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Jeffrey D. Newman  
*Marquette University*

Ronald J. Diebold  
*University of Cincinnati Medical School*

Bruce W. Schultz  
*Marquette University*

K. Dale Noel  
*Marquette University, dale.noel@marquette.edu*

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## Infection of Soybean and Pea Nodules by *Rhizobium* spp. Purine Auxotrophs in the Presence of 5-Aminoimidazole-4-Carboxamide Riboside

JEFFREY D. NEWMAN,<sup>†</sup> RONALD J. DIEBOLD,<sup>‡</sup> BRUCE W. SCHULTZ,<sup>§</sup> AND K. DALE NOEL\*

Department of Biology, Marquette University, Milwaukee, Wisconsin 53233

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Purine auxotrophs of various *Rhizobium* species are symbiotically defective, usually unable to initiate or complete the infection process. Earlier studies demonstrated that, in the *Rhizobium etli*-bean symbiosis, infection by purine auxotrophs is partially restored by supplementation of the plant medium with 5-aminoimidazole-4-carboxamide (AICA) riboside, the unphosphorylated form of the purine biosynthetic intermediate AICAR. The addition of purine to the root environment does not have this effect. In this study, purine auxotrophs of *Rhizobium fredii* HH303 and *Rhizobium leguminosarum* 128C56 (bv. *viciae*) were examined. Nutritional and genetic characterization indicated that each mutant was blocked in purine biosynthesis prior to the production of AICAR. *R. fredii* HH303 and *R. leguminosarum* 128C56 appeared to be deficient in AICA riboside transport and/or conversion into AICAR, and the auxotrophs derived from them grew very poorly with AICA riboside as a purine source. All of the auxotrophs elicited poorly developed, uninfected nodules on their appropriate hosts. On peas, addition of AICA riboside or purine to the root environment led to enhanced nodulation; however, infection threads were observed only in the presence of AICA riboside. On soybeans, only AICA riboside was effective in enhancing nodulation and promoting infection. Although AICA riboside supplementation of the auxotrophs led to infection thread development on both hosts, the numbers of bacteria recovered from the nodules were still 2 or more orders of magnitude lower than in fully developed nodules populated by wild-type bacteria. The ability of AICA riboside to promote infection by purine auxotrophs, despite serving as a very poor purine source for these strains, supports the hypothesis that AICAR plays a role in infection other than merely promoting bacterial growth.

Root nodules of leguminous plants develop from cell division foci triggered within young root cortical layers by signals from bacteria of the genera *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium*. The bacteria penetrate from the root epidermis to the interior of the developing nodule by means of an infection thread, composed of a growing linear bacterial colony and the surrounding tubular cell wall structure produced by the plant. Eventually, numerous plant cells in the nodule interior become infected by endocytosis of the bacteria from unwall tips of the infection thread (17). These released bacteria then differentiate into bacteroids that commence nitrogen fixation.

The process of infection thread formation remains obscure, especially at the molecular level. Very little is known about the contribution of the microsymbiont, although it is clear that certain deficiencies of the bacteria can prevent or severely limit infection. Mutations in crucial bacterial *nod* genes prevent infection, but since they also prevent the induction of any nodule tissue formation, it is likely that *nod* mutations act epistatically by blocking nodule development at stages before infections can form. Potentially more revealing for understanding infection are deficiencies that allow nodules or pseudonodules to form but lead to poorly developed infection threads. This phenotype has been found mainly in polysaccharide-

deficient mutants (5, 7, 12, 22, 25, 32) and auxotrophs (2, 6, 9, 10, 16, 18, 23, 26–28, 30). Perhaps the most universally deleterious such defect is purine auxotrophy.

Purine auxotrophs (Pur<sup>-</sup>) of *Rhizobium etli* CFN42 (bv. *phaseoli*) elicit pseudonodules on bean plants (*Phaseolus vulgaris* L.) (23). These mutants cause root hair curling and root cortical cell division but do not elicit infection threads (35), and no bacteria can be isolated from the resulting pseudonodules (23). Ex planta, growth of the auxotrophs is restored by 5-aminoimidazole-4-carboxamide (AICA) riboside, the unphosphorylated derivative of the purine precursor AICAR, as well as by several purines, purine nucleosides, and bean root exudate. However, supplementing the root medium with purines or purine nucleosides has no effect on the nodulation phenotype, even at 100-fold higher concentrations than those required to provide full growth ex planta. The addition of AICA riboside to the root environment, on the other hand, significantly enhances nodule development (21, 23).

Bean nodules elicited by AICA riboside supplementation of Pur<sup>-</sup> rhizobia are the same size as those elicited by the wild type, and they have infection threads and anatomical features characteristic of nodules formed with wild-type bacteria, such as peripheral vasculature rather than the central vasculature of the pseudonodules. Also, unlike the pseudonodules, they contain the nodule-specific protein leghemoglobin. However, the concentration of leghemoglobin is much lower than normal, and the nodules lack detectable nitrogenase activity. The bacteria that can be recovered from these nodules after full development are 1,000-fold fewer than in nodules elicited by the wild type, and the bacteria have been observed only in infection threads. Large cells in the interior of the nodule, presumably corresponding to those that are normally infected,

\* Corresponding author. Mailing address: Department of Biology, Milwaukee, WI 53233. Phone: (414) 288-1475. Fax: (414) 288-7357.

<sup>†</sup> Present address: Agronomy Department, University of Kentucky, Lexington, KY 40546-0091.

<sup>‡</sup> Present address: University of Cincinnati Medical School, Department of Molecular Genetics and Biochemistry, Cincinnati, OH 45206.

<sup>§</sup> Present address: University of Wisconsin Medical School, Madison, WI 53706.

contain extensive internal membranes but appear to lack bacteroids (21). It seems, therefore, that AICA riboside restores almost complete infection thread development but cannot overcome a second limitation that prevents the proliferation of bacteroids.

Purine auxotrophs of other species of *Rhizobium* are also defective in symbiosis with their normal hosts (5, 6, 9, 10, 16, 18, 26, 27, 30, 33). *Pur<sup>-</sup> Rhizobium leguminosarum* bv. *viciae* strains have been described as noninfective (26) or nonnodulating (27). A purine auxotroph of the broad-host-range *Rhizobium* strain NGR234 elicits root hair curling and nodule meristem initiation on siratro, but no infection threads are formed (9). This mutant also elicits poorly developed nodules on *Leucaena leucocephala* and *Lablab purpureus* (5). On soybeans, *Pur<sup>-</sup> Rhizobium fredii* induces pseudonodules that do not contain bacteria (18). Although anatomical development is not blocked at an identical stage in these different symbioses, the underlying molecular basis of arrested development may be quite similar and the *Pur<sup>-</sup>* defect seems specifically to affect the infection process. A possible exception is the *Rhizobium meliloti*-alfalfa symbiosis; although nodules induced by *Pur<sup>-</sup> R. meliloti* do not fix nitrogen (6, 16, 30, 33), it has been reported that they do have infection threads (33) and do infect host cells (6).

When nutrient supplementation was tested in the studies cited above, the addition of purines to the root environment did not restore or enhance the symbiotic proficiency of the purine auxotrophs, with one exception (27). In that study, it was reported that adenine enhanced nodulation of peas by a *Pur<sup>-</sup>* mutant. However, adenine addition to the root medium had no effect on the nodulation of peas by 31 purine auxotrophs studied by Pain (26).

Since AICA riboside was uniquely effective in bean symbiosis, the present study was undertaken to determine whether AICA riboside can partially restore the symbioses of *Rhizobium* purine auxotrophs with two other legume hosts: peas and soybeans. *Pur<sup>-</sup>* microsymbionts of peas analyzed in this study were constructed by genetic recombination between rhizobial strains having different host ranges. The *Pur<sup>-</sup>* microsymbionts of soybeans chosen for study had been isolated from *R. fredii* HH303 by Kim et al. (18). On both hosts, supplementation of the root medium with AICA riboside stimulated infection and enhanced the development of nodules elicited by these purine auxotrophs, even though the auxotrophs grew very poorly with AICA riboside as the purine source.

## MATERIALS AND METHODS

**Bacterial growth media.** Rich (TY) medium (containing tryptone, yeast extract, and calcium chloride) and minimal (Y) medium (containing succinate and glutamate as carbon and nitrogen sources) have been described previously (24). TY medium was solidified with 1.5% Bacto agar, and purified agar (BBL) was used to solidify Y medium.

**Construction of *Pur<sup>-</sup> R. leguminosarum* strains.** Previously isolated strains CE106 and CE110 (23) are *Str<sup>r</sup> Ery<sup>s</sup>* derivatives of *R. etli* CFN42 (bv. *phaseoli*) that carry *pur::Tn5* mutations (Table 1). Plasmid pJB3, which mobilizes chromosomal DNA at low frequency during conjugation (4, 15), was transferred into these strains. These donor strains were then crossed with *Ery<sup>r</sup>* recipient strain *R. leguminosarum* 128C56 (bv. *viciae*) on nitrocellulose membrane filters incubated on TY agar at 30°C for 24 h. The bacteria on the filter were suspended in 3 ml of 0.1 mM MgSO<sub>4</sub>, and 0.1 ml of this suspension was spread on TY agar containing erythromycin (10 mg/liter) and kanamycin (30 mg/liter). (All antibiotics were from Sigma Chemical Co.,

St. Louis, Mo.) Purified *Ery<sup>r</sup> Km<sup>r</sup> (Str<sup>s</sup>)* transconjugants RL106 and RL110 (Table 1) were verified as having the 128C56 genetic background on the basis of the patterns of proteins revealed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of SDS extracts of washed cultured bacteria (25).

**Tests for growth of bacterial auxotrophs.** Growth was tested on agarose, purified agar, or liquid minimal (Y) medium with the indicated supplements, as described previously (23). For quantitative comparison of growth, overnight cultures of each strain grown in TY liquid were washed in sterile 0.1 M MgSO<sub>4</sub> and resuspended in Y liquid. Each strain was inoculated at a final dilution of 10<sup>-4</sup> into Y liquid, with supplements as noted. After incubation overnight at 30°C, an aliquot was removed from each tube for serial dilution and plating onto TY medium to determine viable bacterial counts.

**[<sup>3</sup>H]AICA riboside uptake and incorporation into nucleic acids.** AICA riboside (supplied by Sigma) was labelled by New England Nuclear Corp. by hydrogen-tritium exchange in tritiated water in the presence of a catalyst. [<sup>3</sup>H]AICA riboside was purified by reverse-phase high-pressure liquid chromatography (HPLC) on a Waters  $\mu$ Bondapak C-18 column. Solvent A was 20 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4, and solvent B was 60% (vol/vol) methanol in water. The chromatogram was developed at a flow rate of 1 ml/min with the following gradient: 0 to 5 min, 0% solvent B; 5 to 15 min, linear increase from 0 to 5% solvent B; 15 to 20 min, linear increase from 5 to 60% solvent B; and 20 to 30 min, 60% solvent B. This purified material gave homogeneous peaks on reverse-phase HPLC eluted with various solvent programs and single spots on two-dimensional thin-layer cellulose chromatography. The radioactivity comigrated with standard commercial AICA riboside in these chromatographic analyses. It was determined that most of the radioactivity was in the base rather than the sugar portion by releasing the AICA portion by hydrolysis in 2 M perchloric acid at 95°C for 1.5 h. After neutralization, more than 90% of the original radioactivity comigrated with standard AICA on reverse-phase HPLC. Standard AICA and AICA riboside gave the absorbance spectra reported in the literature and reacted in an assay for diazotizable amines (14) to give the appropriate color and absorbance spectra.

For measuring uptake, aliquots from overnight cultures of each strain grown in Y liquid were diluted 5- to 10-fold into fresh Y liquid and incubated for 3 to 5 h at 30°C. Aliquots of 25  $\mu$ l were removed to determine CFU on TY agar. [<sup>3</sup>H]AICA riboside (211  $\mu$ Ci/ $\mu$ mol) was added to various concentrations, and 0.3-ml aliquots were removed after 0.25, 1.0, 3.0, and 10.0 min of incubation at 30°C. Immediately after removal, each aliquot was vacuum filtered on a 0.45- $\mu$ m-pore-size GN-6 filter (Gelman) and rinsed with 5 ml of Y liquid prepared without FeCl<sub>3</sub>. When incorporation into nucleic acids was measured, 0.3 ml of the suspension remaining after filtration of the last aliquot was added to 0.6 ml of ice-cold 15% trichloroacetic acid to precipitate nucleic acids. The samples were kept on ice for at least 30 min, filtered on Whatman GF/A glass fiber filters, and rinsed twice with 5 ml of ice-cold 15% trichloroacetic acid and once with 5 ml of 95% ethanol. The filters were air dried, and the amount of tritium taken up or incorporated into nucleic acids was determined by scintillation counting of the filters.

**Nodulation assays.** Tests with pea plants (*Pisum sativum* cv. Wando [Olds Seeds, Madison, Wis.]) in 500-ml Erlenmeyer flasks (3) containing 300 ml of nitrogen-free RBN nutrient medium (36) solidified with 0.7% purified agar were conducted. After being autoclaved, the medium was supplemented with filter-sterilized solutions of AICA riboside, adenine, or

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics <sup>a</sup>	Complementing cosmid	Reference(s)
<b>Bacterial strains</b>			
<i>R. etli</i> bv. phaseoli			
CE3	Str <sup>r</sup> , derivative of CFN42, Fix <sup>+</sup>		24, 31
CE106	<i>purF106<sup>b</sup>::Tn5</i> derivative of CE3, Ndv <sup>-</sup>	pCOS106	24
CE110	<i>purY110<sup>b</sup>::Tn5</i> derivative of CE3, Ndv <sup>-</sup>	pCOS110	24
<i>R. leguminosarum</i> bv. viciae			
128C56 <sup>c</sup>	Ery <sup>r</sup> Fix <sup>+</sup>		7
RL106	<i>purF106<sup>b</sup>::Tn5</i> derivative of 128C56, Ndv <sup>-</sup>	pCOS106	This work
RL110	<i>purY110<sup>b</sup>::Tn5</i> derivative of 128C56, Ndv <sup>-</sup>	pCOS110	This work
<i>R. fredii</i>			
HH303	Fix <sup>+</sup>		18
RfK1105	<i>purQ1105::Tn5</i> derivative of HH303, Ndv <sup>-</sup>	pCOS110	18
RfK1107	<i>purL1107::Tn5</i> derivative of HH303, Ndv <sup>-</sup>	pCOS110	18
<i>E. coli</i> HB101	<i>supE44 hsdS20(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1</i>		29
<b>Plasmids</b>			
pCOS106	pLAFR1 containing CE3 <i>purF<sup>b</sup></i> region		23
pCOS110	pLAFR1 containing CE3 <i>purY</i> , <i>purQ</i> , and <i>purL<sup>b</sup></i> regions		23
pJB3	R68.45 derivative, Tc <sup>r</sup>		4
pJN110A	pCOS110, <i>purY110<sup>b</sup>::Tn5</i>		20
pJN170A	pCOS110, <i>purL170<sup>b</sup>::Tn5</i>		20
pJN382A	pCOS110, <i>purL382<sup>b</sup>::Tn5</i>		20
pJN390A	pCOS110, <i>purQ390<sup>b</sup>::Tn5</i>		20
pJN391A	pCOS110, <i>purQ391<sup>b</sup>::Tn5</i>		20
pRK2013	Km Tra <sup>+</sup> helper plasmid		11
pRK600	Cm Tra <sup>+</sup> pRK2013 Km::Tn9		13

<sup>a</sup> Abbreviations: Str, streptomycin; Fix, symbiotic nitrogen fixation; Ndv, nodule development; Ery, erythromycin; Tc, tetracycline; Km, kanamycin; Tra, RK2 transfer functions; Cm, chloramphenicol.

<sup>b</sup> Gene designations correspond to those of *E. coli* and *Bacillus subtilis* and are based on partial sequence analysis of DNA adjacent to Tn5 insertions (20).

<sup>c</sup> In keeping with previous literature, strain 128C56 and its derivatives RL106 and RL110 are designated *R. leguminosarum* throughout this report; however, the ribosomal RNA sequences of strain 128C56 have not been analyzed. It is conceivable that it eventually will be classified as *R. etli* or even assigned to a third rhizobial species. Strains 128C56 and CFN42 are both very similar to strains now defined as *R. leguminosarum* (31); for instance, they have apparently identical acidic exopolysaccharide, lipopolysaccharide core oligosaccharide, and lipid A sugar compositions.

inosine as specified. Seeds were surface sterilized with 50% commercial bleach for 15 min, rinsed five times in sterile water, and aseptically germinated on moistened filter paper. Two germinated seedlings were placed into each flask, the mouths of the flasks were covered with sponge plugs, and the plants were grown in continual darkness for 7 to 12 days to allow etiolation of the plant stems. The etiolated stems were then pulled outside of the flask so that the shoots could be exposed to light, while aluminum foil covering the flask maintained the roots in darkness. At this time, 0.5 ml of a bacterial culture grown to full density in TY broth was inoculated onto each seed, the mouth of the flask was sealed with the cotton plug, and the plants were allowed to grow for an additional 3 to 4 weeks. Alternatively, peas were grown in modified Leonard jars (1), with AICA riboside or inosine supplements present in the RBN nutrient reservoir throughout the growth of the plant.

Soybean (*Glycine max* cv. Williams-82 [Wilkin Seed & Grain, Pontiac, Ill.]) nodulation tests with plastic growth pouches (Vaughn's Seed Company, Downer's Grove, Ill.) were conducted as described previously for bean plants (24). AICA riboside or inosine was added to the RBN solution at the time of planting and to watering solutions used during the experiment.

**Microscopy.** Nodules were harvested and processed for microscopy as described by VandenBosch et al. (35).

## RESULTS

**Construction of purine auxotrophs of *R. leguminosarum* 128C56.** By R plasmid-mediated conjugation, *pur*<sup>+</sup> alleles of strain 128C56 were replaced with *pur::Tn5* alleles from mutants of *R. etli* CFN42. The resulting transconjugants (RL106 and RL110) had the Ery<sup>r</sup> phenotype and the SDS-PAGE protein profile of strain 128C56 but did not grow on minimal medium unless a purine source, such as adenine, adenosine, hypoxanthine, or inosine, was added. Mutant RL106, but not RL110, also required thiamine for growth, consistent with the respective phenotypes of the *R. etli* mutant donor strains (23). The transconjugants grew very poorly when supplemented with AICA riboside (Table 2). The latter result was unexpected, because the donor strains (*R. etli* mutants CE106 and CE110) grew nearly as well with AICA riboside as they did with purines (Table 2) (23); however, a presumptive explanation was suggested later during experiments that compared AICA riboside uptake in *R. etli* CFN42 and *R. leguminosarum* 128C56 (see the description below).

Previously isolated cosmids carrying the corresponding *pur*<sup>+</sup> alleles of strain CFN42 (23) were transferred from *Escherichia coli* into the Pur<sup>-</sup> transconjugants (RL106 and RL110) by triparental matings, with pRK2013 as a helper plasmid (8). Cosmids pCOS106 and pCOS110 restored prototrophic

TABLE 2. Relative growth levels of purine auxotrophs in liquid minimal medium with or without supplements

Supplement (concn)	Growth <sup>a</sup> of strain:		
	CE106	RL106	RfK1105 <sup>b</sup>
None	1	1	1
AICA riboside (0.1 mM)	40.4	1.7	2.3
AICA riboside (1.0 mM)	45.7	1.7	2.6
Inosine (0.1 mM)	68.1	48.3	171.4
Adenine (0.1 mM)	86.2	93.1	242.9

<sup>a</sup> Entries indicate the ratio of CFU 18 h after inoculation to CFU in minimal Y medium alone in one representative experiment.

<sup>b</sup> RfK1107 exhibited responses similar to those of RfK1105 with these supplements.

growth to strains RL106 and RL110, respectively, as expected, and allowed wild-type nodulation on pea plants, whereas the uncomplemented mutants were symbiotically defective (see the description below).

**Genetic and nutritional characterization of *R. fredii* purine auxotrophs.** Pur<sup>-</sup> strains RfK1105 and RfK1107 grew well when inosine, hypoxanthine, adenosine, or adenine was present, but very poorly on minimal medium supplemented with AICA riboside (Table 2). Strain RfK1105 did not require thiamine, but strain RfK1107 did, indicating that the latter mutant was blocked in the purine pathway before the production of 5-aminoimidazole ribotide, a precursor of thiamine as well as purines. Both strains were complemented to prototrophy by cosmid pCOS110, which contains the purine biosynthetic genes *purY*, *purQ*, and *purL* (Table 1). Strain RfK1105 appeared to be mutated in *purQ*, because mutations *purQ390* and *purQ391* prevented complementation of this mutant (Table 3). Likewise, mutations within the *purL* gene of pCOS110 eliminated the ability to complement RfK1107, suggesting that RfK1107 is mutated in *purL*. Both *purL* and *purQ* specify steps in purine biosynthesis before AICAR production (20). The thiamine requirement of strain RfK1107 and the thiamine independence of strain RfK1105 matched the phenotypes of *R. etli* strains mutated in *purL* and *purQ* (20). The implications of these contrasting phenotypes in terms of PurL and PurQ protein function are discussed elsewhere (20).

**Uptake and incorporation of AICA riboside.** As noted above, Pur<sup>-</sup> mutants derived from *R. fredii* HH303 or *R. leguminosarum* 128C56 grew very poorly with AICA riboside as a purine source, even though all of the mutants should be blocked before and not after AICAR synthesis. For a purine auxotroph to be capable of growth with AICA riboside as a purine source, it must be taken into the cell and converted to AICAR, the actual intermediate in the purine pathway. A block in either of these steps would prevent the utilization of AICA riboside as a purine source.

TABLE 3. Complementation of *R. fredii* purine auxotrophs

Plasmid	Gene mutated	Complementation <sup>a</sup>	
		RfK1105	RfK1107
pCOS110		+	+
pJN110A	<i>purY</i>	+	+
pJN170A	<i>purL</i>	+	-
pJN382A	<i>purL</i>	+	-
pJN390A	<i>purQ</i>	-	+
pJN391A	<i>purQ</i>	-	+

<sup>a</sup> +, restoration to prototrophy; -, failure to restore to prototrophy.

TABLE 4. Uptake of [<sup>3</sup>H]AICA riboside and incorporation into nucleic acids

Strain	<i>K<sub>m</sub></i> (nM)	<i>V<sub>max</sub></i> (pmol min <sup>-1</sup> [10 <sup>7</sup> CFU] <sup>-1</sup> )	Incorporation <sup>a</sup> (% of total uptake)
CE3	380	86	59
128C56	13	6.4	41
HH303	350	6.9	12

<sup>a</sup> Incorporation of tritium into trichloroacetic acid-precipitable material measured after 10 min of incubation in the presence of 1 μM [<sup>3</sup>H]AICA riboside.

To determine whether the poor growth of the mutants on AICA riboside was due to a defect in transport or metabolism of this compound, the prototrophic strains from which they were derived were assayed for uptake of exogenous [<sup>3</sup>H]AICA riboside and incorporation of the tritium into nucleic acids (Table 4). Strain CE3 (Str<sup>-</sup> CFN42) is the wild-type *R. etli* bv. phaseoli strain whose Pur<sup>-</sup> derivatives grow well with AICA riboside as a purine source (Table 2) and which therefore represents a positive control for AICA riboside utilization.

Strains 128C56 and HH303 exhibited maximum rates of uptake (*V<sub>max</sub>*) of AICA riboside that were less than 10% the *V<sub>max</sub>* calculated for strain CE3 (Table 4). The *K<sub>m</sub>* for AICA riboside uptake by *R. fredii* HH303 was similar to that observed in CE3, whereas *R. leguminosarum* 128C56 displayed an extremely low *K<sub>m</sub>* for AICA riboside uptake. In separate experiments, the incorporation of tritium into acid-precipitable material was examined. After 10 min of incubation in the presence of 1 μM AICA riboside, the portion of the total uptake that was acid precipitable (presumably in nucleic acids) was much lower in HH303 than in the other two wild types (Table 4). Together, these results suggested that both *R. fredii* HH303 and *R. leguminosarum* 128C56 are deficient in AICA riboside uptake and that *R. fredii* HH303 may also be deficient in AICA riboside metabolism.

**Symbiotic properties.** Pea plants were grown in two ways, in modified Leonard jars (1) and in Erlenmeyer flasks containing nutrient agar. In both systems, nodules elicited by Pur<sup>-</sup> strains RL106 and RL110 were slow to emerge and 4 weeks after inoculation were still very small bumps (Fig. 1A). No bacteria could be recovered from these pseudonodules by crushing after surface sterilization (Table 5). On some plants inoculated with the mutants, not even very small bumps were visible. Within 4 weeks, roots inoculated with wild-type 128C56 had large, nitrogen-fixing nodules (Fig. 1E) that contained an average of 3.0 × 10<sup>7</sup> bacteria per nodule (measured as recovered CFU).

Despite poorly supporting the growth of mutants RL106 and RL110 ex planta, 0.1 mM AICA riboside enhanced the development of nodules elicited by these mutants (Fig. 1D). Some roots were virtually covered with nodules that were about one-third the size of nodules elicited by the wild type. Increasing the AICA riboside concentration to 1.0 mM had no additional visible effect. When sampled 26 days postinoculation, nodules elicited by the mutants in the presence of AICA riboside were white and contained 100 to 1,000 CFU per nodule. One week later, up to 10<sup>5</sup> CFU could be recovered from these nodules. Examination of such nodules by light microscopy revealed the presence of infection threads filled with bacteria (Fig. 2A). Infected cells like those formed by the wild type (Fig. 2B) were not apparent. Instead, cells filled with starch granules and what appeared to be vesicles or small vacuoles were common. Bacteria recovered from the mutant-induced nodules retained purine auxotrophy.

Nodule development was enhanced also when 0.1 mM

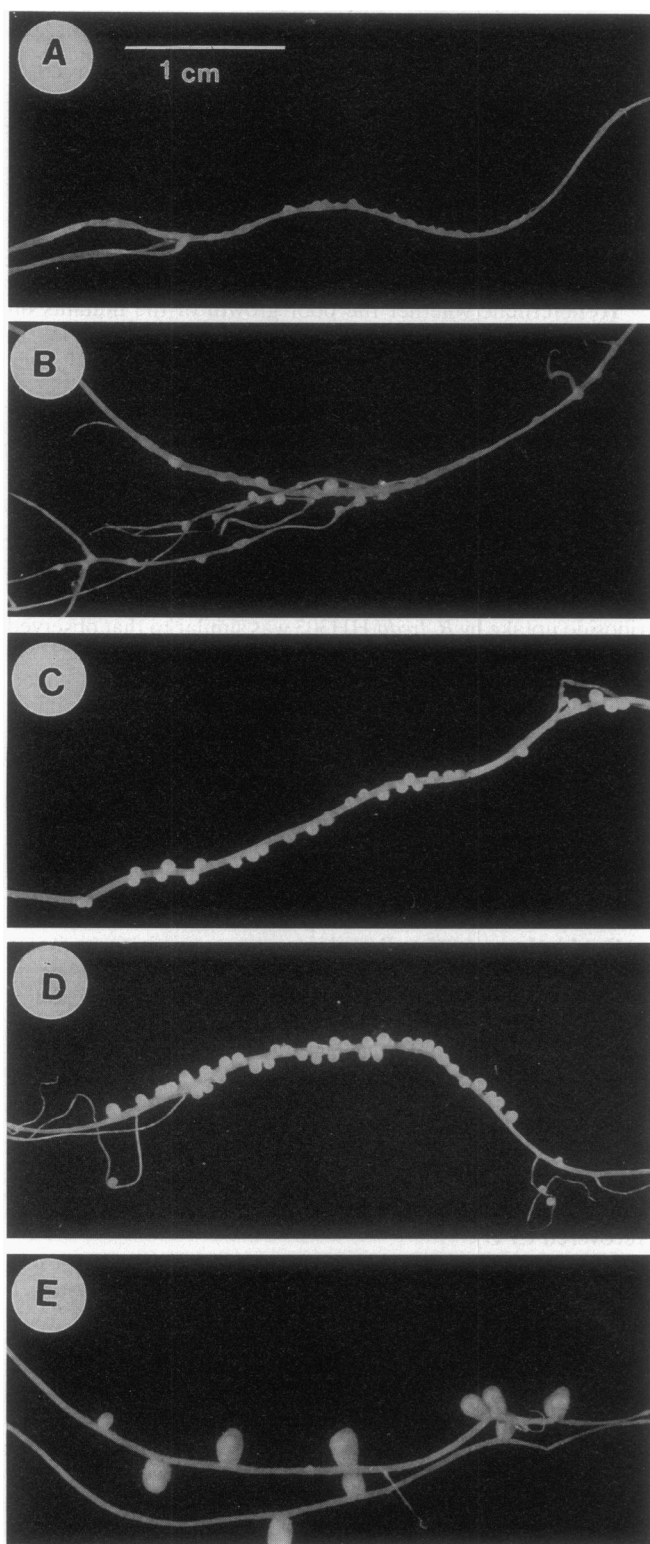


FIG. 1. Nodulation of peas by an *R. leguminosarum* bv. *viciae* purine auxotroph. Pea roots are shown 4 weeks after inoculation with purine auxotroph RL106 (A to D) or wild-type strain 128C56 (E). Supplements (0.1 mM) added to the root medium were as follows: no supplement (A and E), inosine (B), adenine (C), and AICA riboside (D).

TABLE 5. Nodulation of peas and soybeans by purine auxotrophs<sup>a</sup>

Strain	Supplement (concn)	No. of nodules per plant <sup>b</sup>	No. of bacteria per nodule (log <sub>10</sub> ) <sup>c</sup>
128C56	None	++	7.48
RL106	None	—	<1
RL106	Inosine (0.1 mM)	++	<1
RL106	Adenine (0.1 mM)	+++	1.52 <sup>d</sup>
RL106	AICA riboside (0.1 mM)	+++	2.64
HH303	None	9.2 ± 3.6 (15)	7.47
RfK1105	None	0 (17)	<1
RfK1105	Inosine (0.5 mM)	0.2 ± 0.4 (13)	<1
RfK1105	AICA riboside (0.5 mM)	8.1 ± 8.3 (12)	5.66

<sup>a</sup> Results of representative experiments in which nodules were sampled 26 (peas) or 21 (soybeans) days after inoculation.

<sup>b</sup> For nodulation of peas: —, no nodules, or too small to be counted reliably; ++, 30 to 60 nodules; +++, >60 nodules. For soybeans, development such as that shown in Fig. 3B or C was counted as a nodule, but bumps such as those in Fig. 3A were not. The entries indicate the means ± standard deviations. The number of plants from which data were collected is indicated in parentheses.

<sup>c</sup> Entries indicate the means from at least four nodules each. Bacteria were counted as CFU (on TY agar) from crushed nodules after surface sterilization.

<sup>d</sup> Only two of six tested nodules yielded CFU.

inosine or 0.1 mM adenine (Fig. 1B and C) was added to the root medium, although the nodules were not as large as those formed in the presence of AICA riboside. Twenty-six days after inoculation, no bacteria were recovered from sampled nodules elicited by the auxotrophs in the presence of inosine. In the presence of adenine, no bacteria were recovered from four of six nodules tested in this manner, but about 100 bacteria were recovered from each of the other two nodules. One week later, up to 1,000 bacteria could be recovered from nodules elicited by the mutant in the presence of adenine or inosine. However, no infection threads were observed by light microscopic examination at this time. No nodules were formed in the presence of AICA riboside or purines on uninoculated plants.

Examination of nodulation by the *R. fredii* purine auxotrophs revealed that soybean roots inoculated with RfK1105 or RfK1107 exhibited either very small bumps (Fig. 3A) or no discernible nodulation at all, confirming previously published results (18). Bacteria could not be recovered from these pseudonodules by crushing after surface sterilization, and none were observed during light microscopic examination.

Adding AICA riboside to the root medium at 0.1 mM had no visible effect on nodulation by either strain, but 0.5 mM AICA riboside provided obvious enhancement, both in appearance (Fig. 3B) and in number (Table 5). Inosine had no effect at 0.1 mM and very little effect at 0.5 mM. Nodules elicited by the mutants in the presence of 0.5 mM AICA riboside lacked detectable nitrogenase activity but contained 10<sup>5</sup> to 10<sup>6</sup> bacteria, which were still purine auxotrophs. Nitrogen-fixing nodules elicited by wild-type HH303 contained between 10<sup>7</sup> and 10<sup>8</sup> bacteria. In control experiments, there was no indication of nodulation when uninoculated plants were supplemented with 0.5 mM AICA riboside or inosine.

Light microscopic examination of mature nodules elicited by RfK1105 in the presence of AICA riboside (Fig. 4C) revealed an anatomy similar to that observed in nodules elicited by the wild type (Fig. 4E). Several vascular bundles (double arrowheads in Fig. 4) and a layer of thick-walled sclerenchyma cells (19) were apparent near the periphery of the nodule. The large central region contained both vacuolate cells and cells filled with what appeared to be vesicles (Fig. 4D), as found previ-



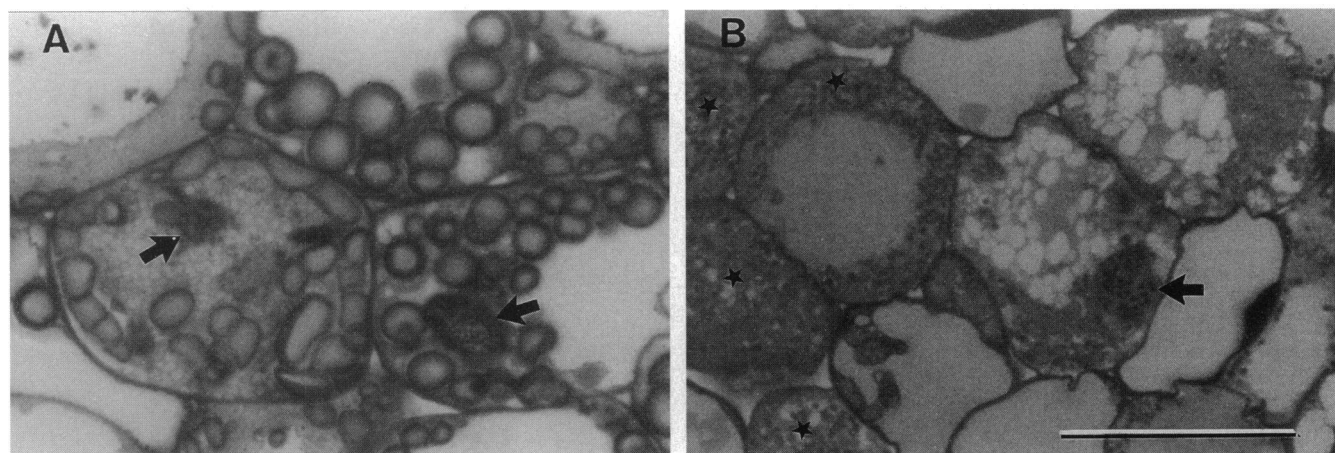


FIG. 2. Infection threads in pea nodules. Infection zone of nodules 30 days after inoculation with RL106 in the presence of AICA riboside (A) or 128C56 (B). The arrows point to infection threads, and stars indicate infected cells. Bar, 30  $\mu$ m.

ously in bean nodules elicited by purine auxotrophs in the presence of AICA riboside (21). Unlike in nodules elicited by the wild type (Fig. 4F), bacteroid-filled cells were not present, although a few bacteroids were visible in some cells.

Since a substantial number of bacteria could be recovered from nodules elicited by the mutant in the presence of AICA riboside, emerging nodules (11 days postinoculation) were examined for the presence of infection threads. Infection

threads (Fig. 5A, arrows) were apparent within a region of densely cytoplasmic cells.

## DISCUSSION

The singular ability of AICA riboside to bring about infection by purine auxotrophs and to enhance the nodule development elicited by these mutants has now been observed in three host-bacterial combinations that represent both determinate (bean and soybean) and indeterminate (pea) nodule development. As argued previously (23), it seems unlikely, even in the bean-*R. etli* symbiosis, that AICA riboside acts merely to restore growth of the auxotrophs. One part of the argument is that supplementation with purines does not have the same symbiotic effect. Furthermore, *R. etli* purine auxotrophs grow as well as the wild-type bacteria in the presence of bean root exudate and in the medium surrounding growing bean roots (23). Recent chromatographic analysis has shown that hypoxanthine, a good purine source for these strains, is abundant in bean root exudate (20). It has been reported that pea root exudate also stimulates the growth of rhizobial purine auxotrophs (27). Therefore, at least at the beginning of infection, purine auxotrophy should not limit growth of the bacteria. In line with this reasoning, it is particularly significant that AICA riboside had its effect on symbiosis in this study, despite supporting almost no growth of these particular auxotrophs ex planta.

While purine auxotrophs derived from *R. etli* CFN42 (23) and *R. meliloti* (33) efficiently utilize AICA riboside as a purine source, the inability to utilize it is not limited to *R. fredii* HH303 and *R. leguminosarum* 128C56. AICA riboside did not restore growth to *Pur*<sup>-</sup> mutants of *Salmonella typhimurium* LT2 when tested in this laboratory. Moreover, *E. coli* K-12 and *S. typhimurium* LT2 are even less capable of transporting AICA riboside than *R. leguminosarum* 128C56 and *R. fredii* HH303 (20).

The low  $V_{\max}$  values for AICA riboside uptake by prototrophic *R. leguminosarum* 128C56 and *R. fredii* HH303 strains relative to the  $V_{\max}$  of *R. etli* CE3 offer a possible explanation for the poor growth of purine auxotrophic derivatives of these strains with AICA riboside as the sole purine source. Even with the relatively high rate of uptake supported by the CE3 genetic background, CE106 did not grow as well with AICA riboside as it did with adenine. Therefore, it is not surprising that the

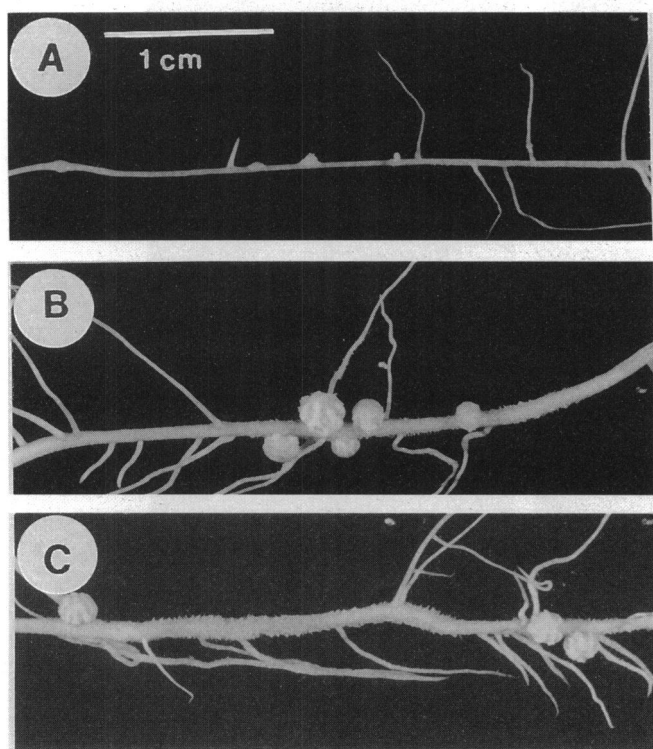


FIG. 3. Nodulation of soybeans by an *R. fredii* purine auxotroph in the presence of AICA riboside. Roots were photographed 3 weeks after inoculation with purine auxotroph RfK1107 (A and B) or wild-type strain HH303 (C). AICA riboside (0.5 mM) was added to the growth medium of the plant shown in panel B.

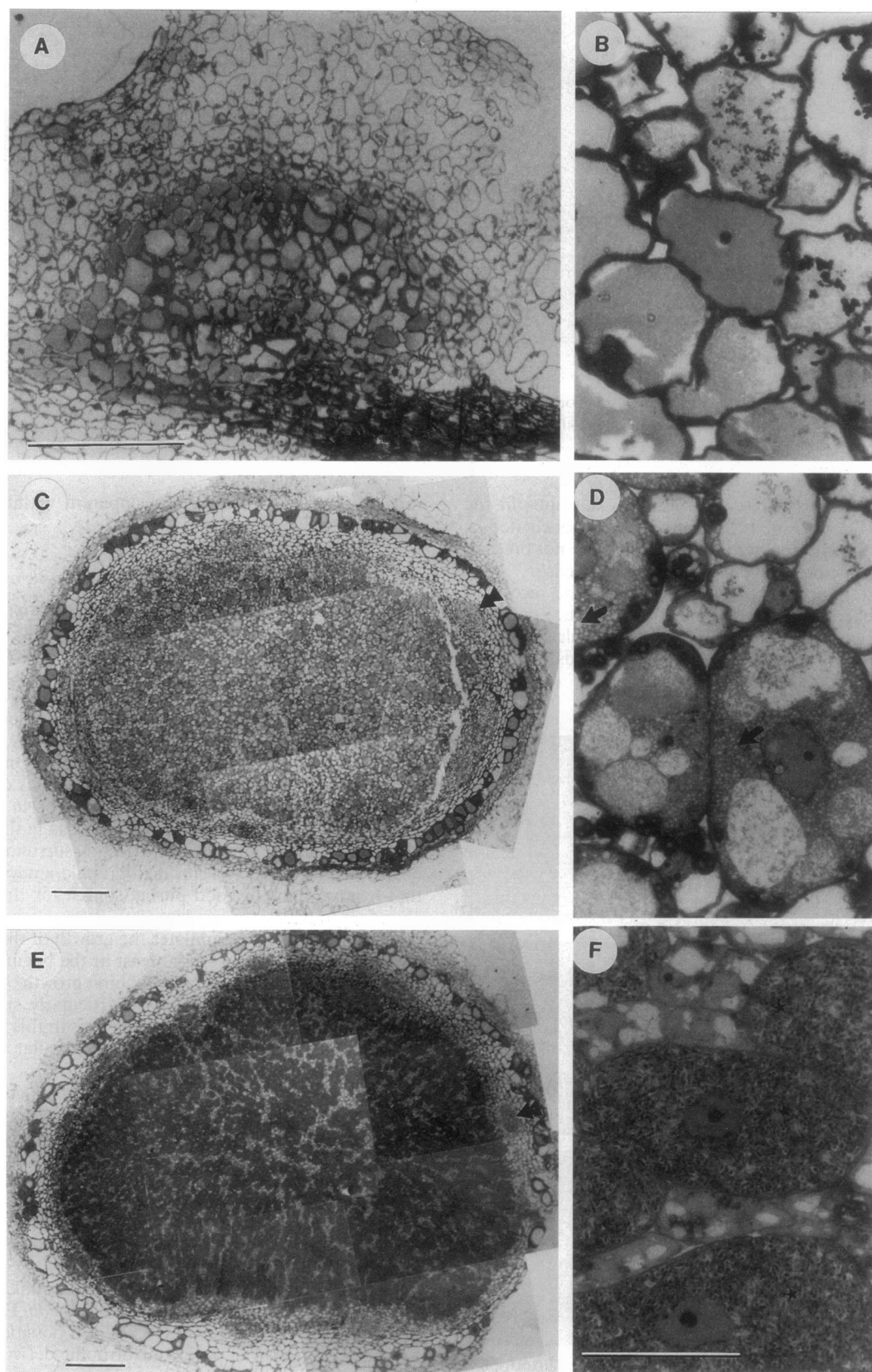


FIG. 4. Morphology of mature soybean nodules. Soybean nodules 3 weeks after inoculation with mutant RfK1105 in the absence (A and B) or presence (C and D) of 0.5 mM AICA riboside or after inoculation with wild-type HH303 (E and F). The double arrowheads in panels C and E point to vascular bundles, the arrows in panel D point to vesicles, and the stars in panel F point to infected cells. Bars A, C, and E, 200  $\mu$ m; bar F, 30  $\mu$ m (applies to B, D, and F).



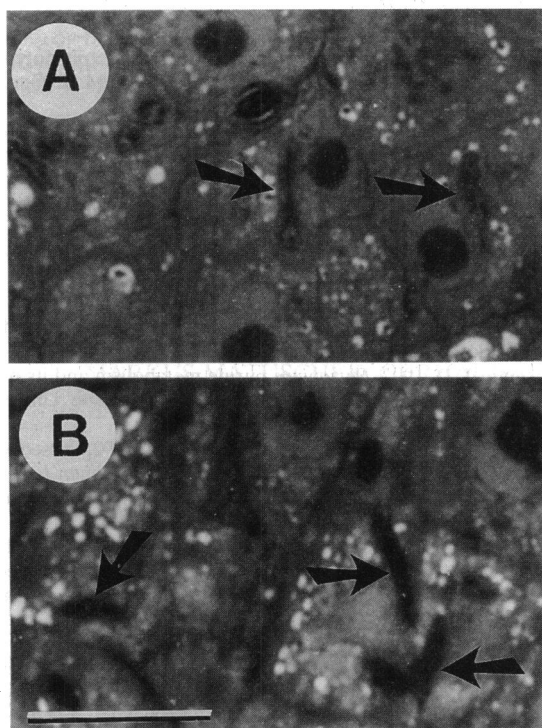


FIG. 5. Infection thread formation in soybeans. Emerging nodules elicited by mutant RfK1105 in the presence of AICA riboside (A) or by wild-type strain HH303 (B) 11 days after inoculation. The arrows point to infection threads. Bar, 10  $\mu$ m.

limitation in AICA riboside transport would curtail growth by *R. fredii* HH303 and *R. leguminosarum* 128C56 purine auxotrophs.

Also significant is the finding that after 10 min, only 12% of the AICA riboside that was taken up by strain HH303 had been converted into nucleic acids. Since this strain is a prototroph, the obstruction cannot lie between AICAR and purine nucleotides, suggesting that *R. fredii* also is deficient in synthesizing AICAR from AICA riboside.

AICA riboside was required at a higher concentration to promote infection of soybeans by *R. fredii* auxotrophs (0.5 mM) than that required on beans by *R. etli* auxotrophs (0.1 mM). This difference may reflect the decreased capacity of *R. fredii* HH303 for AICA riboside uptake and the inferred inefficiency in conversion of AICA riboside to AICAR. The concentration of AICA riboside that is added to the root medium is very much higher than the  $K_m$  for uptake. Therefore, it might seem paradoxical that raising the AICA riboside concentration should have any effect. However, it is likely that AICA riboside diffusion is restricted within the root tissue. If, as in the *R. etli*-bean symbiosis, AICA riboside is required throughout most of the infection thread development (21), then the AICA riboside concentration available to bacteria deep within the narrow and crowded infection thread may be lower than the  $K_m$  for uptake. During infection of beans by *R. etli* purine auxotrophs, an exogenous AICA riboside concentration of 0.1 mM appears to be sufficient for maximal effect, but at 10-fold lower concentrations even partial enhancement of development is not observed (20). Given that the rate of uptake by *R. fredii* is about 10-fold lower at any concentration of AICA riboside, the higher exogenous threshold concentration required for the effect seems reasonable, particularly since different hosts are involved.

In comparing the symbiotic phenotypes of various purine auxotrophs, the different developmental programs of different hosts should be considered. For example, *R. etli* bv. phaseoli and *R. fredii* purine auxotrophs have very similar symbiotic phenotypes on bean (21) and soybean plants, respectively, both of which form determinate nodules. While nodule development can be initiated, the deficiency in infection leads to the formation of pseudonodules, structures lacking anatomical features characteristic of nodules. On soybeans, they exhibit very limited and disorganized development; on beans, they have centrally located vasculature and other features reminiscent of lateral roots and, indeed, lateral roots often develop from their tips (35). Supplementation with AICA riboside, but not purines, restores infection and leads to the formation of a true root nodule structure, which has the peripheral vasculature typical of nodules and what appear to be two distinct cell types in the central region of the nodule (Fig. 4D).

On beans, diversion from pseudonodule to nodule morphology requires that AICA riboside be available until 2 days before release of rhizobia from infection threads into infected cells would begin, i.e., during most of the period of infection thread development (21). After this point, AICA riboside need not be supplied exogenously to achieve the full effect. It seems that some event occurs at this point to commit cells generated by meristematic activity to a nodule-specific developmental program rather than that of a pseudonodule or lateral root (21). The results of the present study corroborate the idea that a certain level of infection is required to trigger true determinate nodule morphogenesis. It would be interesting to repeat these studies with siratro and lotus, two other determinate nodule producers.

Infection may not play the same role in development of indeterminate nodules, if alfalfa serves as a representative model. Application of the nodulation signal, NodRm-1, to uninoculated alfalfa roots elicits what has been interpreted as true nodule organogenesis (34). It may be that adenine and inosine supplementation of the auxotrophs enhanced the growth of pea nodule tissue in this study by promoting more growth of the bacteria in the rhizosphere than that provided by exudate alone and thereby fostered a higher concentration of the Nod factor. The effect of AICA riboside evidently occurred by another mechanism; as a very poor growth substrate, it should not have augmented the rhizosphere population. On the other hand, whereas infection threads were not detected after the auxotrophs were supplemented with purines, AICA riboside led to easily detected infection threads and much greater bacterial populations within the nodules. Even in this indeterminate symbiosis, the unique effect of AICA riboside was to promote infection.

Since mutants that cannot use AICA riboside as a purine source nevertheless are restored to infection by its addition, it is possible that AICA riboside is acting directly upon the plant. However, this compound does not promote infection of beans by an *R. etli* purine auxotroph that, because of a second mutation, is unable to convert AICA riboside to AICAR (20). This evidence suggests that AICA riboside first must be converted to AICAR by the bacteria in order to exert its effect on the symbiosis. It also argues against the possibility that AICA riboside exerts its effect by being converted by bean plants into a purine that is utilized for growth by the auxotrophs. Still, one cannot formally exclude that possibility in the pea and soybean symbioses until the same kind of experiment can be performed with these systems.

In summary, AICA riboside promotes infection by *Rhizobium* purine auxotrophs on three legume hosts, including plants with either determinate or indeterminate nodule devel-

opment. In each of these cases, infection does not proceed beyond a certain extent of infection thread development; and there are no or very few bacteroids. Restoration of infection to this point does not depend on whether the bacteria can utilize AICA riboside efficiently for growth. All that has been observed with these symbioses is consistent with the hypothesis that a factor needed specifically for infection thread development is derived from AICAR by a pathway that deviates from purine biosynthesis.

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