MPMI Vol. 9, No. 2, 1996, pp. 114–124. Publication no. M-1995-1127-01R.

The Addition of 5-Aminoimidazole-4-Carboxamide-Riboside to Nodulation-Defective Purine Auxotrophs of NGR234 Restores Bacterial Growth but Leads to Novel Root Outgrowths on Siratro

Steven P. Djordjevic, Jeremy J. Weinman, John W. Redmond, Michael A. Djordjevic, and Barry G. Rolfe

Plant Microbe Interaction Group, Research School of Biological Sciences, The Australian National University, P.O. Box 475, Canberra City, Australian Capital Territory 2601, Australia

Received 10 July 1995. Accepted 13 October 1995.

The transposon Tn*5*-induced purine auxotrophs of NGR234 (ANU2861 and ANU2866) are defective for nodule formation on a wide variety of legumes and the nonlegume *Parasponia*. Complementation studies and DNA sequence analysis of regions, on either side of the insertion of Tn*5* in ANU2861, revealed two open reading frames with strong sequence homology to the *purM* and *purN* genes of *Escherichia coli*. Strain ANU2861 contained a single copy of Tn*5* inserted into the 3′ region of the putative *purM* gene. On *Macroptilium atropurpureum* (siratro) ANU2861 rapidly induces a reaction analogous to a hypersensitive response at the site of infection (S. P. Djordjevic, R. W. Ridge, H. Chen, J. W. Redmond, M. Batley, and B. G. Rolfe, 1988, J. Bacteriol. 170:1848-1857). However, when the plant growth medium was supplemented with 0.1 mM 5-aminoimidazole-4-carboxamide riboside (AICA-riboside), strains ANU2861 and ANU2866 induced the formation on siratro of root outgrowths that resembled Fix⁻ nodules. These outgrowths do not contain bacteria, lack infection threads, and possess centrally located vascular bundles in contrast to the peripheral location in functional nodules induced by wild-type strain NGR234. When mutant ANU2861 was spot inoculated onto the roots of siratro, or coinoculated onto the roots with the ExoY mutant ANU2811, a thick and short root (Tsr) phenotype was induced. The roots of siratro plants inoculated with parent strain ANU280 do not induce a Tsr-like phenotype. Complementation of ANU2866 for nodulation and prototrophic growth was achieved by a cosmid containing *purY, purQ,* and *purL* genes. The examination of ANU2861 and ANU2866 lipopolysaccharide (LPS) showed that only ANU2861 possessed a defective LPS that was corrected by the addition of AICA-riboside to the growth medium. These results indicated that both mutants are defective in the purine biosynthetic pathway and that metabolic flow through this pathway is essential for nodule morphogenesis.

Corresponding author: Barry G. Rolfe;
E-mail: rolfe@rsbs-central.anu.edu.au

Present address of Steven P. Djordjevic: Microbiology and Immunology Section, NSW Agriculture, Elizabeth Macarthur Agricultural Institute, PMB 8, Camden, NSW, Australia.

Purine auxotrophic mutants of *Rhizobium* fail to effectively nodulate their host plants, forming pseudonodules that (i) lack bacterial content, (ii) show centrally located vascular tissue, and (iii) fail to reduce atmospheric nitrogen (Pankhurst and Schwinghamer 1974; Pain 1979; Scherrer and Denarie 1971; Noel et al. 1986; Noel et al. 1988; Newman et al. 1992). *Rhizobium* strain NGR234 successfully nodulates a wide variety of legume species (Trinick and Galbraith 1980). Transposon-induced purine auxotrophs (ANU2861 and ANU2866) fail to nodulate *Macroptilium atropurpureum* (Moc. & Sessé ex DC.) Urb. and *Desmodium uncinatum* Desv. and differ in their ability to nodulate a variety of other host legumes although the overwhelming response is a poor nodulation phenotype (Chen et al. 1985; Djordjevic et al. 1988). Strains ANU2861 and ANU2866 produce less acidic exopolysaccharide (EPS) and cyclic β-1,2-glucans per gram of cells than does the parent strain ANU280 (Batley et al. 1987) although they produce colony types that appear excessively mucoid (Exo⁺⁺) on complex agar medium (Chen et al. 1985). Ultrastructural examination of the infection sites of strain ANU2861 on siratro showed the presence of root hair curling (Hac⁺ phenotype) and some cortical cell division (Noi⁺), but no infection threads (Inf⁻) (Djordjevic et al. 1988). In addition, micrographs of ANU2861 infection sites on siratro plants showed necrosis in root hair and surrounding epidermal cells with an accumulation of osmiophilic droplets along the plasmalemma and in the cell walls (Djordjevic et al. 1988).

Purine auxotrophs of *R. etli* bv. *phaseoli* induce root hair curling, nodule meristems, and pseudonodules on beans (*Phaseolus vulgaris* L.) (Noel et al. 1988). The addition to purine auxotroph CE106 of 5-aminoimidazole-4-carboxamide riboside (AICA-riboside), the unphosphorylated form of the purine precursor 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), promotes the formation of ineffective nodules (Noel et al. 1988; Newman et al. 1991, 1992, 1994), which had peripheral vascular bundles and cortical cell layers characteristic of normal nodule development. These structures lack the high vacuolate cell content and central vasculature of lateral roots that are typical of pseudonodules induced in the absence of AICA-riboside (Noel et al. 1988; Newman et al. 1992). Noel et al. (1988) and Newman et al. (1992) suggested that the ability of rhizobia to produce AICAR is essential for

the initiation and/or sustained infection thread growth and subsequent nodule development (Newman et al. 1992) and proposed that AICAR is diverted from the purine pathway and metabolized in a manner that is central to the effective development of a nodule.

In this study, we show that ANU2861 is mutated in *purM* while ANU2866 is complemented by a cosmid containing *purY, purQ,* and *purL.* We found that AICA-riboside did not complement ANU2861 or ANU2866 for nodulation and induced the formation of root outgrowths that resembled nodules. These root outgrowths possessed central vascular tissue but were devoid of bacteria. These data support the hypothesis that flow through the purine biosynthesis pathway is essential for complete nodule development.

RESULTS

Genetic and sequence analysis of the ANU2861 mutant locus.

We have previously reported that pSD1 (wild-type 6.2-kb *Eco*RI fragment cloned from parent strain ANU280 into pWB5a) corrected the purine auxotrophy and restored both a wild-type colony morphology and effective nodulation capacity on siratro (Djordjevic et al. 1988). This demonstrated that all the coding and regulatory sequences that impart the ANU2861 phenotype are encoded on this fragment. Further analysis (Fig. 1 and Table 1) showed that a 3.3-kb partial *Pst*I fragment (pSD3) is capable of restoring a complete wild-type phenotype to the mutant strain ANU2861, whereas other subcloned fragments (pSD2, pSD5, and pSD7) failed to complement the mutant phenotype. However, two distinct colony morphologies (wild type and mutant) were observed with approximately 50% frequency when pSD2 or pSD5 was transferred into strain ANU2861. Marker exchange of *Rhizobium* DNA fragments present in plasmids pSD2 and pSD5 and the fragment from the recipient strain ANU2861 was responsible for the formation of dual colony morphologies (data not shown), indicating that subclones pSD2 and pSD5 did not contain all the coding and regulatory sequences required to restore a wild-type phenotype.

DNA sequence analysis of fragments spanning regions either side of the insertion site of transposon Tn*5* (see Figure 1 for sequencing strategy) have identified homology with the *purM* and *purN* coding sequences of *Escherichia coli* (Smith and Daum 1987). The positions of these genes (Fig. 1) are based on the size of the *E. coli* genes, and the location of the homologous NGR234 sequences. On the basis of sequence homology (for the sequence corresponding to *purM*, 57.6% homology was obtained in 396 bases; for the *purN* homologue, 52.9% homology was found over 493 bases, data not shown), the purine auxotrophy of the ANU2861 Tn*5* mutant, and the ability to correct the ANU2861 mutation with AICA-riboside, we have designated these as the putative strain NGR234 homologues of the *purM* and *purN* genes. Thus, the Tn*5* is located in the 3′ region of the putative *purM* gene and these two purine genes are likely to form part of an operon (Fig. 1).

Complementation of mutant strains ANU2861 and ANU2866 with *Rhizobium phaseoli* cosmids.

Noel et al. (1988) constructed cosmids (pCOS106, pCOS110, and pCOS115) that complemented *R. phaseoli* purine auxotrophs. These cosmids were conjugatively transferred into mutant strains ANU2861 and ANU2866 and the transconjugants tested for complementation ability, growth, normal EPS production, and nodulation on siratro. None of the three cosmids was capable of restoring mutant strain ANU2861 to the parent strain ANU280 phenotype. However, pCOS110 (known to contain the purine biosynthetic genes *purY, purQ,* and *purL*) restored a wild-type phenotype to

Table 1. Subcloned regions of the 6.2-kb *Eco*RI fragment of pSD1 and subsequent complementation analysis on siratro

Plasmid	Colony morphology	Complementation	Symbiotic response
pSD1	Exo$^+$, Wild-type	+	Nod$^+$, Fix$^+$, nodules
pSD2	Two colony types		
	(a) Exo$^+$, Wild-type	–[a]	Nod$^+$, Fix$^+$
	(b) Mutant type	–	Nod$^-$ nodules
pSD3	Exo$^+$, Wild-type	+	Nod$^+$, Fix$^+$,
pSD5	Two colony types		
	(a) Exo$^+$, Wild-type	–[a]	Nod$^+$, Fix$^+$
	(b) Mutant type	–	Nod$^-$ nodules
pSD7	Mutant type	–	Nod$^-$ nodules

[a] Correction to the wild-type phenotype occurs as a result of homogenotization via marker exchange.

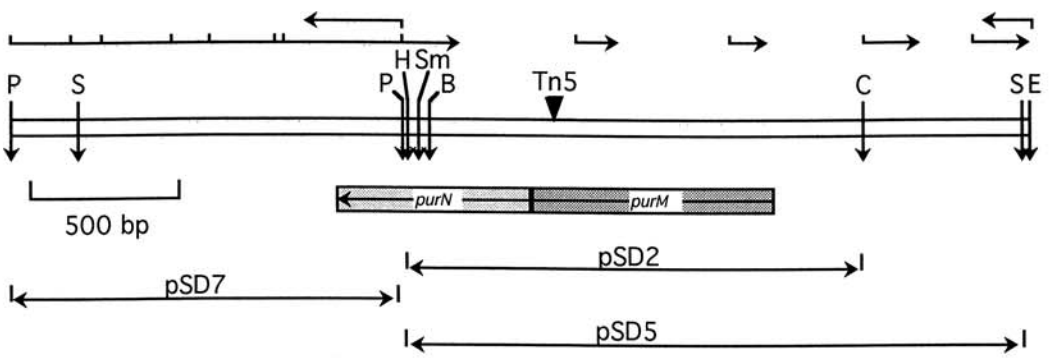

Fig. 1. Physical map of the 3.3-kb region subloned in plasmid pSD3 is shown and the position of the 2861 Tn*5* insertion is indicated. Additional subcloned regions are identified and the predicted position and direction of transcription of the putative *Rhizobium* strain NGR234 *purM* and *purN* genes shown. The start points and direction of the regions sequenced are indicated by the ticks and arrows above the map. Restriction enzyme sites are as described, E, *Eco*RI; P, *Pst*I; C, *Cla*I, S, *Sal*I, H, *Hin*dIII, B, *Bam*HI, and S*, *Sma*I. The triangle denotes the insertion site of Tn*5*.

mutant strain ANU2866, confirming that the ANU2866 Tn*5* locus affected purine biosynthesis.

Comparison of growth of mutant strains ANU2866 and ANU2861 in the presence of AICAR.

Since *purM, purN, purQ,* and *purL* occur before AICA-riboside in the biosynthetic pathway for purines, this compound was used to complement the purine metabolic defect of ANU2861 and ANU2866 phenotypes. Adenine addition has previously been shown (Djordjevic et al. 1988) to restore growth on laboratory media to both strains. Mutant strains ANU2861 and ANU2866 failed to form colonies on MM plates (prepared using electrophoresis grade agarose) after 5 days incubation at 29°C. A range of concentrations (0.01 to 1.0 mM) of adenine and AICA-riboside were tested for their ability to restore colony formation. The addition of 0.1 mM (or higher concentrations) of AICA-riboside or adenine to MM agarose restored growth to both strains enabling them to form mucoid colonies as quickly as parent strain ANU280 (Fig. 2). However, the colonies produced by ANU2861 and ANU2866 on minimal medium (MM) containing AICA-riboside or adenine still appeared excessively mucoid typical of these mutants. Slower growth of both mutants was observed on MM agarose supplemented with only 0.04 mM AICA-riboside. Both mutant strains were capable of scavenging trace quantities of nucleoside contaminants from laboratory grade agar, resulting in slow growth. The addition of AICA to the minimal medium plates at various concentrations did not restore colony growth to the two mutants (data not shown). No difference was observed in colony growth time and morphology of parent strain ANU280 in the absence (Fig. 2A) or presence (Fig. 2D) of AICA-riboside or adenine on MM-agar plates.

AICA-riboside addition alters the nodulation capacity of the mutant strains.

We tested the capacity of AICA-riboside to restore the nodulation-defective phenotype of strains ANU2861 and ANU2866 on siratro. The addition of 0.1 mM AICA-riboside to the growth medium enabled strains ANU2861 and ANU2866 to induce root outgrowths on siratro that resembled nodules. However, ultrastructural examination showed that vascular bundles were centrally located, in contrast to their peripheral location in nodules induced by the wild-type strain ANU280 (Fig. 3). No bacteroids were present in these mutant-induced structures sampled 30 days after inoculation. *Rhizobium* bacteria could not be seen when these structures were examined under the microscope and were not usually recovered from these root outgrowths. On the rare occasion when mutant strain ANU2861 was recovered from root out-

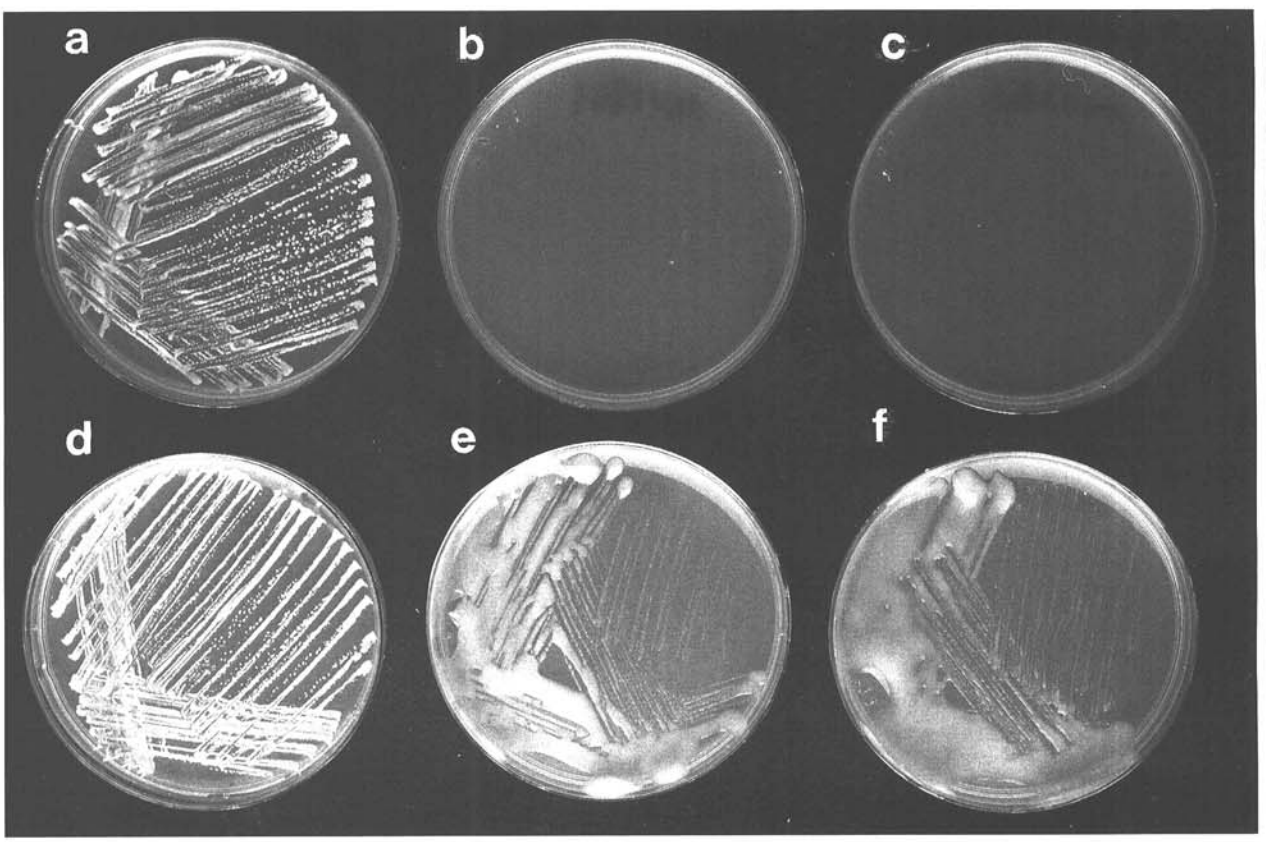

Fig. 2. Colony morphologies of wild-type strain ANU280 and mutant strains ANU2861 and ANU2866 on minimal medium. **A, B,** and **C,** Strains ANU280, ANU2861, and ANU2866 on MM agarose. The growth of parent strain ANU280 was not affected by the addition of 5-aminoimidazole-4-carboxamide riboside (AICA-riboside). *Rhizobium* Tn*5* mutant strains ANU2861 and ANU2866 failed to form colonies on MM-agarose plates (electrophoresis grade agarose) after 5 days incubation at 29°C. The addition of 0.1mM AICA-riboside to MM agarose facilitated formation of mucoid colonies by strains ANU2861 and ANU2866 as quickly as parent strain ANU280 appeared on identical plates. **D, E,** and **F,** ANU280, ANU2861, and ANU2866 grown on MM agarose supplemented with 0.1mM AICA riboside.

growths, it was assumed to have been located between cells in the interstitial spaces as the numbers retrieved were very low (<5 colonies). This indicated that ANU2861 possessed a poor reproductive potential in the root environment of siratro, irrespective of the addition of AICA-riboside. Concentrations of AICA-riboside higher than 0.1 mM had no additional nodulation effects in our plant assay system. The addition of adenine to the plant growth medium failed to alter the Nod$^-$ phenotype of mutants ANU2861 and ANU2866 on siratro. Furthermore, the addition of either AICA-riboside or adenine

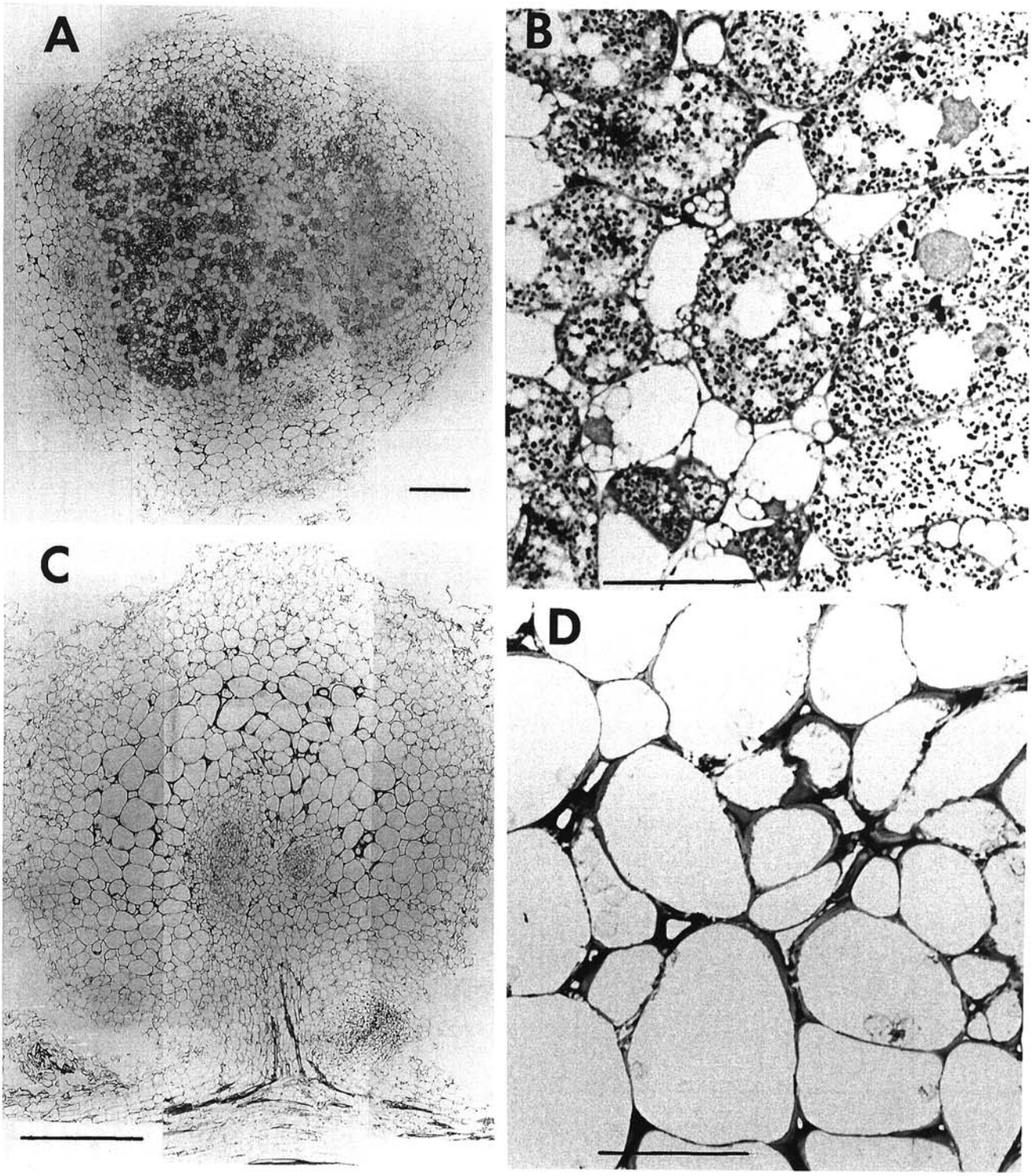

Fig. 3. Light microscopic examination of a siratro nodulelike structure and a normal nitrogen-fixing nodule 4 weeks postinoculation. **A,** A cross section through a functional nitrogen-fixing nodule induced by wild-type strain ANU280. **B,** Clear presence of bacteroids in cells of nodules induced by ANU280. **C** and **D,** A cross section through an empty nodulelike structure induced by mutant strain ANU2861 in the presence of 0.1 mM AICAR. Bacteroids absent and centrally located vascular bundles present. **A** and **C,** Bar = 100 μm; **B** and **D,** Bar = 35 μm.

at various concentrations had no obvious effect on uninoculated siratro plants or plants inoculated with the wild-type strain ANU280.

Coinoculation studies.

The failure of AICA-riboside to restore bone fide nodulation ability to the mutant strains suggested that the mutants could not be sufficiently cross-fed to initiate normal infection. Coinoculation studies were done to assess the ability of prototrophic strains to rescue strain ANU2861. The results show that strain ANU2861 could not be rescued for nodulation ability in any combination of bacteria used (Table 2). Coinoculation of freshly germinated siratro seedlings with a localized low density inoculant (approximately 3,000 cells in 1 μl) of strain ANU2861 and strain ANU2811 induces a distinctive root morphology (Fig. 4) similar to the "thick short root" (Tsr) phenotype originally described by van Brussel et al. (1982) on *Vicia sativa* roots. The formation of heavily branched, short, distorted roots that grow in multiple directions away from the point of inoculation is often observed with mutant strain ANU2861 infections (Fig. 4B) and contrasts with the long threadlike appearance of siratro inoculated with parent strain ANU280 (Fig. 4A). Neither of these stunted root morphologies is observed when siratro is coinoculated with parent strain ANU280 and either ANU2861 or ANU2811. Furthermore, siratro inoculated with strain ANU2861 grown in the presence of 0.1 mM AICA-riboside showed a greatly reduced Tsr phenotype.

Coinoculation of parent strain ANU280 with either strain ANU2861 or ANU2811 (*exoY* mutant) produced healthy, nitrogen-fixing siratro plants, while coinoculation of strain ANU2861 and Sym⁻ strain ANU265 failed to nodulate siratro. Strain ANU2861 has not been recovered from the nodule contents of over 140 nodules. This finding contrasts with the results of coinoculations of parent strain ANU280 and mutant strain ANU2811 in which approximately 30% of nodules examined contained significant numbers of strain ANU2811 (Table 2).

SDS-PAGE of LPS of ANU280 and mutant strain ANU2861.

Previous studies have shown that the structure of EPS of mutant strain ANU2861 is identical to the EPS produced by wild-type strain ANU280 (Djordjevic et al. 1988). We were interested to determine if the lipopolysaccharide (LPS) of mutant strain ANU2861 is different than that of parent strain ANU280. Figure 5 shows a silver-stained 12.5% polyacrylamide gel of LPS isolated from wild-type *Rhizobium* strain ANU280, mutant strain ANU2861 and *E. coli* strain O11184 (Sigma, St. Louis, MO). The parent strain ANU280 LPS is composed of a heavy concentration of core LPS with very little saccharide-substituted LPS. LPS extracts from mutant strain ANU2861 show small quantities of core LPS with heavier concentrations of larger saccharide-substituted LPS. Similar high molecular weight bands are present in the *E. coli* LPS extracts but are absent from LPS isolated from ANU280, ANU2866, and ANU843 (*Rhizobium trifolii*). All LPS banding profiles are unaltered if the LPS samples were predigested with proteinase K or Ribonuclease A prior to electrophoresis (data not shown). This alteration to LPS biosynthesis is corrected by the addition of AICA-riboside to the growth medium (Fig. 5).

DISCUSSION

Rhizobium strain ANU2861 was generated by the insertion of a single copy of transposon Tn*5* into strain NGR234 resulting in a pleiotropic mutant. Molecular analysis of the region surrounding the Tn*5* insertion site of mutant ANU2861 indicated that the transposon was located in the 3′ end of the putative *purM* gene. In *E. coli* (Smith and Daum 1987) and *B. subtilis* (Ebbole and Zalkin 1987), these genes are co-tran-

Table 2. Coinoculation studies with different transposon mutants of ANU280 on siratro[a]

Inoculant	Fix phenotype[b]	Nodule no. per plant	Nodules crushed	Rhizobium identified	Avg. no. Rhizobium recovered	Range	No. of plants
ANU280	+	Avg. of 13 nod (5 to 20) and 5 to 10 calli	80	ANU280, Sm^r, Rif^r, Exo^+ colonies	5×10^7 /nodule	1.5×10^6 to 1.1×10^8	>150
ANU2861	–	15 calli/plant; range 0 to 40	30 calli	Empty	0	NA[c]	>150
ANU2811	–	Average of 6	50	ANU2811 Sm^r, Rif^r, Kan^r, Exo^-	NM	NM	80
ANU265	– (Nod^-)	0	NA	NA	NA	NA	40
ANU280/ANU2861 (1:1)	+	Avg. of 6	65	ANU280, Sm^r, Rif^r, Exo^+	5.8×10^6/nodule	1.3×10^6; 9.0×10^7	60
ANU280/ANU2861 (1:10)	+	11	10	ANU280, Sm^r, Rif^r, Exo^+	NM	NM	20
ANU280/ANU2861 (10:1)	+	11	10	ANU280 Sm^r, Rif^r, Exo^+	NM	NM	20
ANU280/ANU2811 (1:1)	+	Avg. of 7	55	ANU280 (100%), ANU2811 (30%)	ANU280 6.2×10^7	ANU280 4.5×10^2 to 4.1×10^8; ANU2811 5 to 5×10^2	50
ANU2811/ANU2861 (1:1)	–	Avg. of 11	55	ANU2811	4.6×10^7	4.6×10^2; 2.4×10^8	55
ANU2861/ANU265	– (Nod^-)	3 plants with small calli	NA	NA	NA	NA	20
ANU2811/ANU2861 (1:10)	–	Avg. of 11	15	ANU2811	NM	NM	20
ANU2811/ANU2861 (10:1)	–	Avg. of 11	15	ANU2811	NM	NM	20

[a] Inoculant size: Approximately 3,000 cells/seedling.
[b] Fix^+ response indicates healthy, green nitrogen-fixing plants. Fix^- response indicates stunted, yellow non-nitrogen-fixing plants.
[c] NA = not applicable; NM = not measured.

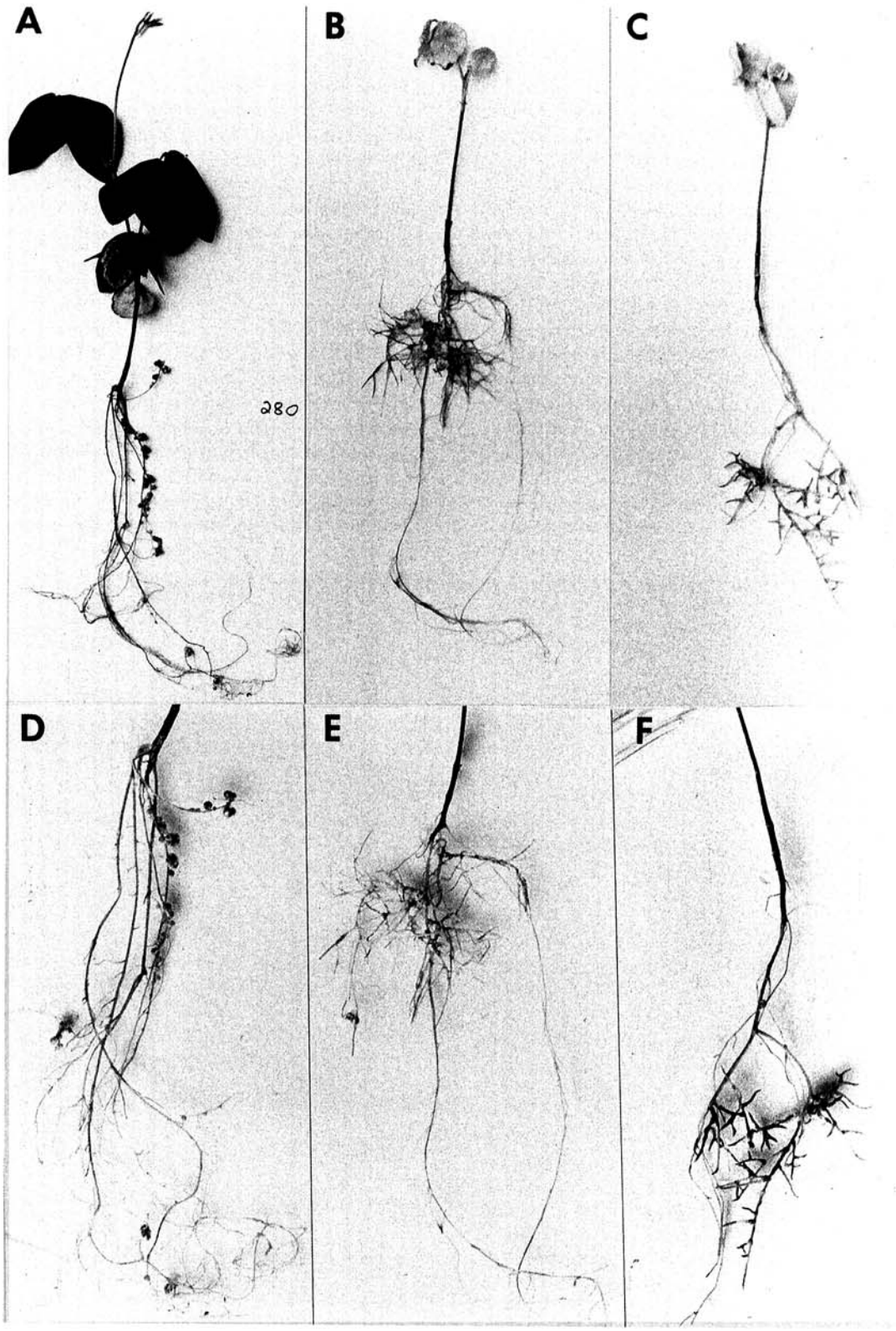

Fig. 4. Root morphology of siratro induced by mutant strain ANU2861. **A, B,** and **C,** Siratro plants inoculated with wild-type strain ANU280, mutant strain ANU2861, and mutant strains ANU2861 and ANU2811, respectively. **D, E,** and **F,** A close-up view of roots 5 weeks postinoculation.

scribed, and their partial sequencing in strain NGR234 indicates that they also may be part of a single operon. Complementation analysis confirmed that mutant ANU2866 was defective in purine biosynthesis. Thus, strains ANU2861 and ANU2866 behave as purine auxotrophs that require the addition of either AICA-riboside or adenine for normal growth on laboratory minimal medium.

Although strain ANU2861 induces root hair curling and some cortical cell division on siratro, a rapid hypersensitive-like response occurs at the site of infection thread initiation (Djordjevic et al. 1988). Mutant strain ANU2861 also induces a Tsr-like phenotype on siratro roots when inoculated alone or, more extensively, when coinoculated with the Exo^- mutant strain ANU2811, but not when coinoculated with parent strain ANU280. van Brussel et al. (1986) reported that wild-type *Rhizobium* strains produce Tsr factor in response to the host plant producing factor A and young legumes growing in the presence of Tsr factor develop the Tsr phenotype. Preparations of extra cellular signals (which include the Nod factors) can inhibit root growth and induce the Tsr phenotype (Stacey et al. 1993) possibly via the induction of ethylene, which can inhibit root growth and nodulation (van Workum et al. 1995). The presence of the Tn*5* in the chromosome of mutant ANU2861 may enhance the production of Tsr factor. Since the Tsr factor is soluble, one might expect to observe the Tsr response in siratro plants coinoculated with mutant ANU2861 and parent strain ANU280, unless the parent strain suppressed the activity of this factor. Perhaps the presence of parent strain ANU280 (and not strain ANU2811) inhibits the synthesis or release of factor A from siratro. The addition of 0.1 mM AICA-riboside repressed the appearance of the Tsr phenotype in siratro plants inoculated with strain ANU2861.

The addition of 0.1 mM AICA-riboside (and not 0.1 mM adenine) to siratro growth medium enables strains ANU2861 and ANU2866 to induce abundant cortical cell divisions resulting in root outgrowths at the site of inoculation. No infection thread formation or bacterial invasion was observed. Sections through these nodules failed to show any bacteroids but revealed centrally located vascular bundles similar to a lateral root structure. That is, the induced root outgrowth is more like a type of hybrid nodule/lateral root structure previously described in some clover-*Rhizobium* infections (McIver et al. 1993). These data imply that the induction of the typical hypersensitive-like response, demonstrated in strain ANU2861 infections (Djordjevic et al. 1988), has been either abolished or greatly diminished. How the addition of AICA-riboside stimulates siratro to induce abundant cortical cell division at the site of inoculation is unknown. The enhanced nodulation of purine mutants of *R. etli* (bv. *phaseoli*) in the presence of AICA-riboside prompted Noel et al. (1988) to suggest that a particular intermediate in the purine biosynthetic pathway, or undiminished metabolic flow through this

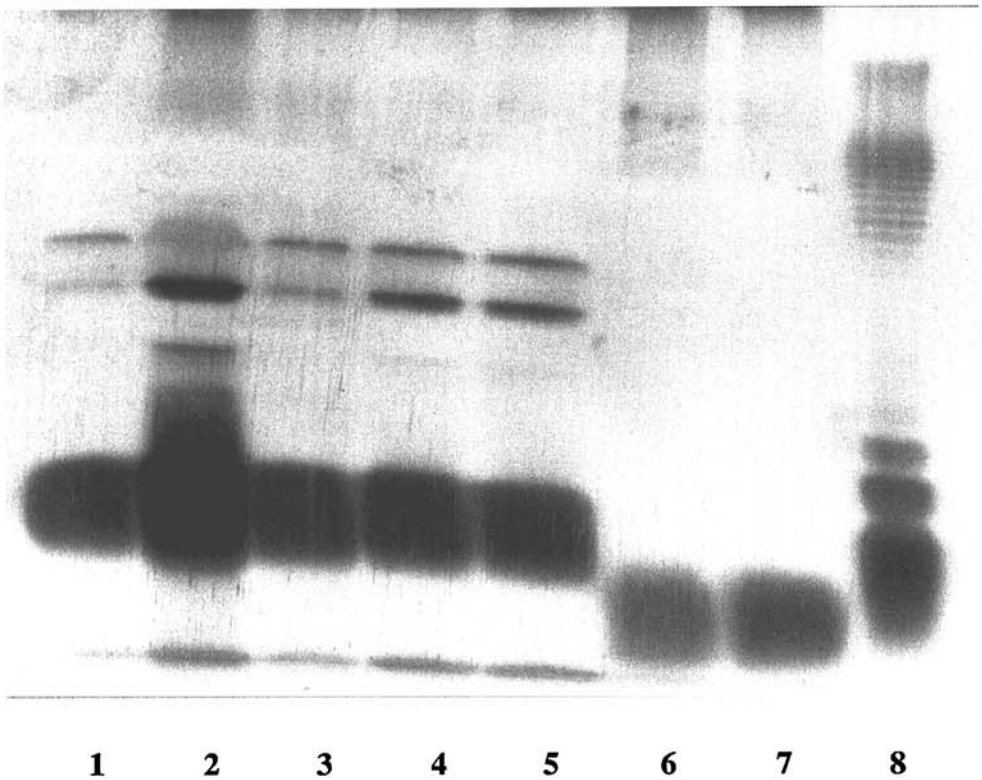

Fig. 5. Silver-stained 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel of lipopolysaccharide (LPS) samples. Each well was loaded with 20 µl of prepared samples (see Materials and Methods). Lanes 1 and 2, loaded with LPS isolated from parent strain ANU280 and mutant ANU2861 (both grown on BMM medium); lane 3, loaded with an LPS sample from mutant ANU2861 grown in BMM with added 5-aminoimidazole-4-carboxamide riboside (AICA-riboside) (0.1 mM); lane 4, an LPS sample from Tn*5*-induced mutant ANU2866 (also an adenine requiring mutant of strain ANU280); lane 5, an LPS sample from mutant ANU2866 grown in BMM with added AICA-riboside (0.1 mM); lanes 6 and 7, loaded with LPS samples from *R. leguminosarum* bv. *trifolii* LPS mutant 224 (F. Dazzo, unpublished); lane 8, an LPS sample from the commercial marker strain *E. coli* strain 011184 (Sigma, St. Louis, MO).

pathway, is essential in the early stages of symbiosis. Our data support this hypothesis. Since AICA-riboside addition can restore growth of both ANU2861 and ANU2866 (although excessively mucoid colonies are still formed) but not the ability to form bone fide nodules on siratro, we support the conclusion that an intermediate in AICAR production, or a side product derived from the pathway, is essential for normal nodule formation (Newman et al. 1992; Noel et al. 1988)

A comparison of purine auxotrophs derived from a variety of *Rhizobium* spp. indicates that the addition of AICA-riboside complements different stages of nodule development in their respective host legumes. Purine auxotrophs of *R. etli* bv. *phaseoli* strain CE3 (Ndv^-) induce non-nitrogen-fixing nodules (devoid of bacteria) on bean without infection thread formation (Noel et al. 1984; VandenBosch et al. 1985; Newman et al. 1992). Purine auxotrophs of *R. leguminosarum* 128C56 (bv. *viciae*) and *R. fredii* HH303 induce small (and delayed in appearance) and sometimes no pseudonodules on their respective host legumes. The addition of AICA-riboside to bean plants inoculated with *R. etli* purine mutants (Ndv^- phenotype) greatly enhanced nodule development with the formation of a true nodule structure (Nod^+ phenotype) (Noel et al. 1988; Newman et al. 1992). Significantly, the number of purine mutants recovered from these AICA-riboside-induced nodules was 1×10^4 greater than that from nodules induced by the purine mutants alone. The addition of AICA-riboside to purine auxotrophs of *R. leguminosarum* 128C56 (bv. *viciae*) and *R. fredii* HH303 significantly enhanced nodule development (presence of peripherally located vascular bundles, and infection threads) and nodule size with up to 1×10^5 of the purine auxotrophs recoverable from these nodules.

Purine auxotrophic mutants of *R. leguminosarum* strain CE3 unable to convert AICA-riboside to AICAR cannot initiate infection, suggesting that AICA-riboside per se does not act directly on leguminous plants but must be further converted to AICAR by the bacteria in order to promote infection (Newman et al. 1992, 1994). Furthermore, if the addition of AICA-riboside to mutant-inoculated plants is delayed, (i) infection and subsequent nodule formation are delayed, shifting the position of infected nodules to younger regions of the root, (ii) the pseudonodules are formed on older portions of the root while infected nodules are forming on younger portions, and (iii) the development of a nodule does not occur unless infection in the presence of AICA-riboside proceeds for at least 6 days, which is 2 days prior to nodule emergence. Collectively, these observations suggest that AICA-riboside is required continuously throughout the course of infection thread development (Newman et al. 1992) and that there is a possible symbiotic role for AICA-riboside separate from its role in purine biosynthesis (Newman et al. 1995).

Several *Rhizobium*-legume interactions have been reported in which two *Rhizobium* strains in coinoculation experiments cooperate in the development of a nitrogen-fixing nodule (Rolfe et al. 1980; Chen and Rolfe 1987). Chen and Rolfe (1987) described numerous coinoculation experiments between Exo^-, Nod^+, and Fix^- mutants of ANU280 and Sym^- Exo^+ strain ANU265 that produced nitrogen-fixing nodules on *Leucaena leucocephala,* another legume variety effectively nodulated by *Rhizobium* strain NGR234. Both *Rhizobium* strains were recovered from the contents of functional nodules. Klein et al. (1988) conducted a detailed examination of the bacterial contents of alfalfa nodules coinoculated with defective but complementary strains of *Rhizobium meliloti.* These authors concluded that one of the coinoculants supplies the Nod^+ phenotype while the other supplies the Exo^+ phenotype. Further, these authors concluded that if the Nod^+ and the Exo^+ phenotypes are provided by a single strain, then the Exo^- participant is excluded from the infection. Coinoculation between an Exo^{++}, Nod^- strain (ANU2861) and an Exo^-, Nod^+ strain (ANU2811) results in a Nod^+ Fix^- response on siratro with strain ANU2811 exclusively occupying the nodule contents.

The electrophoretic profile of LPS of strain ANU2861 differs from that of the wild-type and mutant strain ANU2866, and this difference is reversed by the addition of AICA-riboside. The chemical structures of the LPSs of these strains have not been determined, but we interpret the electrophoretic results as indicating the synthesis of an extended core oligosaccharide by strain ANU2861. On the other hand, the acidic EPS produced by strains ANU2861 and ANU2866 is indistinguishable from that of the parent strain ANU280. The synthesis of the necessary activated sugar precursors is obviously not impaired. Thus, the alteration in the LPS synthesis of strain ANU2861 is likely to be due to an alteration in the synthesis of an activated monosaccharide precursor, or the specificity of a glycosyl transferase. The question is whether this altered LPS is the primary cause of the host plant hypersensitive response or whether there are additional factors resulting from altered purine metabolism. Mutant ANU2866 also has a Nod^- phenotype (Chen et al. 1985) but with no apparent alteration in LPS synthesis. We therefore conclude that the inability of transposon mutants ANU2861 and ANU2866 to induce normal nodules indicates that the defect in purine biosynthesis plays a crucial role in the ability of *Rhizobium* strain NGR234 to nodulate a variety of legumes and the nonlegume *Parasponia.*

MATERIALS AND METHODS

Media and growth of bacteria.

Liquid and solid media—Bergersen's modified medium (BMM), Luria broth (LB), and Fåhraeus medium (F)—were prepared as described previously (Rolfe et al. 1980). Tryptone yeast (TY) medium was prepared as described by Beringer (1974). *Rhizobium* parent strain ANU280 (Rif^r and Sm^r NGR234) and transposon Tn*5* mutants (Table 3) were grown at 28°C on minimal mannitol (MM) medium (Djordjevic et al. 1988). Electrophoresis grade agarose was substituted for laboratory grade agar (Difco, Detroit, MI) in the preparation of solid MM medium where indicated. Purine auxotroph strains ANU2861 and ANU2866 scavenge trace amounts of purine from laboratory grade agar and form small translucent colonies after 4 days incubation. *Escherichia coli* was grown on liquid and solid L broth at 37°C. Antibiotics were used at the following concentrations for *Rhizobium*: 200 mg of ampicillin ml^{-1} and 10 mg of tetracycline ml^{-1}, 50 mg of kanamycin ml^{-1} for *E. coli.* Adenine and AICA-riboside were added at a final concentration of 0.1 mM. AICA was also used at 0.004 mM (1 mg ml^{-1}) where noted.

Recombinant DNA techniques.

DNA was isolated, detected, and manipulated using standard procedures (Maniatis et al. 1982). Restriction endonu-

clease and DNA modifying enzyme reactions were performed according to the manufacturer's recommendations or in TA buffer (O'Farrell et al. 1980).

DNA sequencing.

Clones for sequencing were prepared after subcloning specific fragments or by Exonuclease III digestion (Henikoff 1984). Sequencing was done with fluorescently labeled primers in a chain termination reaction (Sanger et al. 1977). Reactions were run on an Applied Biosystems 373A (Forster City, CA) DNA sequencer and analyzed with SEQ, a suite of sequencing programs available in the RSBS and detailed in Arioli et al. 1994.

Strains and plasmids.

Bacterial strains and plasmids are listed in Table 3. Plasmids pSD1 and pSD2 were described previously (Djordjevic et al. 1988). Plasmid pSD1 was originally described as carrying a 6.3-kb *Eco*RI insert. Detailed restriction mapping of this region indicates that the fragment is about 6.2 kb. Plasmids pSD2, 3, 5, and 7 were constructed by subcloning regions contained within the right-hand portion of the 6.2-kb *Eco*RI fragment of pSD1 into broad host vectors pWB5a (a gift from W. Buikema) and pLAFR3 (Fig. 1). Plasmid pSD3 also contains some polylinker sequence (*Eco*RI-*Pst*I) from its parental clone. *Escherichia coli* strain ANU1073 supplied the necessary *tra* functions in triparental matings (Ditta et al. 1980).

Plant microscopy.

Sections for light microscopy were longitudinally bisected and fixed in 2.5% glutaraldehyde/3.5% formaldehyde in 30 mM PIPES [piperazine-*N*-*N*′-bis(2-ethanesulfonic acid)] buffer (pH 6.8) under vacuum (20 mm Hg) for 2 h, post-fixed (after three rinses in buffer) in 2% osmium tetroxide in distilled water for 2 h, rinsed, and rehydrated in a graduated acetone series (50, 70, 95, and 100%). Specimens were then infiltrated with Spurr's resin, gradually over 3 days, embedded in fresh resin, and polymerized overnight at 65°C.

Sections for light microscopy were cut at 0.5 mm with a glass knife. Some sections had the resin removed by means of a saturated solution of sodium hydroxide in ethanol. All sections were stained with Toluidine Blue (pH 11.1) and viewed with a Nikon Optiphot with Plan Apo objectives.

Plant nodulation assays.

Siratro nodulation assays were adapted for plastic cylinders (Bunzyl) that contained 80 ml of F agar (pH 7.2) prepared as a slope as described by Chen et al. (1985) for *Leucaena* nodulation assays. Siratro seedlings were germinated as described previously (Rolfe et al. 1980). Healthy axenic seedlings were individually transferred to plastic cylinders containing the F agar, covered with sterile plastic bags, and maintained in a plant growth chamber with the following growth condition settings: 25°C, 16 h day, and a 22°C, 8 h night with 80% humidity. Light intensity was 250 to 350 μE s^{-1} m^{-2}. *Rhizobium* strains were in log phase growth prior to harvesting. Bacterial cell numbers were estimated spectrophotometrically (A_{650}) and diluted to approximately 3,000 cells/μl. Cell numbers were confirmed by serial titration and determination of colony forming units. Plastic bags were reapplied immediately after the application of 1 μl of *Rhizobium* inocula to the advancing root tip. Where coinoculation of two *Rhizobium* strains was performed, the total number of bacteria remained at 3,000 per seedling (1,500 cells of each strain per plant). As few as 30 viable *Rhizobium* cells will reliably nodulate siratro in this assay system. Plastic bags were maintained on top of the Bunzyl containers until seedlings had developed a stem approximately 2.5 cm in height from the top of the container (after approximately 4 days growth). Plastic bags were removed and the tops of the cylinders covered with Nescofilm leaving the emerging stem free to grow toward light for the remainder of the experiment. The bacterial contents within a nodule were determined as described previously (Gresshoff et al. 1980; Rolfe et al. 1980).

Isolation of *Rhizobium* LPSs.

Bacterial LPSs were isolated by modification of a procedure described previously (Westphal and Jann 1965; Chen et al. 1993). The methods we have used for the comparison of the LPS samples were essentially those of Hitchcock and

Table 3. Bacterial strains and plasmids

Strain	Relevant properties	Source or reference
Rhizobium strain		
NGR234	Wild-type, broad-host-range cowpea *Rhizobium*	Trinick and Galbraith 1980
ANU280	Sm^r, Rif^r derivative of NGR234	Chen et al. 1985
ANU2861	ANU280 Tn*5*, Pur^-, Exo^{++}	Chen et al. 1985
ANU2811	ANU280 *exoY11*::Tn*5*	Chen et al. 1985
ANU2866	ANU280 Tn*5*, Pur^-, Exo^{++}	Chen et al. 1985
ANU265	A heat-cured $pSym^-$, Sm^r derivative of NGR234	Morrison et al. 1983
224	Lipopolysaccharide (LPS) mutant of *R. leguminosarum* bv. *trifolii* strain ANU843	F. Dazzo (unpublished)
Escherichia coli strain		
DH5	Transformation recipient	Hanahan 1985
NM522	Transformation recipient	Gough and Murray 1983
HB101	*rpsL20 proA2 recA13 hsdS20 (rB-mB-) supE44*	Bolivar et al. 1977
011184	Commercially available wild-type *E. coli* strain	Sigma (St. Louis, MO)
Plasmids		
pSD1	6.2-kb wild-type *Eco*RI fragment cloned into pWB5a	Djordjevic et al. 1988
pSD2	1.3-kb *Hin*dIII-*Cla*I subclone in pWB5a	Djordjevic et al. 1988
pSD3	3.3-kb *Pst*I partial subclone in pWB5a	This work
pSD5	2.1-kb *Hin*dIII-*Eco*RI subclone in pLAFR3	This work
pSD7	1.2-kb *Pst*I subclone in pWB5a	This work
pCOS106, pCOS110, pCOS115	*R. leguminosarum* strain CE3 cosmid clones in pLAFR1	Noel et al. 1988

Brown (1983). Parent *Rhizobium* strain ANU280, and mutant strains ANU2861 and ANU2866 were grown in 200 ml of TY or BMM liquid medium for 48 h at 29°C, shaking at 150 rpm (New Brunswick Scientific Co. Inc., New Brunswick, NJ). Cultures were pelleted by centrifugation (Sorval Centrifuge, Dupont, Wilmington, DE) for 20 min at 8,000 rpm using a Sorval GSA head. Bacterial pellets were resuspended in 10 ml of hot, sterile, distilled water (65 to 68°C) and 10 ml of phenol (65 to 68°C) was added with vigorous vortexing. Bacterial extracts were maintained for 15 min at this temperature, cooled to 10°C by placing on ice for 10 min, and centrifuged at 8,000 rpm for 30 to 45 min in a GSA rotor head. The aqueous layer was removed by pipetting and stored at 4°C. The lower phenol phase containing bacterial residues was further extracted as described and the aqueous phases combined. After dialysis for 3 days against distilled water, the pooled aqueous phases were freeze-dried and resuspended in 5 ml of sterile distilled water for electrophoretic analysis.

Gel electrophoresis.

The sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) methods used were as described elsewhere (Chen et al. 1993). LPS extracts were suspended in SDS sample buffer and heated in boiling water for 3 min. Samples were run on 12.5% polyacrylamide gels (Phast-System, Pharmacia, Uppsala, Sweden) and silver stained using the Phast-Gel silver kit (Pharmacia).

ACKNOWLEDGMENTS

We appreciate and thank Dominique Barnard for the preparation and staining of sections for light microscopy, R. Ridge for photographic assistance, Dale Noel for cosmids, and Frank Dazzo for the LPS mutant 224. We gratefully acknowledge the excellent technical assistance of Anne Moten and Elena Gartner, and the students A. Gartner, M. Shoobridge, E. Hall, A. Wyndham, K. Pickett, L. Capezio, J. Mansour, V. Barlow, and J. Baker from the 1992 C52 class for technical assistance with subcloning and DNA sequencing of parts of pSD1.

LITERATURE CITED

Arioli, T., Howles, P. A., Weinman, J. J., and Rolfe, B. G. 1994. In *Trifolium subterraneum*, chalcone synthase is encoded by a multigene family. Gene 138:79-86.

Batley, M., Redmond, J. W., Djordjevic, S. P., and Rolfe, B. G. 1987. Characterisation of glycerophosphorylated cyclic β-1,2-glucans from fast-growing *Rhizobium* species. Biochem. Biophis. Acta 901:119-126.

Beringer, J. E. 1974. R factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. 84:188-198.

Bolivar, F., Rodriguez, R., Greene, P. J., Betlach, M., Heynecker, H. L., Boyer, H. W., Crosa, J., and Falkow, S. 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene 2:95-113.

Chen, H., and Rolfe, B. G. 1987. Cooperativity between *Rhizobium* mutant strains: Induction of nitrogen-fixing nodules on the tropical legume *Leucaena leucocephala*. J. Plant. Physiol. 127:307-322.

Chen, H. M., Batley, Redmond, J., and Rolfe, B. G. 1985. Alteration of the effective nodulation properties of a fast growing-broad-host range *Rhizobium* due to changes in exopolysaccharide synthesis. J. Plant. Physiol. 120:331-349.

Chen, H., Gartner, E., and Rolfe, B. G. 1993. Involvement of genes on a megaplasmid in the acid-tolerant phenotype of *Rhizobium leguminosarum* biovar *trifolii*. Appl. Environ. Microbiol. 59:1058-1064.

Ditta, G., Stanfield, S., Corbin, D., and Helinski, D. R. 1980. Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA 77: 7347-7351.

Djordjevic, S. P., Ridge, R. W., Chen, H., Redmond, J. W., Batley, M., and Rolfe, B. G. 1988. Induction of pathogenic-like responses in the legume *Macroptilium atropurpureum* by a transposon-induced mutant of the fast-growing, broad-host-range *Rhizobium* strain NGR234. J. Bacteriol. 170:1848-1857.

Ebbole, D. J., and Zalkin, H. 1987. Cloning and characterization of a 12-gene cluster from *Bacillus subtilis* encoding nine enzymes for de novo purine nucleotide synthesis. J. Biol. Chem. 262:8274-8287.

Gough, J. A., and Murray, N. E. 1983. Sequence diversity among related genes for recognition of specific targets in DNA molecules. J. Mol. Biol. 166:1-19.

Gresshoff, P. M., Skotnicki, M. L., Eadie, J. F., and Rolfe, B. G. 1980. Viability of *Rhizobium trifolii* bacteroids from clover root nodules. Plant Sci. Lett. 10:299-304.

Hanahan, D. 1985. Techniques for transformation of *E. coli*. Pages 109-135 in: DNA Cloning, Vol. 1. D. M. Glover, ed. IRL Press, Oxford.

Henikoff, S. 1984. Unidirectional digestion with exonuclease III creates target breakpoints for DNA sequencing. Gene 28:351-359

Hitchcock, P. J., and Brown, T. M. 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gel. J. Bacteriol. 154:269-277.

Klein, S., Hirsch, A. M., Smith, C.A., and Signer, E. R. 1988. Interaction of *nod* and *exo Rhizobium meliloti* in alfalfa nodulation. Mol. Plant-Microbe Interact. 1:94-100.

Maniatis, T. A., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

McIver, J., Djordjevic, M. A., Weinman, J. J., and Rolfe, B. G. 1993. Influence of *Rhizobium leguminosarum* biovar *trifolii* host specific nodulation genes on the ontogeny of clover nodulation. Protoplasma 172:166-179.

Morrison, N. A., Cen, Y. H., Trinick, M. J., Shine, J., and Rolfe, B. G. 1983. Heat curing of a Sym plasmid in a fast-growing *Rhizobium* sp. that is able to nodulate legumes and the nonlegume *Parasponia* sp. J. Bacteriol. 153:527-531.

Newman, J. D., Diebold, R. J., Schultz, B. W., and Noel, K. D. 1994. Infection of soybean and pea nodules elicited by *Rhizobium* purine auxotrophs in the presence of 5-aminoimidazole-4-carboxamide riboside. J. Bacteriol. 176:3286-3294.

Newman, J. D., Rosovitz, M. J., and Noel, K. D. 1995. Requirement for Rhizobial production of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) for infection of bean. Mol. Plant-Microbe Interact. 8:407-414.

Newman, J. D., Schultz, B. W., Rosovitz, M. J., and Noel, K. D. 1991. Aminoimidazolecarboxamide riboside as a determinant for infection of bean by *Rhizobium leguminosarum* CFN42. North Am. Symbiotic Nitrogen Fix. Conf., 13th.

Newman, J. D., Schultz, B. W., and Noel, K. D. 1992. Dissection of nodule development by supplementation of *Rhizobium leguminosarum* biovar *phaseoli* purine auxotrophs with 5-aminoimidazole-4-carboxamide riboside. Plant Physiol. 99:401-408.

Noel, K. D., Diebold, R. J., Cava, J. R., and Brink, B. A. 1988. Rhizobial purine and pyrimidine auxotrophs: Nutrient supplementation, genetic analysis, and the symbiotic requirement for de novo purine biosynthesis. Arch Microbiol. 149:499-506.

Noel, K. D., Sanchez, A., Fernandez, L., Leemans, J., and Cevallos, M. A. 1984. *Rhizobium phaseoli* symbiotic mutants with transposon Tn*5* insertions. J. Bacteriol. 158:148-155.

Noel, K. D., VandenBosch, K. A., and Kulpaca, B. 1986. Mutations in *Rhizobium phaseoli* that lead to arrested development of infection threads. J. Bacteriol. 168:1392-1401.

O'Farrell, P. H., Kutter, E., and Nakanaishi, M. 1980. A restriction map of the bacteriophage T4 genome. Mol. & Gen. Genet. 179:421-435.

Pain, A. N. 1979. Symbiotic properties of antibiotic-resistant and auxotrophic mutants of *Rhizobium leguminosarum*. J. Appl. Bacteriol. 47: 53-64.

Pankhurst, C. E., and Schwinghamer, E. A. 1974. Adenine requirement for nodulation of pea by an auxotrophic mutant of *Rhizobium leguminosarum*. Arch. Microbiol. 100: 219-238.

Rolfe, B. G., Gresshoff, P. M., and Shine, J. 1980. Rapid screening for symbiotic mutants of *Rhizobium* and white clover. Plant Sci. Lett. 19: 277-284.

Sanger, F., Nicklen, S., and Coulson, A. R. 1977. DNA sequencing with

chain terminating inhibitors. Proc Natl. Acad. Sci. USA 74:5463-5467.
Scherrer, A., and Denarie, J. 1971. Symbiotic properties of some auxotrophic mutants of *Rhizobium meliloti* and of their prototrophic revertants. Pages 39-45 in Plant Soil (special vol.).
Smith, J. M., and Daum, H. A. 1987. Identification and nucleotide sequence of a gene encoding 5′-phosphoribosylglycinamide transformylase in *Escherichia coli* K12. J. Biol. Chem. 262:10565-10569.
Stacey, G., Sanjuan, J. Spaink, H., van Brussel, T., Lugtenberg, B. J. J., Glushka, J., and Carlson, R. W. 1993. *Rhizobium* lipo-oligosaccharides: Novel plant growth regulators. Pages 45-58 in: Plant Responses to the Environment. P. M. Gresshoff, ed. CRC Press, Boca Raton, FL.
Trinick, M. J., and Galbraith, J. 1980. The *Rhizobium* requirements of the non-legume *Parasponia* in relationship to the cross-inoculation concept of legumes. New Phytol. 86:17-26.
van Brussel, A. A. N., Tak, T., Wetselaar, A., Pees, E., and Wijffelman, C. A. 1982. Small leguminosae as test plants for nodulation of *Rhizobium leguminosarum* and other rhizobia and agrobacteria harbouring a leguminosarum sym-plasmid. Plant Sci Lett. 27:317-325.
van Brussel, A. A. N., Zaat, S. A. J., Canter Cremers, H. C. J., Wijffelman, C. A., Pees, E., Tak, T., and Lugtenberg, B. J. J. 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. 165:517-522.
van Workum, W. A. T., van Brussel, A. A. N., Tak, T., Wijffelman, C. A., and Kijne, J. W. 1995. Ethylene prevents nodulation of *Vicia sativa* ssp. *nigra* by exopolysaccharide-deficient mutants of *Rhizobium leguminosarum* bv. *viciae.* Mol. Plant-Microbe Interactions 8:278-285.
VandenBosch, K. A., Noel, K. D., Kaneko, Y., and Newcomb, E. H. 1985. Nodule initiation elicited by non-infective mutants of *Rhizobium phaseoli.* J. Bacteriol. 162:950-959.
Westphal, O., and Jann, K. 1965. Bacterial lipopolysaccharides. Methods Carbohydr. Chem. 5:83-91.