MPMI Vol. 8, No. 3, 1995, pp. 407–414.

Requirement for Rhizobial Production of 5-Aminoimidazole-4-Carboxamide Ribonucleotide (AICAR) for Infection of Bean

Jeffrey D. Newman, Mary Jo Rosovitz, and K. Dale Noel

Department of Biology, Marquette University, Milwaukee, Wisconsin 53233 U.S.A.
Received 27 October 1994. Accepted 22 February 1995.

Exogenous application of the purine precursor 5-aminoimidazole-4-carboxamide (AICA) riboside restores infection and enhances the development of bean (*Phaseolus vulgaris*) root nodules elicited by *Rhizobium etli* purine auxotrophs. The uniqueness of AICA riboside as an effector was shown by testing varying concentrations of AICA riboside and purines for this effect, and by examining several mutants defective in various pathways of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) synthesis. The maximum effect on nodule development was achieved at 0.1 mM AICA riboside. The purines adenine, adenosine, or hypoxanthine did not enhance nodule development at any concentration, but at very high concentrations (1.0 mM), inosine did promote infection. Studies with a double mutant indicated that the histidine biosynthetic pathway (in which AICAR is a byproduct) was required for the effect of inosine, but not for the effect of AICA riboside. A mutant resistant to pyrazofurin, a toxic AICA riboside analog, was isolated and found to be defective in conversion of AICA riboside to AICAR. Transfer of a *purF*::Tn*5* mutation into this strain yielded a purine auxotroph that did not grow with AICA riboside as a purine source. AICA riboside failed to promote infection by this double mutant, indicating that AICA riboside does not act directly upon the plant, but rather must be converted to AICAR by rhizobia in order to be effective. In summary, these results indicate that infection of bean by *R. etli* requires rhizobial production of AICAR, whether via purine biosynthesis, histidine biosynthesis, or conversion from AICA riboside.

Additional keywords: nucleoside transport, purine salvage.

The interaction between rhizobia and legumes to form nitrogen-fixing root nodules involves several communication steps to coordinate gene expression and development between the two organisms. The initial exchange of signals involves the activation of rhizobial *nod* gene expression by plant-produced flavonoid compounds (Peters et al. 1986; Rossen et al. 1987; Zaat et al. 1989; Hungria et al. 1991). The *nod* gene products are responsible for the production of host-specific lipooligosaccharide signal molecules that cause root hair curling and nodule meristem initiation (Lerouge et al. 1990; Truchet et al. 1991; Spaink et al. 1991). Root hair curling entraps rhizobia (Bauer 1981) and is often followed by a localized degradation of the root hair cell wall and deposition of new cell wall material and membrane. This process initiates the formation of a narrow tubular structure known as an infection thread, in which the bacterial colony grows inwardly, apparently against the turgor pressure of the host. The infection thread branches and extends into the root cortex, transporting the dividing bacteria to the nodule meristem (Newcomb et al. 1979; Bauer 1981; Calvert et al. 1984; Turgeon and Bauer 1985). Unlike the bacterial signals specified by *nod* genes, signal molecules that may be required to initiate and maintain infection thread development are poorly understood. Studies with rhizobial mutants defective in exopolysaccharide (EPS) or lipopolysaccharide (LPS) indicate that these polysaccharides are important for infection of various legumes (Noel 1992; Leigh and Walker 1994; Perotto et al. 1994). The phenotypes of various *Rhizobium* auxotrophs have also provided interesting clues to the physiology of nodule development. One intriguing type of finding is that in some biosynthetic pathways, intermediates, rather than endproducts, may be required for the bacteria to elicit nodule development or infection. Studies with *R. meliloti* tryptophan auxotrophs suggest that anthranilate synthesis is important for bacteroid development in this symbiosis (Barsomian et al. 1992). *Bradyrhizobium japonicum* tryptophan auxotrophs blocked before the production of indole glycerol phosphate (IGP) are symbiotically defective, while those defective in tryptophan synthetase form normal nitrogen-fixing root nodules (Wells and Kuykendall 1983). This observation suggests that rhizobial production of IGP is important for soybean nodule development. It also implies that the reason tryptophan auxotrophs blocked earlier in the pathway are symbiotically defective is not because they are unable to produce tryptophan, but rather because they are unable to synthesize IGP. These studies set a precedent for the hypothesis, described below, that purine auxotrophs fail to elicit infection threads due to a deficiency in the production of the purine precursor 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR).

The symbiotic defect of *Rhizobium* purine auxotrophs has been observed in numerous laboratories using rhizobia with

Corresponding author: J. D. Newman; E-mail: newman@lycoming.edu

Present Address of M. J. Rosovitz: Department of Bacteriology, University of Wisconsin, Madison, WI 53706 U.S.A.

different host ranges. With the exception of the *R. meliloti*–alfalfa symbiosis (Dickstein et al. 1991; Kerppola and Kahn 1988; Scherrer and Denarie 1971; Swamynathan and Singh 1992), purine auxotrophy appears specifically to affect the infection process (Djordjevic et al. 1988; Kim et al. 1988; Newman et al. 1994; Noel et al. 1988; Pain 1979; Pankhurst and Schwinghamer 1974). The failure of purine auxotrophs to infect does not seem due to a requirement for purines because the mutants grow in the presence of root exudates from at least pea and bean (Pankhurst and Schwinghamer 1974; Noel et al. 1988) and exogenous addition of purines does not correct the symbiotic defect (Djordjevic et al. 1988; Kim et al. 1988; Newman et al. 1994; Noel et al. 1988; Pain 1979). However, while purines are ineffective, the riboside of the purine biosynthetic intermediate AICAR does restore infection by purine auxotrophs on bean, pea, and soybean (Noel et al. 1988; Newman et al. 1992; Newman et al. 1994).

On *Phaseolus vulgaris* bean plants, *R. etli* purine auxotrophs cause root hair curling and nodule meristem initiation but do not induce infection thread formation. In the absence of infection, the meristem develops into a pseudonodule whose anatomy resembles a lateral root rather than a true nodule (Ndv^- phenotype) (VandenBosch et al. 1985; Newman et al. 1992). Restoration of infection by AICA riboside enhances nodule development such that a true root nodule structure forms (Ndv^+ phenotype). These root nodules are the same size as those induced by the wild type and have typical nodule features such as peripheral vascular bundles, leghemoglobin, and a central zone containing two cell types, perhaps corresponding to the normally infected and uninfected cell types. Mutant bacteria are observed in infection threads; however, there is no evidence of bacterial release into plant cells to become bacteroids, and these nodules do not fix nitrogen. It has been argued that the effect of AICA riboside on nodule development arises because it promotes infection thread development to a stage at which development becomes committed to a nodule-type differentiation (Newman et al. 1992).

Our working hypothesis is that the inability to synthesize AICAR, rather than a lack of purine nucleotide synthesis, causes the symbiotic defect in the purine auxotrophs we have studied. In previous studies, purine supplements had been tested at only one concentration. To challenge the hypothetical uniqueness of AICA riboside, varying concentrations of AICA riboside or purines were analyzed for the ability to enhance nodule development. In addition, a mutant was sought to determine if AICA riboside acted directly on the plant or if rhizobial metabolism was required for the effect of AICA riboside.

RESULTS

Concentration of supplements required to restore Ndv^+ phenotype.

In earlier work (Noel et al. 1988), AICA riboside and purines had been tested only at 0.1 mM for the ability to enhance the development of bean nodules elicited by *R. etli* purine auxotrophs such as CE106 (Table 1). Therefore, the lowest effective concentration of AICA riboside was determined and purines were tested at concentrations higher than those used previously (Table 2). For these experiments, enhanced nodule development was defined by overall size approximately matching that of nodules induced by the wild type and by the characteristic surface ridges of parenchyma cells (Baird and Webster 1982) that form during the culmination of the development of mature determinate nodules (Fig. 1). Earlier microscopic analysis has correlated these superficial features with differentiation resembling true root nodule morphology, including peripheral vasculature, infection threads filled with bacteria, and distinct tissue zones characteristic of nodules, whereas the superficially distinct pseudonodule structure that occurs in the absence of AICA riboside (Fig. 1) lacks infection threads, exhibits central vasculature, and otherwise is filled with vacuolate parenchyma cells (Newman et al. 1992). At the highest concentrations (5.0 mM, data not shown), each of the tested compounds inhibited root development. When the root medium was supplemented with AICA riboside, a concentration of 0.1 mM was required to achieve the full effect on nodule development. Increasing the concentration of AICA riboside to 1.0 mM had no additional visible effect and did not increase the number of CFU that could be recovered from the nodules (10^5 to 10^6 CFU/nodule). The presence of 50 μM AICA riboside enhanced the development of some nodules, but many pseudonodules were still present. At concentrations of AICA riboside below 50 μM, no enhancement of nodulation was observed (data not shown).

The development of nodules elicited by CE106 was not enhanced by hypoxanthine, adenine, adenosine, or AICA at any concentration, and no CFU could be recovered from these nodules after surface sterilization. At concentrations that did not inhibit root development, these compounds had no effect

Table 1. Bacterial strains and plasmids used in this study

Strain	Relevant characteristics[a]	Reference
Rhizobium etli[b]		
CE3	Str^r, derivative of wild isolate CFN42	Noel et al. 1984
CE8	Ery^r, derivative of wild isolate CFN42	Noel et al. 1984
CE106	*purF106*::Tn*5*; Str^r, Km^r, Pur^-	Noel et al. 1984
CE230	*his-2*; Ery^r, His^-	Noel et al. 1988
CE263	*his-2*, *purF106*::Tn*5*; Ery^r, Km^r, His^-, Pur^-	Noel et al. 1988
CE362	*aru-1*; Str^r, Pzf^r, Aru^-	This work
CE363	*purF106*::Tn*5*, *aru-1*; Str^r, Km^r, Pzf^r, Pur^-, Aru^-	This work
Plasmids		
pJB3	Tc^r, R68.45 derivative	Brewin et al. 1980

[a] Str = streptomycin, Ery = erythromycin, Km = kanamycin, Pzf = pyrazofurin, Aru = AICA riboside utilization, Tc =tetracycline.
[b] Formerly known as *Rhizobium leguminosarum* biovar phaseoli (Segovia et al. 1993).

on nodulation by the wild-type strain CE3. Inosine enhanced nodule development induced by purine auxotrophs in a concentration-dependent manner similar to that observed with AICA riboside, although 10-fold higher concentrations were required (Table 2). The nodules formed in the presence of 1.0 mM inosine contained 10^5 to 10^6 CFU per nodule and were indistinguishable visually from those formed when 0.1 mM AICA riboside was used as the supplement. Like the AICA riboside-enhanced nodules, nodules enhanced by inosine emerged from the root at approximately the same time, in the same locations, and became the same size as nodules induced by the wild type, had the characteristic surface ridges of mature nodules, and did not fix nitrogen.

Table 2. Nodulation phenotype of CE106 in the presence of AICA riboside or purine supplements[a]

	Concentration of supplements in root medium			
Supplement	**50 μM**	**100 μM**	**0.5 mM**	**1.0 mM**
AICA		–		Toxic
AICA riboside	+/–	+		+
Hypoxanthine		–		–
Inosine		–	+/–	+
Adenine		–		–
Adenosine		–		Toxic

[a] Entries: – = no nodules had enhanced development; +/– = fewer than half of the nodules showed enhanced development; + = most nodules showed enhanced development; toxic = supplement inhibited root development.

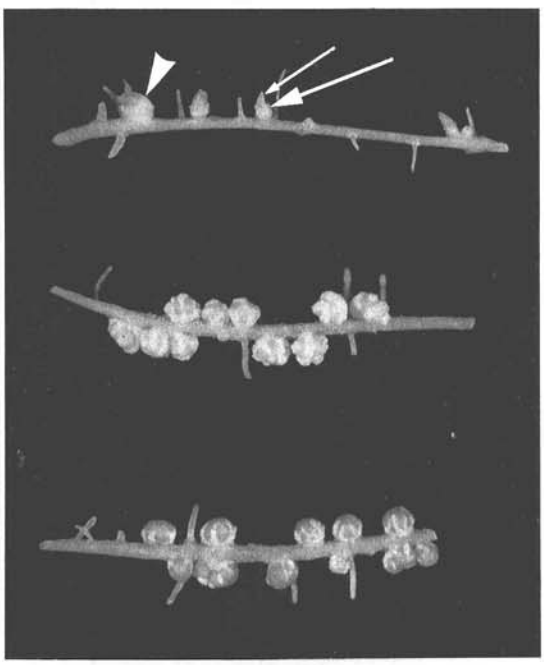


Fig. 1. Enhanced nodule development elicited by a purine auxotroph in the presence of AICA riboside. Comparably located root segments were excised from three plants 22 days after inoculation with *Rhizobium etli* purine auxotroph CE106 (top and middle segments) or wild-type strain CE3 (bottom segment). AICA riboside (0.1 mM) was added to the growth medium of the plant represented by the middle segment. Short lateral roots that eventually had emerged from the pseudonodules of the top root segment were trimmed for the photograph. The emergent lateral root portion (small arrow) is indicated above one pseudonodule (large arrow). What appears to be a large pseudonodule (arrowhead) is a fusion of two pseudonodules. The straightened length of the middle root segment was 2.0 cm.

Histidine biosynthetic requirement for inosine to enhance nodule development.

The ability of inosine to enhance nodule development suggested that AICAR synthesis might not be essential for infection. However, the requirement for such a high concentration was puzzling, since CE106 grows slightly better ex planta with inosine than with AICA riboside (Table 3) and the growth limiting concentrations of each compound are very similar (J. D. Newman, unpublished data). We speculated that perhaps the high concentration of inosine acted indirectly by stimulating AICAR production via an alternative pathway. This could occur if the high concentration of inosine increased IMP levels to some level at which bulk flow would cause AICAR to be synthesized by the reversal of the two steps between these compounds (Fig. 2). Alternatively, the high concentration of inosine could somehow stimulate flow through the histidine biosynthetic pathway, in which AICAR is produced as a byproduct.

R. etli histidine auxotroph CE230 did not accumulate AICAR, as measured by the Bratton–Marshall assay (Gots and Gollub 1957), suggesting that it was blocked early in the histidine pathway. In agreement with earlier results (Noel et al. 1988), this strain induced Fix$^+$ nodules. This result implies that there is sufficient histidine in the root environment for the mutant to grow and that histidine auxotrophy does not cause a symbiotic defect.

Table 3. Relative growth of purine auxotrophs in liquid minimal medium with or without supplements[a]

	Strain	
Supplement	**CE106**	**CE363**
None	1	1
0.1 mM AICA riboside	40.4	1.11
1.0 mM AICA riboside	45.7	1.02
0.1 mM inosine	68.1	45.5
0.1 mM adenine	86.2	114

[a] Entries indicate the CFU 18 h after inoculation relative to the CFU in minimal Y medium alone in one representative experiment.

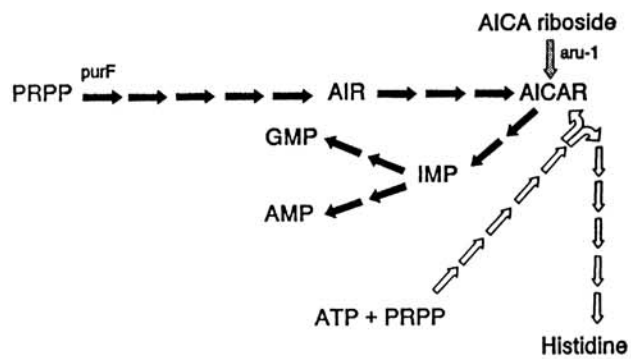


Fig. 2. Routes of AICAR biosynthesis. Filled arrows represent steps of purine biosynthesis, open arrows indicate steps of histidine biosynthesis, shaded arrow corresponds to the AICA riboside salvage pathway. PurF designates the step blocked by the *purF*::Tn*5* mutation in strains CE106 and CE363. Aru-1 indicates the blocked conversion of exogenous AICA riboside into intracellular AICAR in mutants CE362 and CE363. Abbreviations: PRPP, 5-phosphoribosyl-1-pyrophosphate; AIR, 5-aminoimidazole ribonucleotide; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; IMP, inosine monophosphate; GMP, guanosine monophosphate; AMP, adenosine monophosphate.

In earlier work (Noel et al. 1988), a strain carrying the his-2 mutation of CE230 and the CE106 *purF*::Tn*5* mutation was constructed. This His⁻ Pur⁻ double mutant, CE263, should be unable to synthesize AICAR from either the purine or histidine biosynthetic pathways. Like other purine auxotrophs, CE263 elicited only pseudonodules in the absence of supplementation. While AICA riboside at 0.1 mM still enhanced the development of nodules elicited by this mutant, 1.0 mM inosine was not effective. Thus, the ability of inosine, but not AICA riboside, to correct the Ndv⁻ phenotype is dependent on a functional histidine biosynthetic pathway.

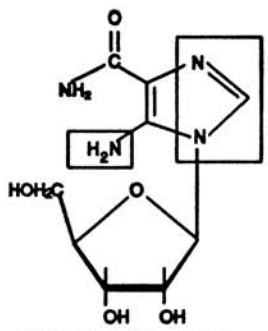
Isolation and characterization of a mutant defective in conversion of AICA riboside to AICAR.

The foregoing studies had suggested that, unless the bacteria could produce AICAR, AICA riboside supplementation was required to achieve infection and nodule development. A key question was whether AICA riboside acted directly on the plant.

With the goal of answering this question by isolating *R. etli* mutants defective in AICA riboside uptake or metabolism, two structural analogs of AICA riboside (Worzalla and Sweeney 1980) were tested for toxicity. Ribavirin (Viratek, Costa Mesa, Calif.) did not inhibit growth on minimal agar when 100 μg was applied to a disk. However, pyrazofurin (Fig. 3) was a potent inhibitor of *R. etli* CE3. In agar disk assays, 50 μg produced a zone of inhibition 5.4 cm in diameter, and in liquid minimal medium, 0.2 mM pyrazofurin resulted in only 2% of normal growth (in CFU) after 24 h of incubation. Incorporation of AICA riboside, inosine, and adenosine into the media at 0.1 mM decreased the diameter of the zone of inhibition by 59, 75, and 57%, respectively, whereas AICA and all other purines and purine nucleosides were ineffective as antagonists. Pyrazofurin inhibited AICA riboside uptake, exhibiting classically competitive kinetics, with a K_i of 7.9 μM.

A spontaneous mutant derivative of CE3 resistant to pyrazofurin was isolated and tested for deficiencies in AICA riboside transport and metabolism. While this mutant strain, CE362, did take up AICA riboside, the amount taken up reached a plateau after the first minute (Fig. 4). In contrast, CE3 continued to take up AICA riboside at a high rate even after 10 min. The apparent K_m and V_{max} values for AICA riboside uptake were 0.53 μM and 25 pmoles min^{-1} (10^7 CFU)-1 for CE3 and 0.06 μM and 4.2 pmoles min^{-1} (10^7 CFU)$^{-1}$ for CE362. The measured AICA riboside uptake by CE362 does not appear to be caused by nonspecific binding of AICA riboside to the bacteria because even this low rate is 10- to 50-fold higher than that observed with *E. coli* (J. D. Newman, unpublished data). Moreover, this level of uptake is similar to that measured with *R. fredii* and *R. leguminosarum* bv. *viciae* (Newman et al. 1994), which must be capable of AICA riboside transport because they grow with it as a purine source, although very poorly. In separate experiments, it was found that after 10 min, strain CE3 had incorporated 59% of the tritium from the total uptake of ^{3}H-AICA riboside into acid-precipitable material (presumably nucleic acids). In strain CE362, on the other hand, the tritium in acid-precipitable material was not significantly above background (<5% of the total uptake after 10 min), indicating that this mutant was unable to convert AICA riboside into purine nucleotides that would be incorporated into nucleic acid.

The conversion of ^{3}H-AICA riboside into nucleotides was measured by separating acid extracts of these strains by anion-exchange HPLC and correlating the elution of tritiated compounds with the emergence of nucleotide standards added to the extracts (Table 4). The CE3 extracts contained a significant amount of tritium that coeluted with ATP, ADP, purine nucleotide monophosphates, NAD^+, and with the solvent front where bases and nucleosides elute. The entire amount of



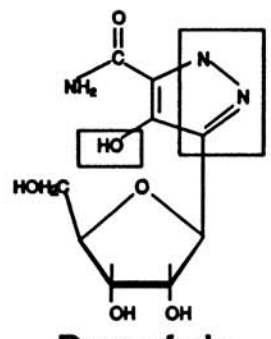


Fig. 3. Structures of AICA riboside and Pyrazofurin. Differences between the two compounds are highlighted by boxes.

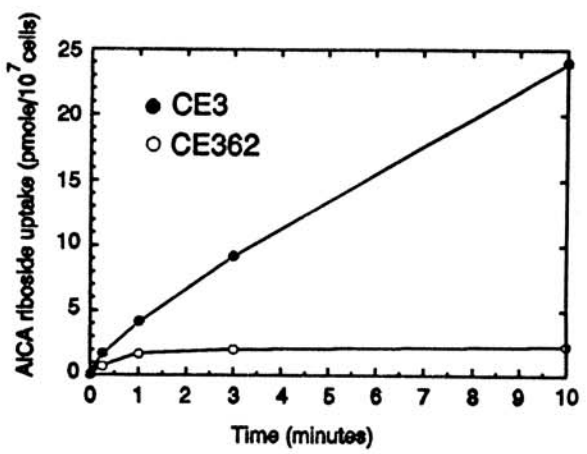


Fig. 4. Uptake of ^{3}H-AICA riboside by CE3 and CE362. AICA riboside uptake was measured after incubation with 0.2 μM ^{3}H-AICA riboside for various lengths of time.

tritium from the CE362 extract eluted with the solvent front, indicating that none of the AICA riboside was converted into nucleotides. Separation of uncharged compounds, such as nucleobases and nucleosides, in the extract by reversed-phase HPLC showed that almost all of the tritium taken up by strain CE362 remained in the form of AICA riboside, with at most 8% as AICA and no other peaks of radioactivity above background. The deficiency of strain CE362 in AICA riboside utilization was designated phenotypically as Aru$^-$, and the responsible mutation was named *aru-1*.

Requirement for conversion of AICA riboside to AICAR for enhancement of nodulation.

Since the mutation of CE362 blocks the conversion of AICA riboside to AICAR, this mutation should prevent the growth of a purine auxotroph supplied with AICA riboside as the sole purine source. To test this prediction, a recombinant strain in which the *purF*$^+$ gene of strain CE362 was replaced with the *purF106*::Tn*5* allele was constructed and designated as strain CE363. The ability of this strain to grow with various purine sources was compared with that of CE106, which contains the *purF106*::Tn*5* allele in an otherwise wild-type genetic background (Table 3). While CE106 grew nearly as well with AICA riboside as with other purine sources, CE363 did not grow at all with AICA riboside as a purine source. Growth of CE363 with adenine or inosine was comparable to that of CE106 (Table 3). In other experiments, CE363 also appeared to grow as well as CE106 on minimal medium plates supplemented with adenosine or hypoxanthine.

The nodulation phenotypes of these strains were examined to determine the effect of the *aru-1* mutation on nodulation and the ability of AICA riboside to promote infection. In the absence of supplementation, CE362 (*aru-1*) formed normal, nitrogen-fixing nodules, as was expected since this mutant is not a purine auxotroph. CE106 (*purF106*) elicited the formation of pseudonodules when the plant was grown in unsupplemented medium and well-developed nodules in the presence of 0.1 mM AICA riboside. CE363 (*purF106 aru-1*) elicited only pseudonodules, even in the presence of 0.1 mM or 1.0 mM AICA riboside, indicating that AICA riboside was unable to promote infection by this mutant.

DISCUSSION

These studies confirmed earlier results (Noel et al. 1988) indicating that adenosine does not promote infection by purine auxotrophs, and extended these observations to demonstrate that adenine and hypoxanthine also are not effective. Even at extremely high concentrations, up to and including those that inhibit root development, adenosine, adenine, and hypoxanthine did not enhance the development of nodules elicited by purine auxotrophs. These observations were not unexpected, because it had been shown previously that the purine auxotrophs grow as well as the wild type in the root environment (Noel et al. 1988). Furthermore, HPLC analysis has shown that hypoxanthine is abundant in root exudate (Newman 1992). The inability of AICA to promote infection also was not surprising, because the wild-type strain from which the auxotroph was derived does not take up AICA, and the mutant cannot use AICA as a purine source (J. D. Newman, unpublished data). Inosine does promote infection, although it is required at a 10-fold higher concentration than AICA riboside.

The ability of histidine auxotroph CE230 to nodulate normally indicates that there is sufficient histidine in the root environment to support the growth of this strain and potentially to repress expression of histidine biosynthetic genes and inhibit flow through the histidine biosynthetic pathway. In auxotrophs unable to synthesize AICAR via the purine pathway, this would result in a negligible amount of AICAR production. Since high concentrations of inosine do not promote infection in CE263, the His$^-$Pur$^-$ double mutant, it appears that a functional histidine pathway is required for inosine to promote infection. The obvious inference is that inosine works indirectly, by stimulating flow through the histidine biosynthetic pathway and thereby stimulating AICAR production. However, the mechanism by which high concentrations of inosine might relieve the suspected inhibition of the histidine pathway is unknown. Moreover, it should be pointed out that AICAR production under these conditions has not been measured directly in planta. It therefore remains to be proven that this is how 1.0 mM inosine promotes infection by purine auxotrophs. Adenosine, adenine, and hypoxanthine apparently do not function in this manner since they do not promote infection, even at high concentrations.

Strain CE362, the pyrazofurin-resistant mutant, was capable of AICA riboside uptake but could not convert AICA riboside to AICAR. Because tritium from ^{3}H-AICA riboside was not detected in the nucleotides or nucleic acids of this strain, it was apparent that the pathway from AICA riboside to nucleotides was blocked. CE362 is not an auxotroph, and therefore could not be blocked at a step between AICAR and AMP or GMP; hence, it was concluded that the defect is in synthesizing AICAR from AICA riboside.

The differences between CE3 and CE362 in the K_m and V_{max} for uptake also might be due entirely to the metabolic block of CE362. AICA riboside taken up by CE3 is rapidly metabolized to phosphorylated compounds that are maintained within the cell. However, because of the restriction in AICA riboside metabolism in CE362, the flux of AICA riboside across the membranes reached equilibrium within 1 min (Fig. 4). This suggests that after only 15 s some of the AICA riboside that had been transported into cell would have been transported back out, leading to an underestimation of the initial rate of transport into the cell.

Based on the defect in AICA riboside utilization, pyrazofurin resistance in CE362 is probably due to a defect in

Table 4. HPLC analysis of ^{3}H-AICA riboside metabolism[a]

Nucleotide	cpm/compound from strain: CE3	CE362
Bases, nucleosides	1762	2892
NAD^+	1124	bkg[b]
AMP, IMP, AICAR[c]	296	bkg
GMP	180	bkg
ADP	331	bkg
ATP	653	bkg

[a] Nucleotides extracted from cells incubated with ^{3}H-AICA riboside were separated by HPLC and the amount of tritium in different nucleotides was measured by scintillation counting of 0.5-ml fractions.
[b] bkg = cpm not significantly above background.
[c] These nucleotides were not sufficiently resolved to assign cpm in fractions to any one compound.

conversion of pyrazofurin to pyrazofurin phosphate, the "nucleotide" form of pyrazofurin. Indeed, in mammalian cells, pyrazofurin phosphate is the active metabolite that inhibits AICAR transformylase and orotidine monophosphate (OMP) decarboxylase (Worzalla and Sweeney, 1980; Sant et al., 1989). To our knowledge, pyrazofurin toxicity and metabolism had not been studied previously in bacteria.

Studies with CE363, the purine auxotroph that cannot convert AICA riboside to AICAR, strongly support the idea that rhizobial AICAR production is essential for infection. The AICA riboside utilization mutation alone has no effect on the symbiosis, since CE362 elicits normal nitrogen-fixing root nodules. However, this mutation does block the ability of AICA riboside to enhance the development of nodules induced by purine auxotrophs, indicating that AICA riboside does not act directly on the plant, but rather must be taken up by the bacteria and converted to AICAR, or possibly some intermediate such as AICA, to be effective.

While caution is imperative in comparing results obtained with different host-symbiont combinations, it is interesting to note that AICA riboside also promotes infection of soybean and pea by purine auxotrophs of *R. fredii* and *R. leguminosarum*, respectively, although higher AICA riboside concentrations are required on soybean (Newman et al. 1994). Unlike *R. etli* auxotrophs, these auxotrophs utilize AICA riboside very poorly as a purine source, allowing only sluggish growth. Thus, assuming that the basis of the effect on symbiosis is the same, it appears that at least the minimal capacity for AICA riboside metabolism that these auxotrophs have, but which CE362 lacks, is necessary and sufficient for AICA riboside to promote infection by purine auxotrophs. This minimal requirement is consistent with a symbiotic role for AICAR separate from its role in purine biosynthesis.

MATERIALS AND METHODS

Bacterial strains and growth media.

Rhizobium strains (Table 1) were grown at 30°C in rich medium (TY) containing tryptone, yeast extract, and calcium chloride, or minimal medium (Y) containing salts, biotin, thiamine, pantothenoic acid, and succinate and glutamate as carbon and nitrogen sources (Noel et al. 1984). TY medium was solidified with 1.5% Bacto Agar, and purified agar (BBL) was used to solidify Y medium. Antibiotics were used at the following concentrations (μg/ml): erythromycin (20), kanamycin (30), nalidixic acid (20), streptomycin (200), tetracycline (5). Purine or AICA riboside supplements were used at 0.1 mM except as noted in the text.

Nodulation assays.

Bean (*Phaseolus vulgaris* cv. Midnight) seeds were obtained from Johnny's Selected Seeds, Albion, Maine. The seeds were surface sterilized, germinated for 2 days, transferred to plastic growth pouches (Vaughn's Seed Company, Downer's Grove, Ill.) containing nitrogen-free RBN plant medium (Wacek and Brill 1976), and inoculated as described previously (Noel et al. 1984). Supplements such as AICA riboside or inosine were added to both the plant medium and watering solutions at the concentrations indicated in the text. A minimum of eight plants were tested for each treatment. Nodules harvested 21 days after inoculation were surface sterilized, crushed, and assayed for viable bacteria as described previously (Noel et al. 1988).

Tests for growth of bacterial auxotrophs.

Growth was tested on agarose, purified agar, or minimal liquid (Y) with the indicated supplements, as described previously (Noel et al. 1988). For quantitative comparison of growth, overnight cultures of each strain grown in TY liquid were washed in sterile 0.1 M $MgSO_4$, and resuspended in Y liquid. Each strain was inoculated at a final dilution of 10^{-4} 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 CFU.

Assay to detect diazotizable amines.

The Bratton–Marshall assay (Gots and Gollub 1957) to detect diazotizable amines was used to determine if *Rhizobium* strain CE230 excreted aminoimidazole compounds when starved for histidine. Overnight cultures grown to full density in TY liquid at 30°C were washed with 0.1 M $MgSO_4$ and resuspended in Y minimal medium to stimulate flow through the histidine pathway up to the blocked step. The cultures were incubated for 2 days at 30°C, the cells were removed by centrifugation, and the culture fluid was analyzed by the Bratton–Marshall assay. *E. coli* His^- mutants were used as controls.

Assays of growth inhibition by pyrazofurin.

Inhibition of agar cultures was measured in two ways. In the first method, 0.3 ml of a culture freshly grown in Y broth was mixed with 3.0 ml of Y top agar (7.5 g/liter purified agar) at 45°C and poured onto Y agar. A sterile paper disk (0.3 cm radius) was placed on the surface of the soft agar overlay and 10 μl of 5 mg/ml pyrazofurin was added to the disk. Zones of inhibition (in which no growth was apparent) were measured after 2 days at 30°C. In tests of reversal of inhibition, the purine supplements were incorporated in the Y agar before the overlay was added. In the second method, the disk with pyrazofurin was placed on Y agar and cells from fresh agar cultures were streaked radially away from the disk with a platinum wire. Both methods gave zones of inhibition of similar size.

For assaying inhibition in liquid, 10^6 bacteria freshly grown in Y broth were diluted into 1 ml of fresh medium containing the specified concentration of pyrazofurin (Calbiochem Co., 99% pure). After 24 h of shaking at 30°C an aliquot was removed for measuring CFU. Inhibited cultures were incubated further to select for resistant mutants.

Isolation of mutant strain CE362.

Strain CE362 was isolated from a liquid culture containing 1 mM pyrazofurin that had been inoculated and incubated as described above. After 3 days, dilutions of the culture were spread on TY agar for single colonies. Purified colonies were tested by disk assay in soft agar and by radial streaking as described above. Some colonies had wild-type or near wild-type sensitivity, but isolate CE362 exhibited no zone of inhibition. In subsequent work, resistant mutants were isolated as survivors of cultures containing 0.1, 0.3, or 1 mM pyrazofurin.

AICA riboside uptake.

Uptake assays were performed as described previously (Newman et al. 1994). The amount of ^{3}H-AICA riboside taken up during the first 15 s of each assay was used to calculate the initial rate, which was plotted as a double reciprocal plot to determine the K_m and V_{max} for AICA riboside uptake under various conditions.

HPLC analysis of ^{3}H-AICA riboside metabolism.

Overnight cultures of CE3 or CE362 grown in Y liquid were diluted fivefold into fresh Y liquid and incubated for 4 h at 30°C. HPLC-purified ^{3}H-AICA riboside (Newman et al. 1994) was added to a final concentration of 2.0 μM and the cultures were incubated for 10 min at 30°C. The cells were vacuum filtered on a 47-mm-diameter 0.45-μm pore GN-6 membrane filter (Gelman), and washed with 10 ml of Y liquid prepared without $FeCl_3$. The filters were placed into a tube containing 5 ml of ice-cold 1 M formic acid for 1.5 h to extract the nucleotides, nucleosides, bases, and other small molecules (Payne and Ames 1982). The filters were removed from the tubes and the formic acid extract was frozen on dry ice and lyophilized overnight. The residues were resuspended in 170 μl of water, insoluble material was removed by centrifugation and the supernatants designated CE3 or CE362 acid extracts. Each acid extract (50 μl) was separated by HPLC on a Whatman Partisil-SAX column. Anion exchange HPLC was performed at a flow rate of 1.0 ml/min with 10 mM KH_2PO_4 as solvent A, and 0.6 M KH_2PO_4 as solvent B. After a 4-min segment with 100% solvent A, curved gradient #7 (Isco) was executed for 36 min, followed by 100% solvent B for 5 min. Fractions of 0.5 ml were collected for scintillation counting.

Construction of CE363.

Conjugative plasmid pJB3 was transferred by mating into CE263, which carries the *purF106*::Tn*5* and *his-2* mutations in an Ery^r, Str^s genetic background. The resulting strain, CE263/pJB3, was mated with CE362, a spontaneous pyrazofurin-resistant derivative of the Str^r strain CE3. The desired transconjugants were then selected on TY agar containing Str, Km, and Nal followed by screening for histidine prototrophs.

ACKNOWLEDGMENTS

This work was supported by grant DCB-8905557 from the National Science Foundation.

LITERATURE CITED

Baird, L. M., and Webster, B. D. 1982. Morphogenesis of effective and ineffective root nodules in *Phaseolus vulgaris* L. Bot. Gaz. 143:41-51.

Barsomian, G. D., Urzainqui, A., Lohman, K., and Walker, G. C. 1992. *Rhizobium meliloti* mutants unable to synthesize anthranilate display a novel symbiotic phenotype. J. Bacteriol. 174:4416-4426.

Bauer, W. D. 1981. Infection of legumes by rhizobia. Annu. Rev. Plant Physiol. 32:407-449.

Brewin, N. J., Beringer, J. E., and Johnston, A. W. B. 1980. Plasmid mediated transfer of host-specificity between two strains of *Rhizobium leguminosarum*. J. Gen. Microbiol. 120:413-420.

Calvert, H. E., Pence, M. K., Pierce, M., Malik, N. S. A., and Bauer, W. D. 1984. Anatomical analysis of the development and distribution of *Rhizobium* infections in soybean roots. Can. J. Bot. 62:2375-2384.

Dickstein, R., Scheirer, D. C., Fowle, W. F., and Ausubel, F. M. 1991. Nodules elicited by *Rhizobium meliloti* heme mutants are arrested at an early stage of development. Mol. Gen. Genet. 230:423-432.

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.

Gots, J. S., and Gollub, E. G. 1957 Sequential blockade in adenine biosynthesis by genetic loss of an apparent bifunctional deacylase. Proc. Natl. Acad. Sci. USA 43:826-834.

Hungria, M., Joseph, C. M., and Phillips, D. A. 1991. *Rhizobium nod* gene inducers exuded naturally from roots of common bean (*Phaseolus vulgaris* L.). Plant Physiol. 97:759-764.

Kerppola, T. K., and Kahn, M. L. 1988. Symbiotic phenotypes of auxotrophic mutants of *Rhizobium meliloti* 104A14. J. Gen. Microbiol. 134:913-919.

Kim, C.-H., Kuykendall, L. D., Shah, K. S., and Keister, D. L. 1988. Induction of symbiotically defective auxotrophic mutants of *Rhizobium fredii* HH303 by transposon mutagenesis. Appl. Environ. Microbiol. 54:423-427.

Leigh, J. A., and Walker, G. C. 1994. Exopolysaccharides of *Rhizobium*: Synthesis, regulation, and symbiotic function. Trends Genet. 10:63-67.

Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Prome, J. C., and Denarie, J. 1990. Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. Nature 344:781-784.

Newcomb, W., D. Sippell, and R. L. Peterson. 1979. The early morphogenesis of *Glycine max* and *Pisum sativum* root nodules. Can. J. Bot. 57:2603-2616.

Newman, J. D. 1992. *Rhizobium* purine metabolism and its role in infection of legumes. Ph.D. thesis. Marquette University, Milwaukee, Wisconsin.

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

Noel, K. D. 1992. Rhizobial polysaccharides required in symbiosis with legumes. Pages 341-357 in: Molecular Signals in Plant-Microbe Communications. D. P. S. Verma, ed. CRC Press, Boca Raton, Florida.

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.

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.

Payne, S. M., and Ames, B. N. 1982. A procedure for rapid extraction and high pressure liquid chromatographic separation of the nucleotides and other small molecules from bacterial cells. Anal. Biochem. 123:151-161.

Perotto, S., Brewin, N. J., and Kannenberg, E. L. 1994. Cytological evidence for a host defense response that reduces cell and tissue invasion in pea nodules by lipopolysaccharide-defective mutants of *Rhizobium leguminosarum* strain 3841. Mol. Plant-Microbe Interact. 7:99-112.

Peters, N. K., Frost, J. W., and Long, S. R. 1986. A plant flavone, luteolin, induces expression of *Rhizobium meliloti* nodulation genes. Science 233:977-980.

Rossen, L., Davis, E. O., and Johnston, A. W. B. 1987. Plant-induced expression of *Rhizobium* genes involved in host specificity and early stages of nodulation. Trends Biochem. Sci. 12:430-434.

Sant, M. E., Lyons, S. D., Kemp, A. J., McClure, L. K., Szabados, E., and Christopherson, R. I. 1989. Dual effects of pyrazofurin and 3-

deazauridine upon pyrimidine and purine biosynthesis in mouse L1210 leukemia. Cancer Res. 49:2645-2650.

Scherrer, A., and Denarie, J. 1971. Symbiotic properties of some auxotrophic mutants of *Rhizobium meliloti* and of their prototrophic revertants. Plant Soil (Special Vol.) Special:39-45.

Segovia, L., Young, J. P. W., and Martinez-Romero, E. 1993. Reclassification of American *Rhizobium leguminosarum* biovar phaseoli type I strains as *Rhizobium etli sp. nov.* Int. J. Syst. Bacteriol. 43:374-377

Spaink, H. P., Sheeley, D. M., van Brussel, A. A. N., Glushka, J., York, W. S., Tak, T., Geiger, O., Kennedy, E. P., Reinhold, V. N., and Lugtenberg, B. J. J. 1991. A novel highly unsaturated fatty acid moiety of lipooligosaccharide signals determines host-specificity of *Rhizobium*. Nature 354:125-130.

Swamynathan, S. K., and Singh, A. 1992. *Rhizobium meliloti* purine auxotrophs are nod$^+$ but defective in nitrogen fixation. J. Genet. 71:11-21.

Truchet, G., Roche, P., Lerouge, P., Vasse, J., Camut, S., de Billy, F., Prome, J.-C., and Denarie, J. 1991. Sulphated lipo-oligosaccharide signals of *Rhizobium meliloti* elicit root nodule organogenesis in alfalfa. Nature 351:670-673.

Turgeon, B. G., and Bauer, W. D. 1985. Ultrastructure of infection-thread development during the infection of soybean by *Rhizobium japonicum*. Planta 163:328-349.

VandenBosch, K. A., Noel, K. D., Kaneko, Y., and Newcomb, E. H. 1985. Nodule initiation elicited by noninfective mutants of *Rhizobium phaseoli*. J. Bacteriol. 162:950-959.

Wacek, T. S., and Brill, W. J. 1976. Simple, rapid assay for screening nitrogen-fixing ability in soybean. Crop Sci. 16:519-522.

Wells, S. E., Kuykendall, L. D. 1983. Tryptophan auxotrophs of *Rhizobium japonicum*. J. Bacteriol. 156:1356-1358.

Worzalla, J. F., and Sweeney, M. J. 1980. Pyrazofurin inhibition of purine biosynthesis via 5-aminoimidazole-4-carboxamide-1-B-D-ribofuranosyl 5′-monophosphateformyltransferase. Cancer Res. 40:1482-1485.

Zaat, S. A. J., Schripsema, J., Wijffelman, C. A., van Brussel, A. A. N., and Lugtenberg, B. J. J. 1989. Analysis of the major inducers of the *Rhizobium nodA* promoter from *Vicia sativa* root exudate and their activity with different *nodD* genes. Plant Mol. Biol. 13:175-188.