel. Lanes: 2, probe alone; 3, probe plus 10 μg of RNA without exonuclease VII; and 4, probe hybridized with RNA and treated with exonuclease VII. Arrowhead indicates the position of the protected fragment, and lane 1 shows the M13mp11 sequence ladder used as a size marker.

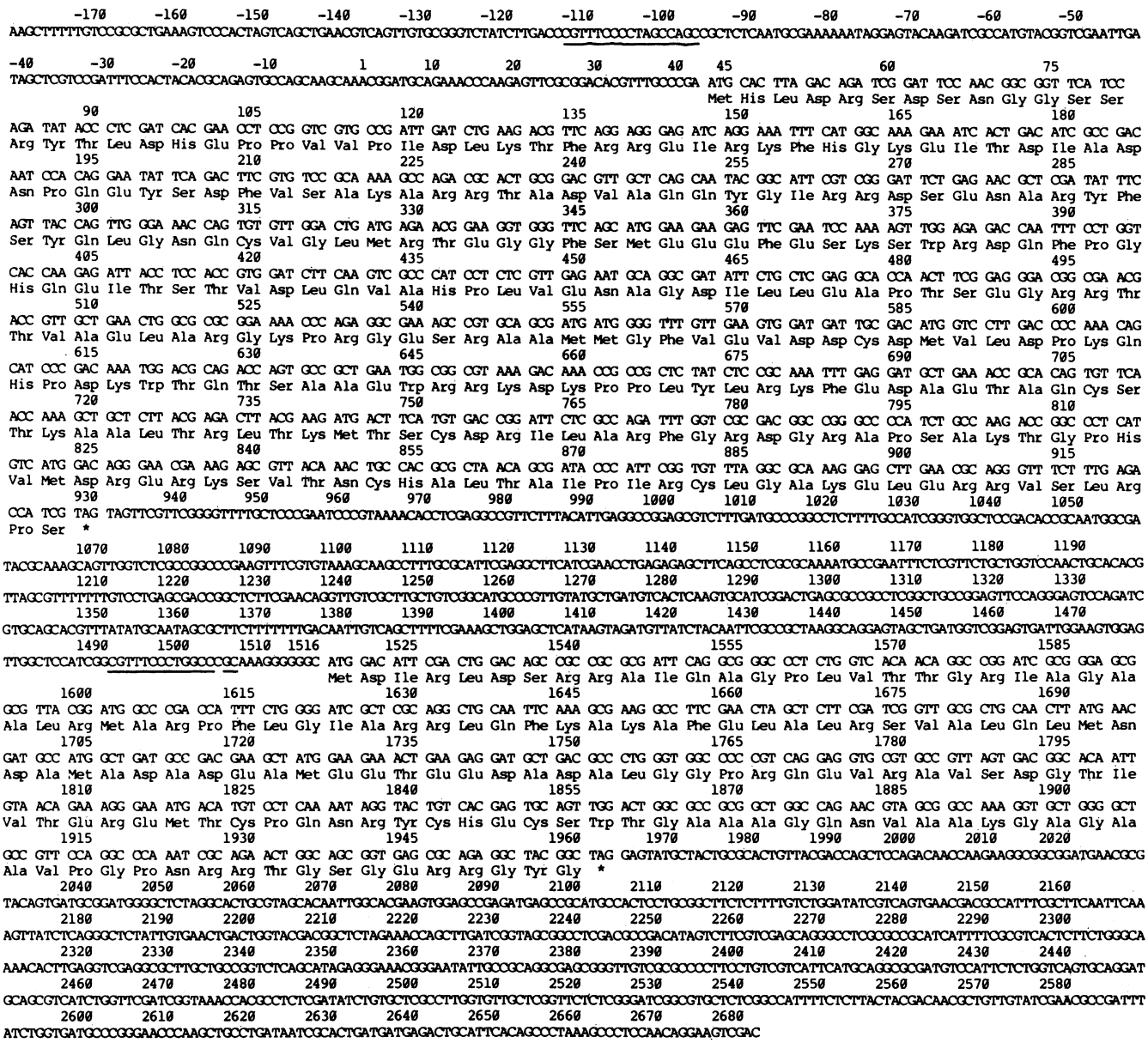

FIG. 5. DNA sequence of the host-inducible genes located on the 2.86-kb *Hin*dIII-*Sal*I fragment of pAC2A4. Stop codons are indicated by the asterisk (*). Initiation site of the first gene is at position +1. The area upstream of the start site of the first transcript was compared with areas proximal to the second transcript and found to contain a common sequence (underlined).

the first fractionation. The first peak eluted with 100% water, while the second peak eluted between 40 and 60% methanol. The second peak was repurified by HPLC with the same column and solvent gradient, and the peak having induction activity was analyzed by gas chromatography-mass spectrometry. The gas chromatography-mass spectrometry results indicated that the HPLC peak was composed of two principal compounds present in a ratio of roughly 10:1 (data not shown). The more abundant of the two compounds had an underivatized molecular weight of 302 and contained three free hydroxyl groups; the other compound had an underivatized molecular weight of 254 with two free hydroxyl groups. The less abundant of the two compounds (molecular weight of 254) was highly aromatic and had a mass spectrum similar in many respects to that of daidzein, an isoflavone which was analyzed in parallel. The mass spectrum of the minor peak is shown in Fig. 6 and was identical to the mass spectrum obtained from an authentic sample of daidzein (4′,7-dihydroxyisoflavone) similarly analyzed as the TMS derivative. In addition, the retention time of the daidzein standard was identical to that of the 254-molecular-weight component. The TMS derivatives of the seed-derived daidzein and synthetic 4′,7-dihydroxyisoflavone each showed the molecular ion at m/z = 398 and major fragmentation ions at 383 and 184 (Fig. 6). These spectra are identical to those obtained by Porter et al. (31). Nuclear magnetic resonance analysis indicated that the B ring had only a single hydroxyl substitution at the *para* position and also suggested a compound similar in structure to 4′,7-dihydroxyisoflavone (data not shown).

Synthetic daidzein and several other commercially available isoflavones, flavones, and flavanones were tested for

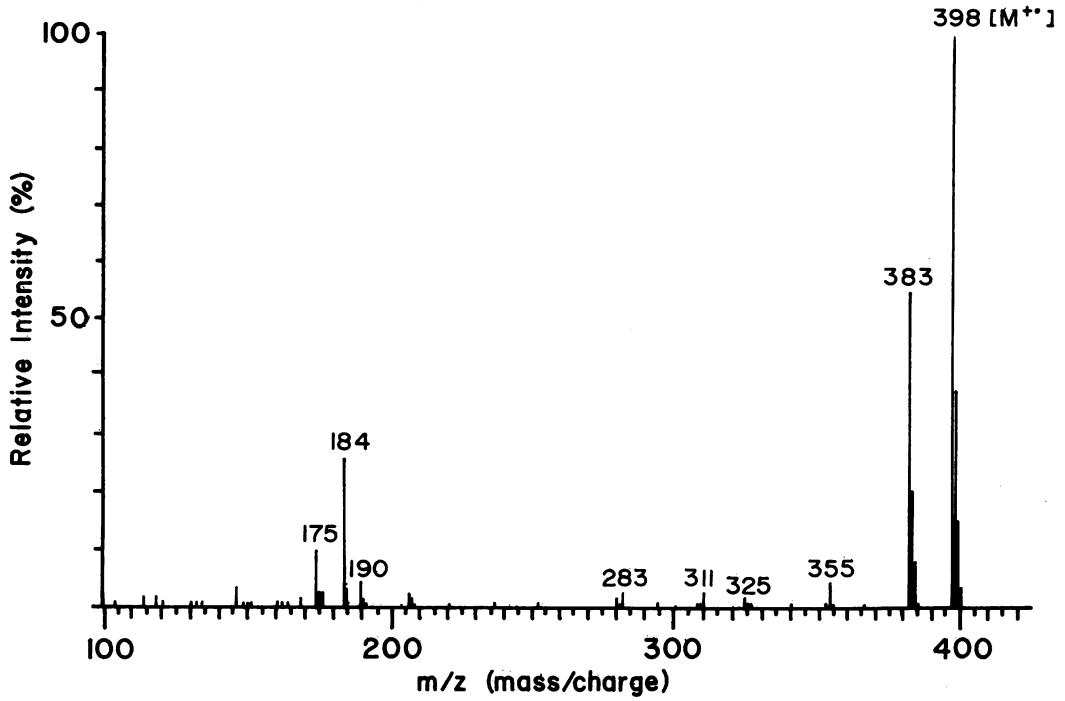

FIG. 6. Electron impact mass spectrum of the bis-TMS derivative of 4',7-dihydroxyisoflavone isolated from soybean seed extract. The spectrum is identical to the mass spectrum for the TMS derivative of synthetic 4',7-dihydroxyisoflavone.

induction activity at two concentrations (0.1 and 1.0 μM). Some of these compounds have previously been shown to induce the common nodulation genes of several fast-growing rhizobia (30, 33, 41, 42, 49) and *B. japonicum* (Kosslak et al., in press). Of the compounds tested at a 0.1 μM concentration, genistein (an isoflavone) and apigenin (a flavone) induced mutant 3F1 to an extent approximately equal to that of daidzein (Table 2). The flavone dihydroxyflavone also induced this gene at a lower level, while the isoflavones formononetin and biochanin A, the flavones luteolin and chrysin, the flavanone naringenin, and the flavanol quercetin showed little or no induction activity. However, when tested at a 1.0 μM concentration (Table 2), several of the compounds previously showing little or no activity induced mutant 3F1 to levels comparable to that induced by daidzein.

Taken together, the analytical and biological data suggested that one of the active ingredients in the root exudate or root extract responsible for promoter induction is 4',7-dihydroxyisoflavone or its derivative.

Symbiotic phenotype of Mu d1 insertion mutant 3F1. It was previously reported (29) that the Mu d1(Kan *lac*) insertion in the induction mutant 3F1 did not cause a loss of nodulating ability and hence that this insertion did not occur in any essential nodulation genes. The induction of these genes by host plant substances suggests that the insertion of Mu d1 disrupted symbiotic genes with no apparent phenotype. In

TABLE 1. Distribution of promoter-inducing substance in *G. max* cv. Peking plants[a]

Plant part	β-Galactosidase activity[b]	% Relative activity[c]
Roots	87.4	100.0
Leaves	3.9	4.4
Hypocotyls	12.5	14.3
Cotyledons	9.7	11.0
Epicotyls	9.9	11.3
Seed coats	18.2	20.8
Seeds[d]	43.7	50.0

[a] Plants were 1 week old at harvest.
[b] A_{420}/mg of protein, assayed as previously described (29).
[c] Relative to that of roots as assayed with USDA 201::Mu d1(Kan *lac*) mutant 3F1.
[d] Seeds were devoid of coats.

TABLE 2. Induction of Mu d1(Kan *lac*) mutant 3F1 by flavanoids

Compound	Units of β-galactosidase activity[a] at a compound concn of (μM):	
	0.1	1.0
Isoflavones		
Daidzein	263 ± 4	480 ± 9
Genistein	126 ± 10	350 ± 3
Formononetin	25 ± 2	56 ± 4
Biochanin A	26 ± 2	32 ± 3
Flavones		
Apigenin	282 ± 19	439 ± 32
4',7-Dihydroxyflavone	94 ± 12	390 ± 14
Luteolin	28 ± 2	282 ± 5
Chrysin	42 ± 4	281 ± 17
Flavanone (naringenin)	28 ± 2	195 ± 16
Flavanol (quercetin)	26 ± 2	41 ± 10
Control[b]	20 ± 1	

[a] Values represent the mean ± standard deviation of three replicates at each concentration tested (0.1 and 1.0 μM).
[b] Control was induction medium plus ethanol without added inducer.

addition, the two host-induced genes remain transcriptionally active throughout the process of symbiosis. Equal concentration, paired competition studies (23) between the mutant and wild-type strains of USDA 201 indicated that the induction mutant is severely out-competed by the wild-type parent strain and only occupies about 1% of the nodules formed. Since two other root substance-inducible mutants behaved like 3F1 in competition assay and since all three have Mu dl inserted into different *Eco*RI fragments of the Sym plasmid (data not shown), it is possible that this phenotype is due to some other common gene(s) being affected (see reference 29).

DISCUSSION

Early bacterium-plant communication is considered to be essential for the establishment of an effective legume-*Rhizobium* symbiosis. Using Mu dl(Kan *lac*)-generated transcription fusions, we previously demonstrated (29) that soybean root exudate-root extract induces several promoters of symbiosis-related genes in *Rhizobium* species. We isolated the region of DNA around the Mu dl insertion from one of the mutants and found two genes, in tandem, present on a 4.2-kb *Hin*dIII fragment of the Sym plasmid of USDA 201. Both of these genes are induced at the same level by host compounds and are transcribed in the same direction.

The area upstream of the transcription start site of the first gene and the sequence upstream to the open reading frame of the second gene contain a common sequence (Fig. 5). This sequence was not found in the promoter regions from *K. pneumoniae nifHDK* (40) and *nifLA* (16), *R. trifolii nifHDK* (38), *R. meliloti nifHDK* and symbiotic promoters P1, P2, P3, and P4 (6), *B. japonicum nifH* and *nifDK* (1), or any known nodulation genes (34, 39, 41, 42).

The inducing substance(s) was found not only in roots but also in seeds, hypocotyls, epicotyls, cotyledons, and seed coats; however, very little or none was found in leaves. If material leaching from seeds and seed coats is capable of inducing these genes, it may provide for very early transcriptional activation and priming of *Rhizobium* cells for infection of a suitable legume host. Since these sequences are induced only by soybean, kidney bean, and cowpea root exudates-root extracts (the hosts nodulated by *R. fredii* USDA 201), we argue that these sequences may be involved in the plant-specific control of *R. fredii* gene expression.

One of the compounds responsible for the induction of the genes in mutant 3F1 containing Mu dl was found to have the properties of daidzein, an isoflavone. Recently, Kosslak et al. (in press) reported that daidzein is also responsible for inducing the common nodulation genes of *B. japonicum* strains. Our results in Table 2 suggest that, like common *nod* gene induction in *B. japonicum*, a hydroxyl group at the C-7 position on the A ring of the tested flavanoids is sufficient for inducing genes in mutant 3F1. It is interesting to note that daidzein also acts as an inhibitor for common *nod* gene expression in *R. leguminosarum* (17). Daidzein has previously been reported to be present in soybean root extracts (31) and root exudate (2), and a glucoside of daidzein occurs in soybean meal (see reference 19). In fact, most isoflavones present in legume species have one or more sugars attached, the position of which may provide a specificity of interaction. Furthermore, the glycosylated form is water soluble, as is the compound found in root exudate, while daidzein is soluble only in alcohol and ether. The two forms may be present in the soybean seed extract eluting as two different peaks on the C-18 HPLC column.

The exact biological function of the root exudate-inducible genes is unknown, and amino acid sequence comparison of the proteins encoded by these two genes with the protein data base of the National Biomedical Research Foundation by using a FASTP computer program (25) showed no significant homologies with known proteins. Since in two other Mu dl(Kan *lac*) induction mutants (3B2 and 6A6) the site of insertion was in different genes on the symbiotic plasmid, a number of host-induced genes may exist in *Rhizobium* species. The exact role of these host-induced genes must await further analysis.

Chemical communication between a microbe (pathogen [43] or symbiont) and a host plant may provide specificity, competitive advantage, or transient expression of genes involved in such interactions. This appears to be a two-way phenomenon as observed in the production of thick short roots by *Rhizobium* species treated with pea root exudate (44).

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LITERATURE CITED

1. **Adams, T. H., and B. K. Chelm.** 1984. The *nifH* and *nifDK* promoter regions from *Rhizobium japonicum* shared structural homologies with each other and with nitrogen regulated promoters from other organisms. J. Mol. Appl. Genet. **2**:392–405.
2. **Arcy-Lameta, A.** 1986. Study of soybean and lentil root exudates. II. Identification of some polyphenolic compounds, relation with plantlet physiology. Plant Soil **92**:113–123.
3. **Ayanaba, A., R. A. Haugland, M. J. Sadowsky, R. G. Upchurch, K. D. Weiland, and R. M. Zablotowicz.** 1986. Rapid colored-nodule assay for assessing root-exudate-enhanced competitiveness of *Bradyrhizobium japonicum*. Appl. Environ. Microbiol. **52**:874–851.
4. **Bauer, W. D.** 1981. Infection of legumes by rhizobia. Annu. Rev. Plant Physiol. **32**:407–449.
5. **Berk, A. J., and P. A. Sharp.** 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. Cell **12**:721–732.
6. **Better, M., B. Lewis, D. Corbin, G. Ditta, and D. R. Helinski.** 1983. Structural relationships among *Rhizobium meliloti* symbiotic promoters. Cell **35**:479–485.
7. **Bhagwatt, A. A., and J. Thomas.** 1982. Legume-*Rhizobium* interactions: cowpea root exudate elicits faster nodulation response by *Rhizobium* species. Appl. Environ. Microbiol. **43**:800–805.
8. **Bhuvaneswari, T. V., and W. D. Bauer.** 1978. Role of lectins in plant-microorganism interactions. III. Influence of rhizosphere/rhizoplane culture conditions on the soybean lectin binding properties of rhizobia. Plant Physiol. **62**:71–74.
9. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
10. **Bisseling, T., C. Been, J. Klugkist, A. van Kammen, and K. Nadler.** 1983. Nodule-specific host proteins in effective and ineffective root nodules of *Pisum sativum*. EMBO J. **2**:961–966.
11. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol.

134:1141–1156.
12. **Cooper, T. G., P. Whitney, and B. Magasanik.** 1974. Reaction of *lac*-specific ribonucleic acid from *E. coli* with *lac* deoxyribonucleic acid. J. Biol. Chem. **249**:6548–6555.
13. **Currier, W. W., and G. A. Strobel.** 1976. Chemotaxis of *Rhizobium* spp. to plant root exudates. Plant Physiol. **57**:820–823.
14. **Currier, W. W., and G. A. Strobel.** 1977. Chemotaxis of *Rhizobium* spp. to a glycoprotein produced by birdsfoot trefoil roots. Science **196**:434–435.
15. **Dale, R. M., B. A. McClure, and J. P. Houchins.** 1985. A rapid single stranded cloning strategy for producing a sequential series of overlapping clones for use in DNA sequencing: application to sequencing the corn mitochondrial 18S rDNA. Plasmid **13**:31–40.
16. **Drummond, M., J. Clements, M. Merrick, and R. Dixon.** 1983. Positive control and autogenous regulation of the *nifLA* promoter in *Klebsiella pneumoniae*. Nature (London) **301**:302–307.
17. **Firmin, J. L., K. E. Wilson, L. Rossen, and A. W. B. Johnston.** 1986. Flavonoid activation of nodulation genes in *Rhizobium* reversed by other compounds present in plants. Nature (London) **324**:90–92.
18. **Halverson, L. J., and G. Stacey.** 1984. Host recognition in the *Rhizobium*-soybean symbiosis: detection of a protein factor in soybean root exudate which is involved in the nodulation process. Plant Physiol. **74**:84–89.
19. **Harborne, J. B.** 1971. Distribution of flavonoids in the leguminosae, p. 31–71. *In* J. B. Harborne, D. Boulter, and B. L. Turner (ed.), Chemotaxonomy of the leguminosae. Academic Press, Inc., New York.
20. **Haugland, R., and D. P. S. Verma.** 1981. Interspecific plasmid and genomic DNA sequence homologies and localization of nif genes in effective and ineffective strains of *Rhizobium japonicum*. J. Mol. Appl. Genet. **1**:205–217.
21. **Holmes, D. S., and M. Quigley.** 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114**:193–197.
22. **Innes, R. W., P. L. Kuempel, J. Plazinski, H. Canter-Cremers, B. G. Rolfe, and M. A. Djordjevic.** 1985. Plant factors induce expression of nodulation and host-range genes in *Rhizobium trifolii*. Mol. Gen. Genet. **201**:426–432.
23. **Kosslak, R. M., B. B. Bohlool, S. F. Dowdle, and M. J. Sadowsky.** 1983. Competition of *Rhizobium japonicum* strains in early stages of soybean nodulation. Appl. Environ. Microbiol. **46**:870–873.
24. **Legocki, R. P., and D. P. S. Verma.** 1980. Identification of "nodule-specific" host proteins (nodulins) involved in the development of *Rhizobium*-legume symbiosis. Cell **20**:156–163.
25. **Lipman, D. J., and W. R. Pearson.** 1985. Rapid and sensitive protein similarity searches. Science **227**:1435–1441.
26. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Mulligan, J. T., and S. R. Long.** 1985. Induction of *Rhizobium meliloti nodC* expression by plant exudate requires *nodD*. Proc. Natl. Acad. Sci. USA **82**:6609–6613.
29. **Olson, E. R., M. J. Sadowsky, and D. P. S. Verma.** 1985. Identification of genes involved in the *Rhizobium*-legume symbiosis by mu-dI(kan, lac)-generated transcription fusions. Bio/Technology **3**:143–149.
30. **Peters, N. K., J. W. Frost, and S. R. Long.** 1986. A plant flavone, luteolin, induces expression of *Rhizobium meliloti* nodulation genes. Science **233**:977–979.
31. **Porter, P. M., W. L. Banwart, and J. J. Hassett.** 1985. HPLC isolation and GC-MS identification of genistein, daidzein, and coumesterol from unhydrolyzed soybean root extracts. Environ. Exp. Bot. **25**:229–232.
32. **Rolfe, B. G., and J. Shine.** 1984. *Rhizobium*-leguminous symbiosis: the bacterial point of view, p. 95–128. *In* D. P. S. Verma and T. H. Hohn (ed.), Genes involved in microbe-plant interactions. Springer-Verlag, New York.
33. **Rossen, L., C. A. Shearman, A. W. B. Johnston, and J. A. Downie.** 1985. The *nodD* gene of *Rhizobium leguminosarum* is autoregulatory and in the presence of plant exudate induces the *nodA,B,C* genes. EMBO J. **4**:3369–3373.
34. **Rostas, K., E. Kondorosi, B. Horvath, A. Simoncsits, and A. Kondorosi.** 1986. Conservation of extended promoter regions of nodulation genes in *Rhizobium*. Proc. Natl. Acad. Sci. USA **83**:1757–1761.
35. **Sadowsky, M. J., and B. B. Bohlool.** 1983. Possible involvement of a megaplasmid in nodulation of soybeans by fast-growing rhizobia from China. Appl. Environ. Microbiol. **46**:906–911.
36. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
37. **Schmidt, E. L.** 1979. Initiation of plant-root microbe interactions. Annu. Rev. Microbiol. **33**:355–376.
38. **Schofield, P. R., and J. M. Watson.** 1985. Conservation of *nif*- and species-specific domains within repeated promoter sequences from fast-growing *Rhizobium* species. Nucleic Acids Res. **13**:3407–3418.
39. **Scott, K. F.** 1986. Conserved nodulation of genes from the non-legume symbiont *Bradyrhizobium* sp. (*Parasponia*). Nucleic Acids Res. **14**:2905–2919.
40. **Scott, K. F., B. G. Rolfe, and J. Shine.** 1981. Biological nitrogen fixation: primary structure of the *Klebsiella pneumoniae nifH* and *nifD* genes. J. Mol. Appl. Genet. **1**:71–81.
41. **Shearman, C. A., L. Rossen, A. W. B. Johnston, and J. A. Downie.** 1986. The *Rhizobium leguminosarum* nodulation gene *nodF* encodes a polypeptide similar to acyl-carrier protein and is regulated by *nodD* plus a factor in pea root-exudate. EMBO J. **5**:647–652.
42. **Spaink, H. P., R. J. H. Okker, C. A. Wijffelman, E. Pees, and B. J. J. Lugtenberg.** 1987. Regulation of the promoters in the nodulation region of the symbiosis plasmid pRL1J1 of *Rhizobium leguminosarum*, p. 244–246. *In* D. P. S. Verma and N. Brisson (ed.), Molecular genetics of plant-microbe interactions. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.
43. **Stachel, S. E., E. Messens, M. van Montagu, and P. Zambryski.** 1985. Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. Nature (London) **318**:624–629.
44. **van Brussel, A. A. N., S. A. J. Zaat, H. C. J. Canter-Cremers, C. A. Wijffelman, E. Pees, T. Tak, and B. J. Lugtenberg.** 1986. Role of plant root exudate and Sym plasmid-localized nodulation genes in the synthesis by *Rhizobium leguminosarum* of TSR factor which causes thick and short roots on common vetch. J. Bacteriol. **163**:517–522.
45. **Verma, D. P. S., and S. Long.** 1983. The molecular biology of *Rhizobium*-legume symbiosis. Int. Rev. Cytol. Suppl. **14**:211–245.
46. **Verma, D. P. S., and K. Nadler.** 1984. Legume-*Rhizobium* symbiosis: the host's point of view, p. 57–93. *In* D. P. S. Verma and T. Hohn (ed.), Genes involved in microbe-plant interactions. Springer-Verlag, New York.
47. **Vincent, J. M.** 1970. A manual for the practical study of root-nodule bacteria. IBP Handbook 15. Blackwell Scientific Publications, Oxford.
48. **Wilbur, W. J., and D. J. Lipman.** 1983. Rapid similarity searches of nucleic acid and protein data banks. Proc. Natl. Acad. Sci. USA **80**:726–730.
49. **Zaat, S. A. J., C. A. Wijffelman, H. P. Spaink, A. A. N. van Brussel, R. J. H. Okker, and B. J. J. Lugtenberg.** 1987. Induction of the *nodA* promoter of *Rhizobium leguminosarum* Sym plasmid pRL1J*T* by plant flavones and flavanones. J. Bacteriol. **169**:198–204.