JOURNAL OF BACTERIOLOGY, Oct. 1989, p. 5458–5466
0021-9193/89/105458-09$02.00/0

Vol. 171, No. 10

Aromatic Aminotransferase Activity and Indoleacetic Acid Production in *Rhizobium meliloti*

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Received 4 April 1989/Accepted 27 June 1989

Bacterial indoleacetic acid (IAA) production, which has been proposed to play a role in the *Rhizobium*-legume symbiosis, is a poorly understood process. Previous data have suggested that IAA biosynthesis in *Rhizobium meliloti* can occur through an indolepyruvate intermediate derived from tryptophan by an aminotransferase activity. To further examine this biosynthetic pathway, the aromatic aminotransferase (AAT) activity of *Rhizobium meliloti* 102F34 (F34) was characterized. At least four proteins were detected on nondenaturing gels of F34 protein extracts that exhibited AAT activity. All four of these AATs were constitutively produced and utilized the aromatic amino acids tryptophan, phenylalanine, and tyrosine as amino substrates. Two AATs were also capable of using aspartate. Plasmids from an F34 gene bank were identified that coded for the synthesis of at least three of these proteins, and the respective gene sequences were localized by transposon mutagenesis. Selected transposon insertions were recombined into the F34 genome to produce strains defective in two of these proteins (AAT1 and AAT2). Characterization of the mutants revealed that neither was essential for the biosynthesis of IAA in the absence of exogenous tryptophan, but that both contributed to IAA biosynthesis when high levels of exogenous tryptophan were present. AAT1 and AAT2 were also not required for the production of a minimal level of aromatic amino acids, but both were able to scavenge nitrogen from the aromatic amino acids during nitrogen deprivation. Neither AAT1 nor AAT2 was essential for symbiosis with alfalfa.

Bacteria in the genus *Rhizobium* reduce atmospheric nitrogen to ammonia when they are located in the root nodules of leguminous plants. The formation of these root nodules is dependent on a complex series of interactions between the bacterium and the plant and requires growth and differentiation on the part of both partners. The plant growth regulator indoleacetic acid (IAA) has long been postulated to play a role in one or more aspects of nodule growth and development (21, 27, 28, 31, 40), and the detection of increased levels of IAA in nodule tissue supports this hypothesis (9, 18, 35, 40). Rhizobia are capable of producing physiologically significant levels of IAA during free-living growth in the absence of exogenous tryptophan (Trp) (2, 11, 44) and could conceivably contribute to the increased IAA accumulation observed in nodules. Recently, Hunter (19) has demonstrated that nodules induced by an IAA-overproducing *Bradyrhizobium japonicum* strain contain a much greater amount of IAA than nodules induced by the parental strain, providing evidence that the bacterium can affect nodule IAA levels.

Understanding the biochemical nature of IAA production in *Rhizobium* spp. is a first step in determining whether bacterially produced IAA is involved in nodule development. Biochemical evidence supports the hypothesis that the first step in IAA production by fast-growing rhizobia is likely to be a transamination of Trp to indolepyruvate (3, 10–12, 14). Indole pyruvate could then either undergo spontaneous degradation to IAA or be specifically decarboxylated to indole acetaldehyde and then converted to IAA. No definitive evidence that the indolepyruvate IAA biosynthetic pathway exists in *Rhizobium* has been obtained, however, and nothing is known about *Rhizobium* tryptophan aminotransferase (TAT), the putative first enzyme in IAA biosynthesis.

Aminotransferases that utilize aromatic amino acids in the presence of a keto acid acceptor have been studied in a variety of other microorganisms (15, 22, 26, 33, 46). It is generally found for a microorganism that there are several proteins capable of aromatic aminotransferase (AAT) activity, with each protein often able to utilize multiple substrate amino acids, including all three aromatic amino acids as well as aspartate (Asp). These AATs, either alone or in combination with other AATs, function in a variety of cellular roles, including biosynthesis of the aromatic amino acids phenylalanine (Phe) and tyrosine (Tyr), catabolism of amino acids to provide nitrogen and/or carbon skeletons under conditions of nitrogen or carbon limitation, and generation of important secondary metabolites (20).

A detailed study of TAT activity in *Rhizobium* spp. is necessary to determine whether such enzymes are involved in IAA biosynthesis and possibly to generate IAA-defective mutants for evaluating the role of bacterially produced IAA in symbiosis. This paper describes a biochemical and molecular characterization of the TATs of *Rhizobium meliloti* 102F34. As in other bacteria, multiple proteins having AAT activity were identified on nondenaturing polyacrylamide gels of crude protein extracts. These proteins were characterized, and the gene sequences for all but one of them were isolated from an *R. meliloti* gene bank. To begin to dissect the cellular functions of these aminotransferase enzymes and to identify which, if any, are particularly important for IAA biosynthesis, mutant strains defective in two aminotransferases were generated and characterized.

MATERIALS AND METHODS

Strains and media. *Rhizobium meliloti* 102F34 (F34) was originally supplied by Nitragin Co., Milwaukee, Wis. A

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spontaneous nalidixic acid-resistant (Nal^r) derivative of F34 isolated in this laboratory was used in these studies. *R. leguminosarum* 128C53, *R. trifolii* 0403, *Bradyrhizobium japonicum* 61A76, and *B. japonicum* USDA110 were supplied by Mac Cantrell, Oregon State University, Corvalis, and *R. leguminosarum* UML2 was supplied by Antonio Leyva, University of Madrid, Madrid, Spain. A Nal^r derivative of *Agrobacterium tumefaciens* A348, *Pseudomonas aeruginosa* PAO1161, *P. putida* 2440, and *Azotobacter vinelandii* UW were obtained from Ross Durland, University of California, San Diego. *Escherichia coli* strains used included HB101 (*pro leu thi lacY endA recA hsdR hsdM ara-14 galK2 xyl-5 mtl-1 supE44* Str^r), HB101::Tn*5*, C2110nal (*his rha polA* Nal^r), and DG30nal (*proA2 aspC13 hisG4 ilvE12 argE3 thi-1 tyrB507 hsdS14 hppT29 lacY1 galK2 xyl-5 mtl-1 rpsL31 tsx33* λ^- *supE44 recB21 recC22 sbcB15* Nal^r). DG30 was obtained from B. Bachmann, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn., and a spontaneous Nal^r derivative was isolated. The *E. coli ilvE* gene sequence was provided on plasmid pKIA-1 (24) by S. Kuramitsu, Osaka University, Toyonaka, Osaka. The *tyrB* gene sequence was supplied on pLC28-33 (47), a plasmid from the Clark and Carbon collection obtained from B. Bachmann, *E. coli* Genetic Stock Center.

E. coli, *A. tumefaciens*, *P. aeruginosa*, and *P. putida* were grown on LB medium (GIBCO Laboratories) and *A. vinelandii* was grown on C medium (0.8 mM $MgSO_4$, 1.7 mM NaCl, 0.25% [wt/vol] yeast extract, 0.05% [wt/vol] casamino acids, 1% [wt/vol] mannitol). The *Rhizobium* and *Bradyrhizobium* species were maintained on TY medium (6 g of tryptone and 3 g of yeast extract per liter plus 9 mM $CaCl_2$) or yeast mannitol broth (YMB) medium (41). The *Rhizobium* minimal medium used to select for *R. leguminosarum* transconjugants was as described by O'Gara and Shanmugam (32). A modified *Rhizobium* minimal medium (Rmin) was used in F34 growth experiments and contained (in grams per liter) Na_2HPO_4, 5.8; KH_2PO_4, 3; NaCl, 5; biotin, 0.0005; $FeCl_3 \cdot 6H_2O$, 0.0004; plus 8 mM $MgSO_4$, 0.1% mannitol, and 0.1% sodium glutamate. Amino acids were included at 50 μg/ml when specified. Nitrogen-limiting Rmin was made by decreasing the glutamate level in Rmin to 0.01% (wt/vol). Amino acids were added at 0.1% when included in nitrogen-limiting Rmin. DG30 minimal medium consisted of medium E salts (42) supplemented with proline, histidine, arginine, isoleucine, leucine, valine, Asp, Tyr, and Phe at 50 μg/ml, asparagine and glutamine at 100 μg/ml, 0.1% glutamate, 0.5% glucose, 0.25% succinate, 0.25% malate, 0.1% 2-oxoglutarate (2-OG), and 0.0005% thiamine. Antibiotics were incorporated into selective media at the following concentrations: nalidixic acid, 10 μg/ml; tetracycline, 15 μg/ml for *E. coli* and 5 μg/ml for other gram-negative bacteria; kanamycin or neomycin, 50 μg/ml; gentamicin, 25 μg/ml; carbenicillin, 50 μg/ml; and penicillin, 250 μg/ml.

AAT assays. An enol-borate spectrophotometric assay originally described by Lin et al. (29) was adapted for the determination of total AAT activity in *R. meliloti* crude protein extracts. Crude protein extracts of log-phase cells were generated by sonication in S buffer (20 mM Tris [pH 8], 10 mM EDTA, 10% glycerol, 0.1 mM dithiothreitol, 20 μM pyridoxal phosphate). The assay mix contained 85 mM Tris (pH 8.0), 10 mM 2-OG, 10 mM amino acid, and 40 μM pyridoxal phosphate in a 400-μl final reaction volume. Then, 60 μg of protein extract was preincubated at 30°C with all the reaction constituents except 2-OG. Addition of 2-OG initiated the reaction, which was allowed to proceed for 20 min before it was terminated by the addition of 0.1 ml of ice-cold 20% (wt/vol) trichloroacetic acid (TCA). Samples were incubated on ice for at least 15 min, after which time they were centrifuged, and 0.25 ml was transferred to 1.2 ml of 2 M arsenate–1 M borate buffer, pH 6.5. After 15 min, the arsenate-borate samples were read in the spectrophotometer to detect the presence of aromatic keto acid-borate complexes. A blank containing the identical reaction components, except that protein extract and 20% TCA were added simultaneously, was used for spectrophotometric calibration. The molar extinction coefficients used to calculate the amount of aromatic keto acids produced were 12,700 for indolepyruvate at 330 nm, 12,400 for hydroxyphenylpyruvate at 310 nm, and 9,150 for phenylpyruvate at 300 nm (29).

Individual AAT enzymes were detected by a nondenaturing gel enzyme activity assay. It was found that 9% acrylamide gels (acrylamide-bisacrylamide ratio, 30:0.8) containing 0.375 M Tris (pH 8.3), 0.05% ammonium persulfate, and 0.033% TEMED (*N,N,N',N'*-tetramethylethylenediamine) that were allowed to cure overnight best resolved the AATs of F34. These gels were run for 1 h in 0.375 M Tris (pH 8.3) in the cold before the Tris-glycine electrophoresis buffer (1 mM Tris, 76.7 mM glycine, pH 8.3) was substituted, and 100 μg of crude protein extract was loaded in each sample well. Electrophoresis was done at 150 to 200 V (never exceeding 40 mA) in the cold for approximately 5 h until the dye reached the bottom of the gel. Gels were stained at room temperature in the dark for aminotransferase activity. The staining mixture contained 12.5 mM 2-OG, 0.2 mM pyridoxal phosphate, 0.6 mM nitroblue tetrazolium, 0.098 mM phenazine methosulfate, 0.1 M KH_2PO_4 (pH 7.5), 10 mM amino acid, 3 mM NAD, 3 to 6 U of glutamate dehydrogenase, and 0.8% agarose in a total volume of 50 ml. When either 2-OG or the amino acid substrates were omitted from the staining reaction mix, the darkly stained protein bands indicative of aminotransferase activity were not detected, confirming that the assay is specific for aminotransferase enzymes.

Transposon mutagenesis and marker exchange. Plasmids from the broad-host-range *Eco*RI F34 gene bank were mutagenized with transposon Tn*5* by the procedure detailed by Ditta (6) or a Tn*3* derivative, Tn*3*HoHo (39). Selected transposon insertions were introduced into the genome by homologous double recombination by the technique of marker exchange as described by Ditta (6).

Plasmid manipulations. Restriction enzymes and T4 DNA ligase were purchased from Bethesda Research Laboratories or New England BioLabs and used according to the manufacturer's instructions. Recombinant DNA procedures were done by the method of Maniatis et al. (30). Plasmids were mobilized from *E. coli* HB101 to other hosts by the triparental mating procedure of Ditta et al. (8). HB101(pRK2073) was the mobilizing strain.

Southern hybridizations. Genomic DNA was prepared by the method of Better et al. (4). DNA fragments, electrophoresed in 0.8% agarose gels, were transferred to nitrocellulose paper (type HA; Schleicher & Schuell) by the method of Wahl et al. (43). Colony blots from ordered plates of the F34 gene bank were prepared by the method of Grunstein and Hogness (17). DNA probes were labeled with [α-^{32}P] dCTP (3,000 Ci/mmol) by the nick translation procedure of Rigby et al. (37). Hybridization of labeled probe to the filters was done in 50% (vol/vol) deionized formamide–5× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate)–100 μg of heparin per ml at 37°C for 18 to 20 h. Low-stringency hybridizations were carried out by decreasing the formamide concentration to 46%. Filters were washed after hybridization in 2× SSC–0.2% sodium dodecyl sulfate (SDS) three

times at 37°C and one time in 0.1× SSC–0.1% SDS at room temperature, air dried, and exposed to Kodak XAR or OG film in the presence of the appropriate intensifying screen at −70°C.

IAA determinations. IAA levels were quantitated by high-pressure liquid chromatography (HPLC). Stationary-phase culture supernatants (25 ml) were acidified with HCl to pH 2.5 to 3 and extracted twice with equal volumes of ethyl acetate (HPLC-grade; EM Sciences). The solvent fraction was evaporated to dryness at 40°C under vacuum. The residue was suspended in 1 ml of methanol, filtered, and analyzed on a SpectraPhysics model 8750 HPLC equipped with an ODS Hypersil C-18 reverse phase column (Shandon Southern Instruments). Isocratic runs of 40% (vol/vol) methanol in 20 mM sodium acetate (pH 3.5) were determined to best separate IAA from other contaminating compounds. IAA was detected at 280 nm with a SpectraPhysics UV/Vis variable-wavelength absorbance monitor. The IAA peak was quantitated by using indole propionic acid as an internal standard (1).

Nitrosoguanidine (NG) mutants were rapidly screened for their IAA production capabilities with Salkowski reagent (2 ml of 0.5 M $FeCl_3$, 60 ml of H_2SO_4, 100 ml of H_2O) (16). Individual colonies were inoculated into 100 μl of Rmin supplemented with tryptophan (300 μg/ml) in a UV-sterilized microtiter dish and grown to stationary phase at 30°C. Then, 100 μl of Salkowski reagent was added directly to each well, and the intensity of pink color development was observed after 30 to 60 min.

NG mutagenesis. Cells were mutagenized with NG as follows. Cultures (10 ml) were grown in Rmin to a Klett value of 25, harvested and washed four times in 0.1 M citrate buffer, pH 5.5, and suspended in 10 ml of citrate buffer. Samples (2 ml) were incubated at 30°C without shaking with NG (50 μg/ml dissolved in citrate buffer) for 30 or 60 min. Mutagenized cells were washed twice with 0.1 M KH_2PO_4 (pH 7) buffer before being grown to stationary phase in YMB.

NG-mutagenized cells were penicillin enriched according to the selection scheme imposed on them. Cultures were grown overnight in YMB, washed repeatedly in saline, and inoculated at low density into the specified selective medium. Cultures were incubated overnight (at least 2 to 3 generations) before the addition of penicillin (5 mg/ml) and lysozyme (10 μg/ml), and incubation was continued for 4 to 5 h. The surviving cells were pelleted and washed before being grown to stationary phase in supplemented medium and plated onto selective plates.

Plant tests. The nodulation capabilities of mutant strains were assessed by inoculation of alfalfa as described previously (5). Nodulation kinetics and competition experiments were done as described (B. L. Kittell, D. R. Helinski, and G. S. Ditta, submitted for publication).

RESULTS

Characterization of AAT activity in F34. Crude protein extracts of log-phase F34 cultures were examined for total AAT activity by an enol-borate spectrophotometric assay (29). AAT activities of similar efficiencies were detected with each of the aromatic amino acids as amino substrate and 2-OG as the keto acid substrate (Table 1). When these protein extracts were electrophoresed on nondenaturing gels and stained for TAT activity, at least four protein bands (Fig. 1, lane 2) that were capable of converting Trp to indolepyruvate in the presence of 2-OG could be detected. On some gels, a fifth band, AAT3b, was also detected (Fig. 1, lane 1). Omitting either Trp or 2-OG from the activity stain completely eliminated all of the bands. Substitution of either Phe or Tyr for Trp in the nondenaturing gel-enzyme activity assay resulted in a staining pattern identical to that seen with Trp, strongly suggesting that the TAT proteins are more general AATs. Aspartate aminotransferase activity could be detected in protein bands that comigrated with AAT1 and AAT4, suggesting that these two proteins have an even broader substrate specificity that includes Asp (Fig. 1, lane 4). None of the AATs identified comigrated with aminotransferases that utilized the branched-chain amino acids, including alanine, valine, leucine, and isoleucine, as substrates.

TABLE 1. AAT activity of F34 protein extracts

Growth conditions[b]	Sp act (nmol/mg per min)[a]		
	Trp	Tyr	Phe
Rmin	38	49	53
Rmin + Trp	46	58	71
Rmin + Phe + Tyr	48	61	72
Rmin + Asp	50	61	NT[c]
Rmin + 0.01% glucose	38	58	62
Bacteroids	8	12	11

[a] AAT activity of F34 protein extracts prepared from log-phase cells or bacteroids isolated from 6-week-old nodules was determined by a modified enol-borate spectrophotometric assay. The specific activity is reported as nanomoles of aromatic keto acid formed per milligram of protein extract per minute with the designated amino acid substrate and 2-OG as the keto acid substrate. The specific activities are the mean of two to four experiments. The standard deviation between experiments done with free-living bacteria averaged 3 and never exceeded 5. The standard deviation between experiments with bacteroid extracts was 0.7.

[b] Growth media are described in Materials and Methods. Rmin + 0.01% glucose is a nitrogen-limiting medium.

[c] NT, Not tested.

Total AAT activity of protein extracts from cells grown under conditions that may be expected to induce or repress one or more of the AATs was determined (Table 1). From these data, there was no indication that any of the AATs were regulated by the presence or absence of substrate amino acids or by conditions of nitrogen limitation. Nonde-

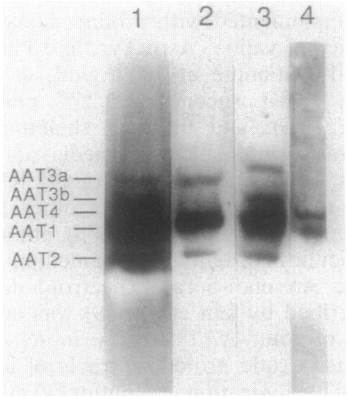

FIG. 1. TAT activity of F34 protein extracts on nondenaturing gels. Protein extracts were prepared by sonication, electrophoresed on nondenaturing 9% acrylamide gels, and stained for aminotransferase activity. Proteins with aminotransferase activity appear as dark bands. Lanes 1 and 2, Protein extract from log-phase F34; lane 3, protein extract from F34 bacteroids. Lanes 1 to 3 were stained with Trp and 2-OG as enzyme substrates. Lane 4, Protein extract from log-phase F34 stained with aspartate and 2-OG. Positions of the AAT proteins are shown.

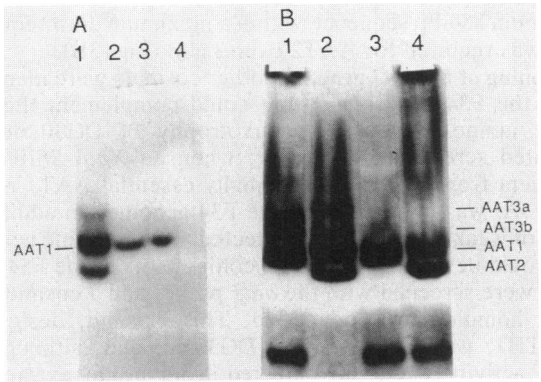

FIG. 2. Expression of F34 AAT proteins from plasmids in heterologous bacteria. Protein extracts were prepared by sonication, electrophoresed on nondenaturing 9% acrylamide gels, and stained for aminotransferase activity with Trp and 2-OG as substrates. Protein extracts from the following cultures are included: (A) Lane 1, F34; lane 2, DG30nal(pAAT1-R1); lane 3, DG30nal(pAAT1-R5); lane 4, DG30nal. (B) Lane 1, *R. leguminosarum*(pAATH1); lane 2, F34; lane 3, *R. leguminosarum*; lane 4, *R. leguminosarum*(pAAT2). The locations of the F34 AATs are marked.

naturing gel enzyme activity assays of the different F34 protein extracts confirmed the presence of all of the protein bands in similar ratios regardless of the conditions under which the cultures were grown.

Protein extracts prepared from F34 bacteroids isolated from 6-week-old alfalfa nodules had a fivefold-lower specific activity than free-living F34 protein extracts (Table 1). However, the bacteroid protein extracts still displayed five bands of AAT activity on nondenaturing gels (Fig. 1, lane 3). Although the bacteroid protein gel pattern was similar to that seen with free-living F34 protein extracts, one protein, AAT3a, appeared to migrate more slowly in the bacteroid crude extracts. Whether this is because the AAT3a protein in bacteroids is a different protein or an altered form of the protein in vegetative cells with a different electrophoretic migration property is unknown.

Cloning of the *aat1* gene. To better understand the functions of the various AATs of F34, their corresponding gene sequences were cloned. The first approach taken to isolate *aat* gene sequences involved complementation of *E. coli* DG30 (15), a mutant strain defective in all three *E. coli* AAT proteins (a Phe, Tyr, and Asp auxotroph), with the F34 *Eco*RI gene bank. The F34 cosmid bank was transferred by conjugal mating to a spontaneous Nal^r DG30 derivative, and the transconjugants were plated onto DG30 minimal plates lacking Phe and Tyr. Two different colony types, large and small, appeared after 7 to 10 days at 30°C, and representative clones from each were examined for plasmid content.

All the large colonies examined contained one of two different plasmids. These two plasmids, designated pAAT1-R1 and pAAT1-R5, had a 4.2-kilobase (kb) *Eco*RI restriction fragment doublet in common. When reintroduced into DG30nal, both plasmids were able to support cell growth on plates lacking Phe, Tyr, and Asp. Furthermore, protein extracts from these DG30 transconjugants contained a protein with AAT activity that comigrated with F34 AAT1 on nondenaturing gels (Fig. 2A).

The *aat1* gene sequence was localized by using transposon mutagenesis and subcloning. Both pAAT1 cosmids were Tn*5* mutagenized, transferred to DG30nal, and tested for their ability to complement DG30 for auxotrophy and to produce functional AAT1 protein. Tn*5* insertions that resulted in the

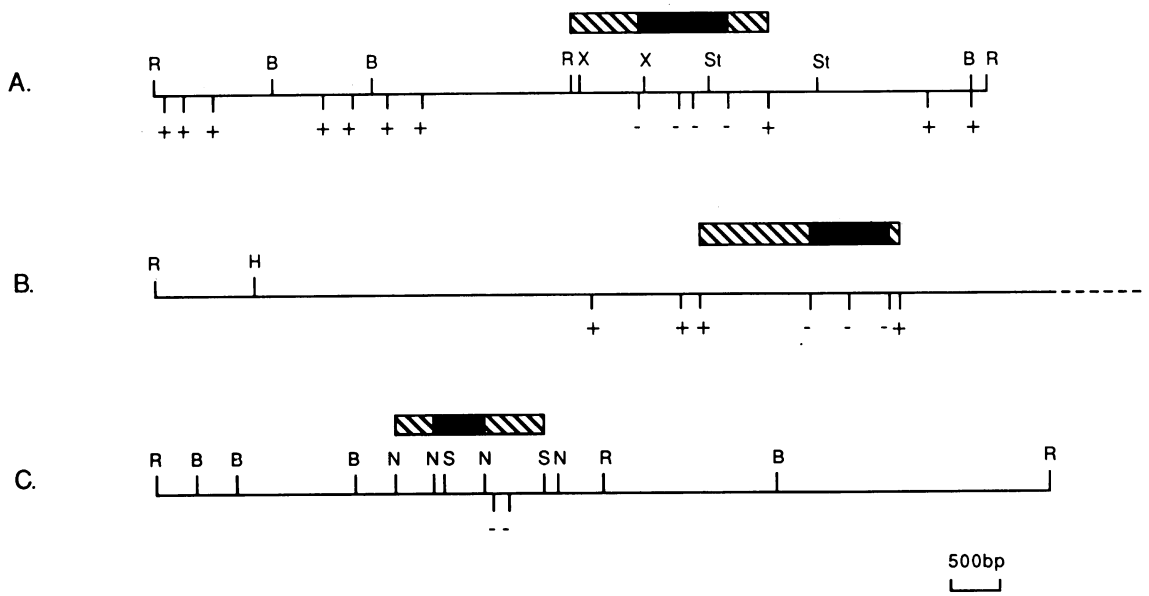

FIG. 3. Restriction maps of the cosmids containing F34 *aat* gene sequences. The restriction maps of the F34 cosmids containing the F34 *aat* gene sequences are diagrammed. Vertical lines beneath each map designate the location of Tn*5* insertions. Tn*5* insertions with a + beneath them did not affect AAT expression; those with a − beneath them eliminated AAT expression. Restriction enzyme sites: B, *Bgl*II; H, *Hin*dIII; N, *Nru*I; R, *Eco*RI; S, *Sal*I; St, *Stu*I; X, *Xho*I. (A) Restriction map of the 8.4-kb region of DNA sequence overlap between the *aat1*-containing cosmids pAAT1-R1 and pAAT1-R5. The bar above the map defines a minimum (solid) and maximum (hatched) sequence required for AAT1 expression. (B) Restriction map of the region of DNA sequence overlap between the three cosmids that carry the *aat2* gene sequence. The bar above the map defines a minimum (solid) and maximum (hatched) sequence required for AAT2 expression. (C) Restriction map diagrams the portion of pAATH1 that carries the *aat3a* and *aat3b* gene sequence(s). The bar above the map defines the minimum (solid) and maximum (hatched) regions of homology to *aat1* as determined by probing Southern blots of plasmid digests with an intragenic *aat1* fragment.

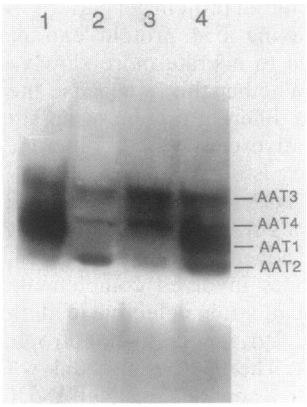

FIG. 4. TAT activity in AAT mutants. Protein extracts were prepared by sonication, electrophoresed on nondenaturing 9% acrylamide gels, and stained for aminotransferase activity with Trp and 2-OG as substrates. Protein extracts: lane 1, AAT2$^-$; lane 2, AAT1$^-$; lane 3, AAT2/1$^-$; lane 4, F34. The positions of the F34 AATs are marked.

loss of these abilities were assumed to be within the operon coding for AAT1 and were mapped by restriction enzyme analysis (Fig. 3A). To verify that these Tn*5* insertions eliminated AAT1 activity, they were introduced into the chromosome by marker exchange. Protein extracts from the chromosomally mutated F34 strains were analyzed with the nondenaturing gel enzyme activity assay. The AAT1 protein band was missing from these extracts when the gels were stained with either Trp (Fig. 4, lane 2), Tyr, or Phe as the amino acid substrate. The *aat1* gene sequence boundary was further localized by subcloning a 4-kb *Eco*RI-*Bgl*II fragment from within the *Eco*RI doublet into pUC18 and demonstrating that this subclone was sufficient for AAT1 activity in DG30. Combining these data, a minimum of 950 base pairs (bp) of DNA essential for AAT1 expression were localized within a 2-kb maximum boundary (Fig. 3A).

Cloning of the *aat2* gene. DG30nal transconjugants that produced small colonies on plates lacking Phe and Tyr contained one of three overlapping plasmids, pAAT2-R7, pAAT2-R8, or pAAT2-R9; these plasmids have a 12-kb *Eco*RI fragment in common. When reintroduced into DG30nal, all the cosmids were able to support cell growth in the absence of Phe and Tyr but not Asp. Such complementation was quite weak, however, and suggested that the *R. meliloti* gene(s) was poorly expressed in *E. coli*. Furthermore, when protein extracts of DG30(pAAT2) transconjugants were assayed on nondenaturing gels, no AAT activity could be detected. One of the pAAT2 cosmids was therefore conjugally transferred to *R. leguminosarum* UML2 for analysis, and protein extracts from this species revealed a new AAT activity comigrating with AAT2 of F34 (Fig. 2B).

The pAAT2 plasmids were Tn*5* mutagenized, transferred to DG30nal, and tested for their ability to complement the DG30 auxotrophy. Mutagenized plasmids that no longer complemented this auxotrophy were identified, and the locations of their Tn*5* insertions were determined by restriction enzyme mapping (Fig. 3B). Confirmation that these Tn*5* insertions eliminated AAT2 expression was obtained by introducing them into the chromosome by marker exchange and assaying protein extracts of the mutagenized strains with the nondenaturing gel enzyme activity assay (Fig. 4, lane 1). The AAT2 protein band was missing from these extracts regardless of the aromatic amino acid donor used in the AAT activity stain. Analysis of these Tn*5* mutants identified a minimum 850-bp sequence within a maximum 2-kb sequence that was required for AAT2 expression (Fig. 3B).

Cloning of the *aat3* gene. No other cosmids were identified from the F34 gene banks that could complement the aromatic amino acid or Asp auxotrophy of DG30 despite repeated screenings. However, when an *Xho*I-*Stu*I DNA fragment from within the minimally essential AAT1 region (Fig. 3A) was used to probe the F34 genome, an additional band of weak homology was detected. To determine whether this could be another *aat* gene, colony blots of the F34 gene bank were screened with the *aat1* probe, and a cosmid with weak homology was identified. This plasmid, designated pAATH1, was transferred to DG30nal, and, although no AAT activity could be detected from crude extracts in nondenaturing gel enzyme activity assays, there was very weak complementation of the DG30 aromatic amino acid auxotrophy. To determine whether this plasmid had gene sequences for an F34 AAT, pAATH1 was conjugally transferred to *R. leguminosarum* UML2, and AAT expression from protein extracts of the transconjugants was examined in the nondenaturing gel enzyme activity assay. In this bacterium, the pAATH1 plasmid was responsible for the production of two new protein bands with AAT activity (Fig. 2B). One of the bands was coincident with AAT3b, and the other migrated somewhat faster than AAT3a. Two new AAT bands were also detected from protein extracts of *Agrobacterium tumefaciens* carrying pAATH1 (data not shown). From these data, it was concluded that pAATH1 indeed carried one or more genes for AAT activity and that these genes likely corresponded to those coding for AAT3a and AAT3b.

By subcloning the 4.2-kb *Bgl*II fragment of pAATH1 into the broad-host-range vector pRK404 (7) and transferring it to *R. leguminosarum* for analysis, it was determined that the coding sequence(s) for AAT3a and AAT3b expression was localized within this fragment. To further localize the gene sequences, the region of homology to the internal *aat1* probe was defined (Fig. 3C). Transposon mutagenesis confirmed that this region was responsible for directing expression of both AAT3a and AAT3b. Two Tn*5* insertions were identified that resulted in loss of expression of the AAT3a and AAT3b proteins in *R. leguminosarum*. The Tn*5* insertions were mapped and, not unexpectedly, found to be within a region that contained homology to *aat1*. Each of these Tn*5* insertions eliminated the expression of both AAT3a and AAT3b. None of the Tn*5* insertions examined that mapped within the 4.2-kb *Bgl*II fragment affected expression of only one of the AAT3 protein bands, suggesting that either AAT3a and AAT3b are coded for in the same operon and a polar Tn*5* mutation in the upstream gene eliminates transcription of both genes simultaneously or AAT3a and AAT3b are coded for by the same gene and represent different forms of the same protein.

Repeated attempts to marker exchange these Tn*5* insertions into the F34 chromosome were unsuccessful, possibly due to noncontiguous and repeated sequences surrounding this region on pAATH1. Attempts to find other copies of *aat3a* and *aat3b* within the *Eco*RI cosmid gene bank and within a *Bgl*II plasmid gene bank (8) were also unsuccessful.

Interspecies homology to *aat1*. An intragenic *Xho*I-*Stu*I fragment of the *aat1* gene was used as a probe to determine DNA homology between the three *aat* gene sequences by hybridization analyses. Although the *aat1* and *aat3* gene sequences were found to be homologous, no homology between *aat1* and *aat2* could be detected, even at lowered stringencies. The *Rhizobium aat1* gene also exhibited limited

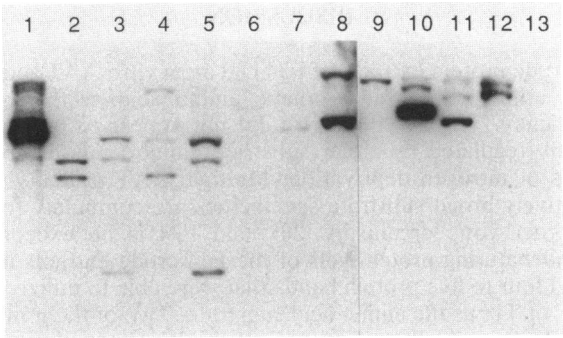

FIG. 5. Homology of the F34 *aat1* gene sequence to genomic DNAs from heterologous bacteria. Genomic DNAs were digested with *Eco*RI, transferred to nitrocellulose, and probed with a ^{32}P-labeled intragenic *aat1* fragment. Hybridization was done under low-stringency conditions. Genomic DNAs are from (lane 1) *R. meliloti* F34, (lane 2) *R. leguminosarum* UML2, (lane 3) *R. leguminosarum* 128C53, (lane 4) *R. trifolii* 0403, (lane 5) *R. phaseoli* 8002, (lane 6) *B. japonicum* USDA 110, (lane 7) *B. japonicum* 61A76, (lane 8) *R. fredii* 193, (lane 9) *E. coli* HB101, (lane 10) *A. vinelandii* UW, (lane 11) *P. putida* 2440, (lane 12) *P. aeruginosa* PAO1161, (lane 13) *A. tumefaciens* A348.

homology to a DNA fragment carrying the *tyrB* gene of *E. coli*, but was not homologous to DNA containing *ilvE*, a gene whose product has both branched-chain and aromatic aminotransferase activity in *E. coli* (data not shown). The *aat1* gene sequence was homologous to multiple bands in both fast- and slow-growing species of *Rhizobium* and to other gram-negative bacteria tested, including *Azotobacter vinelandii*, *Pseudomonas aeruginosa*, *P. putida*, and *A. tumefaciens* (Fig. 5).

Characterization of AAT$^-$ mutants. Transposon mutagenesis of the *aat1* and *aat2* gene sequences facilitated the generation of F34 mutant strains AAT1$^-$ and AAT2$^-$, defective in AAT1 and AAT2 proteins, respectively, as described above. Transposon mutagenesis of pAAT2 was repeated with Tn*3*HoHo, a Tn*3* derivative that carries a carbenicillin resistance marker, and a carbenicillin-resistant AAT2$^-$ strain was generated by marker exchange with the Tn*3*HoHo-mutagenized plasmid. By using marker exchange again to recombine the pAAT1::Tn*5* sequence into the carbenicillin-resistant AAT2$^-$, the double AAT mutant AAT2/1$^-$ was constructed. As in the case of the AAT single mutants, AAT2/1$^-$ was characterized by the loss of the appropriate AAT protein bands on nondenaturing gels stained for AAT activity (Fig. 4, lane 3).

Protein extracts of the AAT mutants were assayed spectrophotometrically to determine the relative contribution that each AAT protein made to the total in vitro AAT activity (Table 2). As expected, AAT1, the protein that produced the most intensely staining band in the nondenaturing gel enzyme activity assay, was responsible for a majority of the total AAT activity in cell extracts. In the case of AAT2, the correlation between protein band intensity in the nondenaturing gel enzyme activity assay and relative contribution to total cell AAT activity was not seen, however, since AAT2$^-$ had total AAT levels equivalent to those of wild-type F34 even though the protein was absent by gel assay. While the reason for this is not understood, it is possible either that AAT2 is actually a minor component of total AAT activity and its relatively intense staining capability is an artifact of the gel assay or that one or more of the remaining AAT proteins compensates for the absence of AAT2 by increasing its expression correspondingly.

The growth properties of the AAT mutants were also assessed (Table 2). All of the AAT mutants grew on minimal medium at rates comparable to wild-type F34, indicating that neither AAT1 nor AAT2 is required for aromatic amino acid biosynthesis. The AAT mutants were defective in their ability to grow on minimal medium supplemented with the toxic Trp analog 5-methyl-Trp or 6-methyl-Trp. They were not altered, however, in their response to α-methyl-Trp. Under conditions of nitrogen limitation, produced in these experiments by lowering the glutamate concentration to 0.01%, the growth rate of wild-type F34 was inhibited and cultures were not able to achieve densities comparable to those reached under nonlimiting conditions. Although the generation times were further decreased, F34 cultures growing in 0.01% glutamate supplemented with aromatic amino acids were able to reach densities similar to those seen under nonlimiting conditions, indicating a weak ability to obtain nitrogen for growth from the aromatic amino acids. When AAT mutants were grown in 0.01% glutamate, their growth was similar to F34 under the same medium conditions. However, the incorporation of Tyr or Phe into this medium did not enhance culture densities as was seen for the wild type. In addition, the growth rate of all three AAT mutants in 0.01% glutamate plus Tyr or Phe was significantly slower than F34 under the same conditions. The growth rate of AAT1$^-$ and AAT2/1$^-$ but not AAT2$^-$ was similarly reduced in medium containing 0.01% glutamate and Trp. These data suggest that AAT1 and, to a lesser extent, AAT2 can function to detoxify toxic Trp analogs and to scavenge

TABLE 2. Characterization of F34 and its AAT mutants

Strain	Relative AAT activity[a]	Resistance to Trp analogs[b]			Final culture density[c]			IAA production[d] (μg/ml)		
		5-MT	6-MT	α-MT	+N	<N	<N + Tyr	Trp_0	Trp_{10}	Trp_{500}
F34	1.0	++	+++	+++	+++	+	+++	0.018	0.45	6.5
AAT1$^-$	0.13	+/−	+	+++	+++	+	+	0.017	0.46	3.2
AAT2$^-$	0.99	+/−	+	+++	+++	+	+	0.017	0.51	4.9
AAT2/1$^-$	0.26	+/−	+	+++	+++	+	+	0.017	0.49	2.9

[a] AAT activity was determined from protein extracts of AAT mutants grown to log phase in Rmin by the enol-borate spectrophotometric assay. The substrates were Trp and 2-OG. AAT activity is expressed relative to the level in F34 and as the mean of four experiments.

[b] Trp analog resistance was assessed by streaking each strain for isolated colonies and comparing colony sizes after 3 days at 30°C. Trp analogs were incorporated into Rmin at the following concentrations: 6-methyl-Trp (6-MT), 75 μg/ml; 5-methyl-Trp (5-MT), 200 μg/ml; α-methyl-Trp (α-MT); 200 μg/ml. Symbols: +++, wild-type colony size; ++, slightly inhibited; +, strongly inhibited; +/−, barely visible growth.

[c] Final culture densities were measured after cultures entered stationary phase. Symbols: +++, wild-type density; +, less than half the wild-type density. Cultures were grown in Rmin (+N); in nitrogen-limiting Rmin (<N); and in nitrogen-limiting Rmin supplemented with Tyr (<N + Tyr).

[d] IAA production, was determined by HPLC as described in Materials and Methods. The standard deviations for IAA measurements are: Trp_0 (Trp at 0 μg/ml), ±0.005; Trp_{10} (Trp at 10 μg/ml), ±0.10; Trp_{500} (Trp at 500 μg/ml), ±1.1.

nitrogen from alternative, less-preferred nitrogen sources during times of deprivation.

IAA accumulation capabilities of the wild-type F34 and the various AAT mutants were assessed by HPLC in the presence and absence of exogenous Trp (Table 2). In the absence of supplemental Trp, stationary-phase cultures of F34 accumulated 17 ng of IAA per ml. This amount of IAA is in good agreement with reported IAA levels in the other fast-growing rhizobia (2, 11, 44) and is within a range of concentrations known to affect plant roots (36, 45). IAA production was markedly increased when Trp was included in the growth medium, with levels increasing over 380-fold in the presence of 500 μg of Trp per ml. At high Trp levels, the AAT mutants were somewhat reduced in IAA production, with AAT2/1$^-$ being the most defective and AAT2$^-$ the least defective. None, however, were totally deficient in IAA production. In the absence of exogenous Trp or in the presence of low levels of supplemental Trp (i.e., 10 μg/ml), the AAT mutants accumulated IAA at levels equivalent to the wild type. Therefore, it appears that although the AAT1 and AAT2 proteins are involved in the synthesis of IAA when cells are grown in the presence of high levels of Trp, they are not the primary enzymes responsible for basal-level IAA production. Likewise, the AAT1$^-$, AAT2$^-$, and AAT2/1$^-$ mutants cannot be considered IAA-defective mutants unless the cells are grown in a high-Trp environment.

Because there is evidence that plant root exudates contain significant levels of Trp (21), it is possible that one or more of the AAT mutants could exhibit a symbiotically defective phenotype in the presence of the plant. Therefore, the nodulation capabilities of the AAT mutants were determined. All three mutants were able to form wild-type numbers of morphologically normal, nitrogen-fixing nodules when inoculated on alfalfa roots, and the kinetics of nodulation paralleled that of F34. When equal numbers of AAT mutants and F34 were coinoculated onto alfalfa, the nodule occupancy ratio was 1:1, indicating that there was no competitive advantage or disadvantage associated with these mutations. It was therefore concluded, that AAT1$^-$, AAT2$^-$, and AAT2/1$^-$ were symbiotically equivalent to wild-type F34.

Other attempts to isolate IAA-defective mutants. Attempts to isolate IAA-defective mutants from NG-mutagenized populations of either F34 or the AAT2/1$^-$ mutant were largely unsuccessful. Mutagenized cells were initially screened for increased sensitivity to the toxic Trp analog 6-methyl-Trp, and potential mutant cultures were tested for IAA production by the Salkowski colorimetric assay (16). Mutant candidates were obtained from each mutagenized population that produced significantly lower levels of IAA in the presence of Trp. Upon further examination, however, it was concluded that all of the F34 mutants were likely to have defects in carbon source utilization rather than specific mutations in IAA biosynthesis, since they grew more slowly on specific carbon sources. It is possible that IAA production in these instances may have been affected because of limiting 2-OG availability for transamination reactions. Derivatives of the AAT2/1$^-$ mutant were also identified that produced significantly less IAA than the wild type when grown in low levels of Trp. However, none of these showed defects in AAT activity, and all produced normal levels of IAA (approximately 18 ng/ml) when grown without Trp supplementation. Some of the phenotypes characterized included Trp uptake mutants, a mutant defective in aromatic amino acid biosynthesis, and a mutant defective in sugar metabolism.

DISCUSSION

Crude protein extracts of F34 had an in vitro AAT activity that utilized all three aromatic amino acids with similar efficiency. This AAT activity did not appear to be significantly regulated by either substrate amino acids or conditions of nitrogen deprivation. Multiple AAT proteins with relatively broad substrate specificities are commonly found in procaryotic organisms (20), and F34 is no exception. Nondenaturing protein gels of the F34 crude extracts identified four to five protein bands that were able to utilize Trp, Phe, or Tyr as the amino acid substrate. Two of the proteins could also use Asp as a substrate. It is not clear why nondenaturing gels containing F34 extract sometimes detected four proteins with AAT activity and at other times revealed five. The presence of four or five bands did not correlate with specific conditions under which the cells were grown or with any obvious variation in assay conditions. In *Klebsiella aerogenes*, the major AAT activity appears to have as many as three bands of various intensities on nondenaturing gels, even after purification (34). The variable F34 band (AAT3b) could similarly be an altered form of AAT3 whose existence is sporadic. Alternatively, F34 extracts could have five unique AATs, one of which is unstable and therefore only detected occasionally.

The isolation of three unique plasmids capable of directing the synthesis of different F34 AATs provides direct evidence for the existence of at least three separate AAT proteins in F34. Genomic mutations in two of the gene sequences correspondingly eliminated two of the protein species. One of the plasmids, pAATH1, contains gene sequences responsible for the expression of both AAT3a and AAT3b. The coding sequences for the two proteins are therefore closely linked, if not identical, and are contained within a single transcriptional operon. AAT3a and AAT3b may represent different forms of the same protein. The existence of AAT4 is strongly suggested by the presence on nondenaturing gels of an AAT protein band not coded for by any of the plasmids isolated thus far.

Tn*5* and subcloning analyses of pAAT1 defined the minimally required gene sequence for AAT1 expression to be 0.95 kb, with a maximum of about 2 kb. A minimum gene sequence of 0.85 kb and a maximum 2-kb region were also determined for AAT2. These sizes are in good agreement with the gene sequence requirements for the synthesis of *E. coli* AATs, which range in size from 927 bp for IlvE to 1,188 bp and 1,181 bp for AspC and TyrB, respectively (12, 23–25).

The *aat1* gene sequence was homologous to that of *aat3*, to a fragment of *E. coli* DNA carrying the *tyrB* gene, and to sequences in other *R. meliloti* strains (data not shown), other *Rhizobium* species of both the fast-growing and slow-growing varieties, and other gram-negative soil bacteria. These data suggest that *aat1* is a member of a relatively highly conserved family of bacterial genes, notwithstanding its apparent dispensability in *R. meliloti* under laboratory growth conditions. There appears to be no homology between *aat2* and either *aat1* or *aat3* even though all three catalyze the same reaction in vitro. It is conceivable that AAT2 has a different cellular function in vivo than do AAT1 and AAT3. In *E. coli ilvE*, the gene for a branched-chain aminotransferase that also utilizes Phe as a substrate, also shows no homology to either *tyrB* or *aspC*, the genes for the two major *E. coli* AATs in the cell which also share sequence homology (23–25).

From a characterization of transposon mutants defective in AAT1, AAT2, or both AAT1 and AAT2, it can be

concluded that neither AAT1 nor AAT2 plays a major role in symbiosis or in aromatic amino acid biosynthesis. Rather, AAT1 and AAT2 appear to be catabolic enzymes that act to scavenge nitrogen from less-preferred sources during times of nitrogen limitation and that can also act to detoxify toxic Trp analogs. Neither is required for the synthesis of basal levels of IAA, although both can contribute to IAA biosynthesis when there are high levels of exogenous Trp in the environment.

The AAT1 and AAT2 mutants that we describe here confirm that the indolepyruvate pathway for IAA biosynthesis functions in *R. meliloti* in the presence of Trp. Unfortunately, however, we were unable to ascertain the source of basal-level IAA production (i.e., in the absence of an external Trp supply). A reasonable possibility is that these very low levels of IAA are also derived from the indolepyruvate pathway through AAT3 and/or AAT4, the two remaining AATs in the AAT2/1$^-$ double mutant. This could occur if either or both enzymes had significantly lower K_m's for Trp than AAT1 or AAT2, enabling them to utilize a limiting intracellular pool of this amino acid. If both enzymes had roughly equivalent activities, our failure to isolate either an AAT3 or AAT4 mutant from a population of AAT2/1$^-$ cells could be explained by the fact that a single mutant showing at best a 50% drop in activity probably would not have been detected with the enrichment and screening procedures used.

On the other hand, the possibility cannot be eliminated that low levels of IAA production occur in *R. meliloti* by an entirely different biochemical pathway. Recent biochemical evidence suggests that some strains of *B. japonicum* can synthesize IAA by more than one pathway, including a pathway utilizing an indoleacetamide intermediate (38), similar to the pathway utilized by *Pseudomonas savastanoi* and the T-DNA loci in *Agrobacterium tumefaciens* after transfer to plant cells. However, in the same work these authors were unable to demonstrate the existence of this pathway in the F34 strain of *R. meliloti* or in any other fast-growing *Rhizobium* species. Similarly, we have determined by HPLC analysis that whole cells and crude extracts of F34 are unable to use indoleacetamide, indoleacetonitrile, or tryptamine as IAA precursors (data not shown).

Identifying the ultimate source(s) of IAA production in *R. meliloti* has proven to be a difficult task. Clearly, the isolation of additional AAT mutants would be of interest. By studying such mutants alone and in combination with existing AAT mutants, it should be possible to resolve the overall role of the indolepyruvate pathway in IAA biosynthesis. If indeed a second pathway for IAA biosynthesis was indicated, an AAT-defective strain would be useful in the investigation of this second pathway, allowing a genetic and biochemical analysis in the absence of the indolepyruvate pathway. Additional AAT mutants would also facilitate further investigation of the role of the individual AATs in cellular metabolism and perhaps provide insight into why the bacterium has maintained multiple enzymes that seemingly have the same function.

ACKNOWLEDGMENTS

We thank Antonio Palomares for his assistance with the *E. coli aat* hybridization data. We also thank Barbara Bachman for providing *E. coli* DG30 and plasmid pLC28-33 and H. Kagamiyama for providing the plasmid containing the *E. coli ilvE* gene sequence.

This work was supported by grant DE-FG03-85ER13355 from the Department of Energy.

LITERATURE CITED

1. **Akiyama, M., N. Sakurai, and S. Kuraishi.** 1983. A simplified method for the quantitative determination of indoleacetic acid by high performance liquid chromatography with a fluorometric detector. Plant Cell Physiol. **24**:1431–1439.
2. **Badenoch-Jones, J., R. E. Summons, M. A. Djordjevic, J. Shine, D. S. Letham, and B. G. Rolfe.** 1982. Mass spectrometric quantification of indole-3-acetic acid in *Rhizobium* culture supernatants: relation to root hair curling and nodule initiation. Appl. Environ. Microbiol. **44**:275–280.
3. **Badenoch-Jones, J., R. E. Summons, B. Entsch, B. G. Rolfe, C. W. Parker, and D. S. Letham.** 1982. Mass spectrometric identification of indole compounds produced by *Rhizobium* strains. Biomed. Mass Spectrom. **9**:429–437.
4. **Better, M., B. Lewis, D. Corbin, G. Ditta, and D. Helinski.** 1983. Structural relationships among *Rhizobium meliloti* symbiotic promoters. Cell **35**:479–485.
5. **Corbin, D., G. S. Ditta, and D. R. Helinski.** 1982. Clustering of nitrogen fixation (*nif*) genes in *Rhizobium meliloti*. J. Bacteriol. **149**:221–228.
6. **Ditta, G.** 1986. Tn*5* mapping of *Rhizobium* nitrogen fixation genes. Methods Enzymol. **118**:519–528.
7. **Ditta, G., T. Schmidhauser, E. Yakobson, P. Lu, X.-W. Liang, D. R. Finlay, D. Guiney, and D. R. Helinski.** 1985. Plasmids related to the broad host range vector, pRK290, useful for gene cloning and for monitoring gene expression. Plasmid **13**:149–153.
8. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 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.
9. **Dullaart, J.** 1967. Quantitative estimation of indoleacetic acid and indolecarboxylic acid in root nodules and roots of *Lupinus luteus* L. Acta Bot. Neerl. **16**:222–230.
10. **Dullaart, J.** 1970. The bioproduction of indole-3-acetic acid and related compound in root nodules and roots of *Lupinus luteus* L. and by its rhizobial symbiont. Acta Bot. Neerl. **19**:573–615.
11. **Ernstsen, A., G. Sandberg, A. Crozier, and C. T. Wheeler.** 1987. Endogenous indoles and the biosynthesis and metabolism of indole-3-acetic acid in cultures of *Rhizobium phaseoli*. Planta **171**:422–428.
12. **Fotheringham, I. G., S. A. Dacey, P. P. Taylor, T. J. Smith, M. G. Hunter, M. E. Finlay, S. B. Primrose, D. M. Parker, and R. M. Edwards.** 1986. The cloning and sequence analysis of the *aspC* and *tyrB* genes from *Escherichia coli* K12. Biochem. J. **234**:593–604.
13. **Garcia-Rodriguez, T., C. Alvarez, and J. Perez-Silva.** 1984. Indole-3-acetic acid production by cell-free extracts of *Rhizobium trifolii*. Soil Biol. Biochem. **16**:677–678.
14. **Garcia-Rodriguez, T., A. M. Gutierrez-Navarro, R. Garcia, and J. P. Silva.** 1982. Indole acetic acid production by *Rhizobium*: effect of 2-ketoglutaric acid. Soil Biol. Biochem. **14**:153–155.
15. **Gelfand, D. H., and R. A. Steinberg.** 1977. *Escherichia coli* mutants deficient in the aspartate and aromatic amino acid aminotransferases. J. Bacteriol. **130**:429–440.
16. **Gordon, S. A., and R. P. Weber.** 1950. Colorimetric estimation of indoleacetic acid. Plant Physiol. **26**:192–195.
17. **Grunstein, M., and D. S. Hogness.** 1975. Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. USA **72**:3961–3965.
18. **Hunter, W. J.** 1986. Free and conjugated IAA content of legume root nodules. Plant Physiol. **80**(Suppl.):135.
19. **Hunter, W. J.** 1987. Influence of 5-methyltryptophan-resistant *Bradyrhizobium japonicum* on soybean root nodule indole-3-acetic acid content. Appl. Environ. Microbiol. **53**:1051–1055.
20. **Jensen, R. A., and D. H. Calhoun.** 1981. Intracellular roles of microbial aminotransferases: overlap enzymes across different biochemical pathways. Crit. Rev. Microbiol. **8**:229–266.
21. **Kefford, N. P., J. Brockwell, and J. A. Zwar.** 1960. The symbiotic synthesis of auxin by legumes and nodule bacteria and its role in nodule development. Aust. J. Biol. Sci. **13**: 456–467.
22. **Kradolfer, P., P. Niederberger, and R. Hutter.** 1982. Tryptophan

degradation in *Saccharomyces cerevisiae*: characterization of two aromatic aminotransferases. Arch. Microbiol. **133**:242–248.
23. **Kuramitsu, S., K. Inoue, T. Ogawa, H. Ogawa, and H. Kagamiyama.** 1985. Aromatic amino acid aminotransferase of *Escherichia coli*: nucleotide sequence of the *tyrB* gene. Biochem. Biophys. Res. Commun. **133**:134–139.
24. **Kuramitsu, S., T. Ogawa, H. Ogawa, and H. Kagamiyama.** 1985. Branched-chain amino acid aminotransferase of *Escherichia coli*: nucleotide sequence of the *ilvE* gene and the deduced amino acid sequence. J. Biochem. **97**:993–999.
25. **Kuramitsu, S., S. Okuno, T. Ogawa, H. Ogawa, and H. Kagamiyama.** 1985. Aspartate aminotransferase of *Escherichia coli*: nucleotide sequence of the *aspC* gene. J. Biochem. **97**:1259–1262.
26. **Lee, C. W., and M. J. Desmazeaud.** 1985. Utilization of aromatic amino acids as nitrogen sources in *Brevibacterium linens*: an inducible aromatic amino acid aminotransferase. Arch. Microbiol. **140**:331–337.
27. **Libbenga, K. R., and J. G. Torrey.** 1973. Hormone-induced endoreduplication prior to mitosis in cultured pea root cortex cells. Am. J. Bot. **60**:293–299.
28. **Libbenga, K. R., F. van Iren, R. J. Bogers, and M. F. Schrang-Lamers.** 1973. The role of hormones and gradients in the initiation of cortex proliferation and nodule formation in *Pisum sativum* L. Planta **114**:29–39.
29. **Lin, E. C. C., B. M. Pitt, M. Civen, and W. E. Knox.** 1958. The assay of aromatic amino acid transaminations and keto acid oxidation by the enol borate-tautomerase method. J. Biol. Chem. **233**:668–673.
30. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Nutman, P. S.** 1955. The influence of the legume in root nodule symbiosis. Biol. Rev. **31**:109–140.
32. **O'Gara, F., and K. T. Shanmugam.** 1976. Regulation of nitrogen fixation by rhizobia. Export of fixed nitrogen as ammonia. Biochim. Biophys. Acta **437**:313–321.
33. **Paris, C. G., and B. Magasanik.** 1981. Tryptophan metabolism in *Klebsiella aerogenes*: regulation of the utilization of aromatic amino acids as sources of nitrogen. J. Bacteriol. **145**:257–265.
34. **Paris, C. G., and B. Magasanik.** 1981. Purification and properties of aromatic amino acid aminotransferases from *Klebsiella aerogenes*. J. Bacteriol. **145**:266–271.
35. **Pate, J. S.** 1958. Studies of the growth substances of legume nodules using paper chromatography. Aust. J. Biol. Sci. **11**: 516–528.
36. **Plazinski, J., and B. G. Rolfe.** 1985. Interaction of *Azospirillum* and *Rhizobium* strains leading to inhibition of nodulation. Appl. Environ. Microbiol. **49**:990–993.
37. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labelling deoxyribonucleic acid to high specific activity in vitro by nick translation with deoxyribonucleic acid polymerase I. J. Mol. Biol. **113**:237–251.
38. **Sekine, M., T. Ichikawa, N. Kuga, M. Kobayashi, A. Sakurai, and K. Syono.** 1988. Detection of the IAA biosynthetic pathway from tryptophan via indole-3-acetamide in *Bradyrhizobium* spp. Plant Cell Physiol. **29**:867–874.
39. **Stachel, S., G. An, C. Flores, and E. W. Nester.** 1985. A Tn*3-lacZ* transposon for the random generation of β-galactosidase gene fusions: applications to the analysis of gene expression in *Agrobacterium*. EMBO J. **4**:891–898.
40. **Thimann, K. V.** 1936. On the physiology of the formation of nodules on legume roots. Proc. Natl. Acad. Sci. USA **22**: 511–514.
41. **Vincent, J. M.** 1970. A manual for the practical study of the root nodule bacteria. International Biological Programs Handbook 15. Blackwell Scientific Publications, Oxford.
42. **Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218**:97–102.
43. **Wahl, G. M., M. Stern, and G. R. Stark.** 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. USA **76**:3683–3687.
44. **Wang, T. L., E. A. Wood, and N. J. Brewin.** 1982. Growth regulators, *Rhizobium* and nodulation in peas. Planta **155**: 345–349.
45. **Wareing, P. F., and I. D. J. Phillips.** 1981. Growth and differentiation in plants, 3rd ed. Pergamon Press, New York.
46. **Whitaker, R. J., C. G. Gaines, and R. A. Jensen.** 1982. A multispecific quintet of aromatic aminotransferases that overlap different biochemical pathways in *pseudomonas aeruginosa*. J. Biol. Chem. **257**:13550–13556.
47. **Yang, J., and J. Pittard.** 1987. Molecular analysis of the regulatory region of the *Escherichia coli* K-12 *tyrB* gene. J. Bacteriol. **169**:4710–4715.