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Metabolism of Tryptophan and Tryptophan Analogs by *Rhizobium meliloti*[1]

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ABSTRACT

The alfalfa symbiont *Rhizobium meliloti* Rm1021 produces indole-3-acetic acid in a regulated manner when supplied with exogenous tryptophan. Mutants with altered response to tryptophan analogs still produce indole-3-acetic acid, but are Fix⁻ because bacteria do not fully differentiate into the nitrogen-fixing bacteriod form. These mutations are in apparently essential genes tightly linked to a dominant streptomycin resistance locus.

Rhizobia are able to convert the amino acid Trp to IAA, which is the primary naturally occurring auxin in plants (2, 15, 17). As an organ, the legume root nodule has a higher level of auxin than the surrounding root tissue (1, 7). It has been speculated, therefore, that IAA produced by bacteria is involved in inducing root cortex dedifferentiation and nodule meristem formation, although this relationship has never been rigorously demonstrated.

Here we describe IAA production by *Rhizobium meliloti* Rm1021 and the isolation of mutants with altered response to Trp analogs.

MATERIALS AND METHODS

IAA Production

Overnight cultures were grown in minimal M9 medium (2) containing glucose (M9G) or succinate (M9SA), diluted to A_{675} ~0.1, and grown for 3 h before addition of Trp to 2 or 4 mM. At 2 to 10 h intervals, cells were pelleted and 2 mL Salkowski reagent (1 mL 0.5 M $FeCl_3$, 50 mL $HC10_4$) (10) was added to 1 mL supernatant, and A_{530} was read after 20 min. Salkowski reagent can detect greater than 10 μg/mL IAA.

Identification of Indolics

For TLC, cells were pelleted and 10 mL supernatant was acidified to pH 3 with HCl. Supernatants were extracted twice with equal volume ethyl acetate, and the aqueous phase was neutralized and extracted again with a third volume of ethyl acetate. The pooled organic phase was dried on a watch glass, the residue was dissolved in 0.5 mL ethanol, and 25 μL was spotted in 5 μL aliquots on plastic backed silica gel TLC plates (Baker-flex, J. T. Baker Chemical Co.). The plates were developed with $CHCl_3$:acetic acid (9:1) or isopropanol:NH_4OH:H_2O (10:1:1) (16), sprayed with Salkowski-Van Urk reagent (8), heated to 60°C for 10 min, and gently washed in H_2O. Standards (Sigma) were IAA, IAM[3], IPA, IAD, indole lactic acid, indole butyric acid, indole acrylic acid, and Trp. Trp, IAA, IAM, and IAD were shown to survive the extraction procedure.

Isolation of Mutants

Independent washed cultures of *Rhizobium meliloti* Rm1021 grown in LB broth were treated with NTG (20, 21) or untreated, and 0.1 mL was plated on M9G (for isolation of strains Rm1021-αMT8 and Rm1021-αMT16) or M9SA (for isolation of Rm1021-αMT9) agar with 400 to 600 μg/mL αMT. Spontaneous resistant colonies arose at 10^{-6} to 10^{-8}, while NTG-treated cultures gave resistant colonies at 10^{-5} to 10^{-6}. Random Tn*5* and Tn*5-233* mutagenesis was done as described (5). Strain Rm1021-5MT60 was obtained from M. Honma and F. Ausubel.

Cloning

A genomic library from Rm1021 in cosmid pLAFR1 (18) was mated into a Gm^rSp^r derivative of Rm1021-5MT60 with selection for cosmid tetracycline resistance. Fast-growing colonies were found among the transconjugants at about 0.5% of Tc^r colonies and were Fix^+. Subclones were isolated from a *Sau*3A partial digest library of pMW10 made in pRK404 (6) and were mated *en masse* into Rm1021-5MT60 or SU47 with selection for Tc^r of pRK404.

Fixation, Staining, and Microscopy

Two and one-half week old nodules induced by Rm1021-αMT8 were prepared for electron microscopy as described by Hirsch *et al.* (12, 13).

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[3] Abbreviations: IAM, indole-3-acetamide; IPA, indole-3-pyruvate; IAD, indole-3-acetaldehyde; NTG, nitrosoguanidine; αMT, α-methyl tryptophan; 5MT, 5-methyl tryptophan; 5FT, 5-methyl tryptophan; Sm, streptomycin.

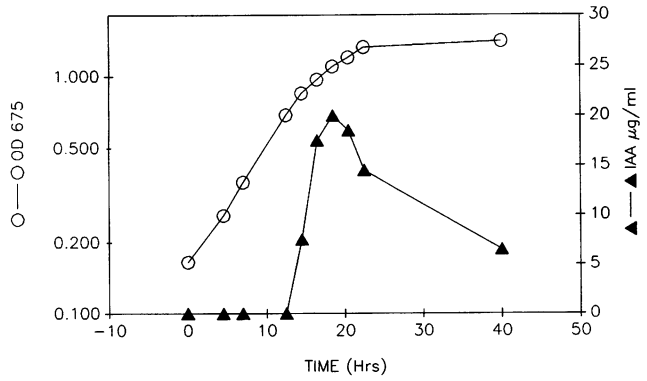

Figure 1. Accumulation of indole acetic acid (as measured by Salkowski reagent; black triangles) in cultures of Rm 1021 with 4 mM Trp. A_{675} of the culture is indicated by circles.

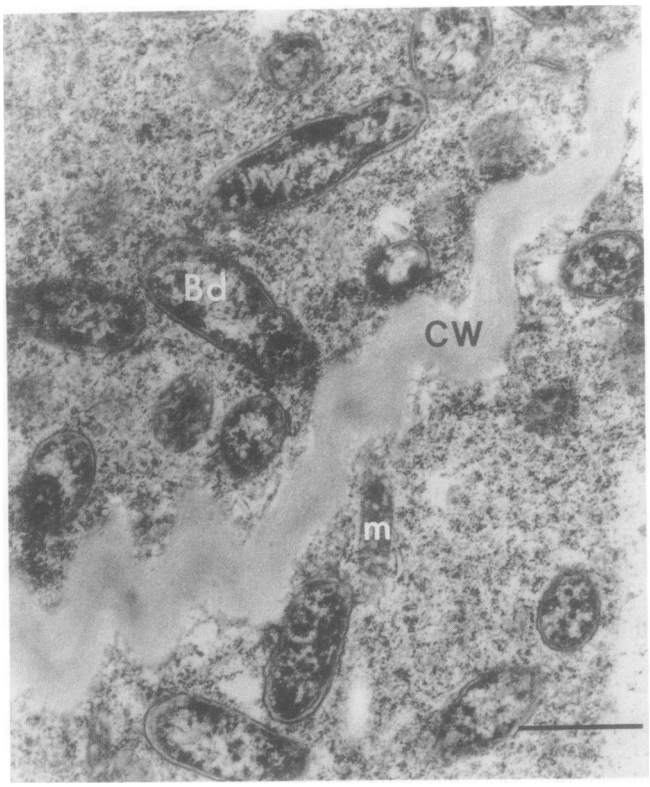

Figure 2. Electron micrograph (×19,000) of the symbiotic zone of a 2-½ week-old Fix⁻ nodule induced by Rm1021-αMT8 (courtesy of Ann Hirsch and Carol Smith). Bar = 1 μM. Bd, bacteroid, CW, cell wall; m, mitochondrion.

RESULTS AND DISCUSSION

Indole Acetic Acid Production

In minimal M9 medium + Trp, IAA first becomes visible when the cells enter stationary phase (OD_{675} ~0.7) and increases over a period of about 6 h before falling again (Fig. 1), possibly due to catabolism which has been observed in *Rhizobium* (23). TLC in two systems (see "Materials and Methods") clearly reveals spots that stain the same color as and comigrate with authentic Trp and IAA (data not shown).

Table I. *Mutant Phenotypes*

Allele	Growth Without Inhibitor	Sensitivity or Resistance of Colonies to Trp Analogs			Fix
		αMT[a]	5MT[b]	5FT[c]	
Rm1021	+++++	S	R	S	+
Rm1021-αMT8	++++	R	R	R	−
Rm1021-αMT9	+++	R/S	S	S	−
Rm1021-αMT16	++++	R	R	S	−
Rm1021-5MT60	+	S	S	R	−

[a] 500 μg/mL. [b] 150 μg/mL. [c] 150 μg/mL.

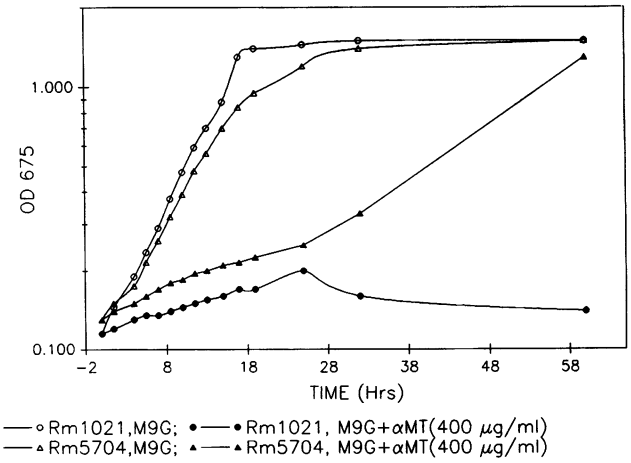

Figure 3. Growth of Rm1021 and Rm1021-αMT8 with and without αMT.

Variable faint spots resembling IPA and IAD are usually present also, consistent with IAA biosynthesis via transamination of Trp to IPA and subsequent decarboxylation to IAD and IAA, which is a pathway commonly seen in plants (27) and suggested for *Rhizobium* (9). However, there is no evidence of a spot for IAM, corresponding to the pathway unique to bacteria (*e.g. Pseudomonas savastanoi*) and crown gall, in which Trp is oxidatively decarboxylated to IAM which is then hydrolyzed to IAA (4). This is surprising because *R. meliloti* is closely related to the crown gall agent *Agrobacterium tumefaciens*, and the IAM pathway does occur in *Bradyrhizobium* sp. (24). In addition, at least one other intensely pink staining (indolic) spot that does not correspond to any of the standards, as well as several other weakly staining spots, are seen.

Nodulation-deficient mutant Rm1027 (18) produces very low levels of IAA at all growth phases, but this IAA⁻ phenotype is due neither to the nodulation deficiency mutation (*nodC*::ISRM1) nor to a Tn5 insertion in the chromosome linked to *pyr-49* in this strain (3). A nod⁺, Tn5-less derivative of Rm1027 is still IAA⁻ (28), and other *nodA, B, C*, or *D1* mutants tested (14) are IAA⁺.

Mutants with Altered Responses to Tryptophan Analogs

In the olive-knot bacterium *P. savastanoi*, Kosuge and associates (4, 25) have demonstrated that bacterially produced

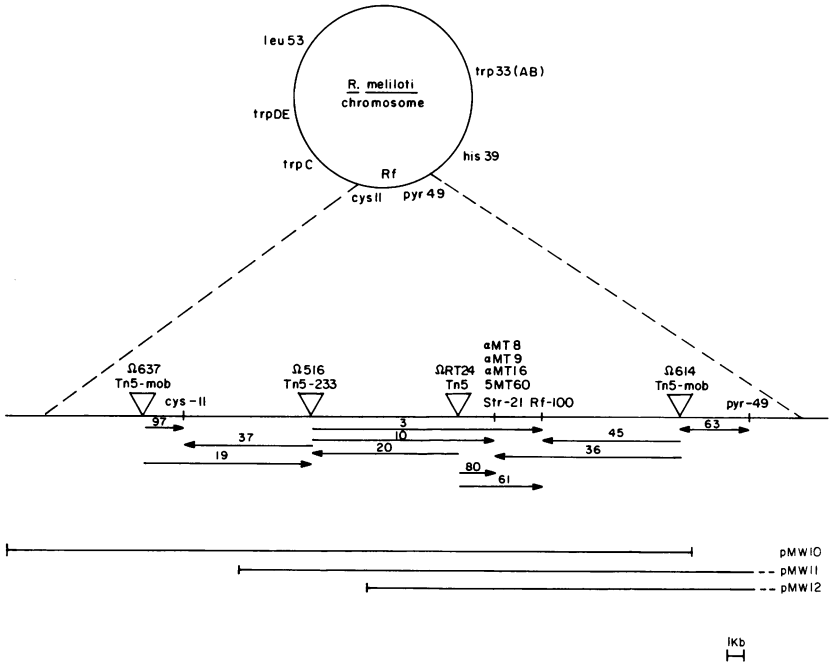

Figure 4. Chromosomal map and transductional linkage map (ϕM12) of the αMT region. Inserts of Tn5 and derivatives are indicated. Arrows run from selected (tail) to unselected (head) marker and show percent cotransduction. *str-21* has not been separated from the *MT* loci by transduction.

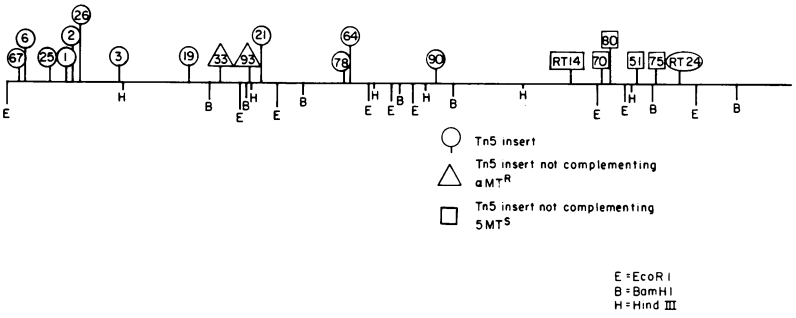

Figure 5. Restriction map of the *αMT* region showing extents of cosmid clones and Tn5 inserts. Inserts with triangular heads, in pMW20, fail to complement Rm1021-αMT8. Inserts with rectangular heads, in pMW10 or pMW11 (RT14), fail to complement Rm1021-5MT60.

Table II. *Complementation of Mutants by pMW10 and pMW11, pMW12*

Chromosomal Allele	pMW10					pMW11, pMW12				
	Growth	Sensitivity or resistance of colonies to Trp analogs			Fix	Growth	Sensitivity or resistance of colonies to Trp analogs			Fix
		αMT[a]	5MT[b]	5FT[c]			αMT[a]	5MT[b]	5FT[c]	
Rm1021	+++++	S	R	S	+	+++++	S	R	S	+
Rm1021-αMT8	+++++	S	R	S	+	+++	R	R	R	−
Rm1021-αMT9	+++++	S	R	NT[d]	+	++	R/S	S	NT	−
Rm1021-αMT16	+++++	S	R	NT	+	+++	R	NT	NT	−
Rm1021-5MT60	+++++	S	R	R	+	+++++	S	R	R	+

[a] 500 μg/mL. [b] 150 μg/mL. [c] 150 μg/mL. [d] Not tested.

IAA is essential for pathogenicity. Mutants isolated as resistant to αMT fail to produce IAA in culture and are avirulent (25), and wild type DNA restores virulence, IAA production and αMT sensitivity (4). Presumably, Trp analogs such as αMT inhibit endogenous Trp biosynthesis, and IAA$^-$ mutants survive because they do not divert the reduced pool of Trp from protein synthesis. Similarly, in *Azospirillum brasilense*, a nitrogen-fixing bacterium found in association with roots of grasses, mutants with altered response to Trp analogs produce an altered spectrum of Trp breakdown products (11).

In *R. meliloti* Rm1021, αMT at 400 μg/mL (1.8 mM) prevents the formation of colonies on minimal M9G medium. Twenty-six spontaneous or nitrosoguanidine induced mutants were isolated as able to form colonies on M9G or M9SA + αMT (500 μg/mL). Although all form nodules on alfalfa, nodules of three mutants (Rm1021-αMT8, Rm1021-αMT9,

Rm1021-αMT16) are large, white, and cylindrical and fail to fix nitrogen. At 2½ weeks, when wild-type nodules are actively fixing nitrogen, these nodules are already senescent. When the nodules are examined in the light and electron microscope, an abnormally large number of starch grains are visible. Bacteroids are not as elongated as those of wild-type, and cytoplasm appears degenerate (Fig. 2).

Growth responses to Trp analogs are shown in Table I for these three mutants and for mutant Rm1021-5MT60, isolated by M. Honma and F. Ausubel by replica plating as sensitive to 5MT and found to be Fix⁻. αMT inhibits growth of both wild-type and αMT^r strains, but the resistant mutants grow to saturation, whereas wild type does not (Fig. 3). Inhibition of wild type is competitively reversed by a 600-fold lower molar concentration of Trp, or by a 300-fold lower concentration of anthranilic acid, the first specific precursor of Trp synthesis, but not by phenylalanine or tyrosine. Thus, αMT makes Rm1021 a conditional auxotroph, probably by inhibiting anthranilate synthetase (22). The 5MT sensitivity of Rm1021-αMT9 is not reversed by added Trp, even at equimolar concentrations. All the MT mutants produce IAA as determined by Salkowski assay, and TLC reveals approximately the same pattern of tryptophan degradation as in Rm1021. Rm1021-αMT9 appears to produce slightly more IAA than Rm1021 by both Salkowski assay and TLC. Both Rm1021-αMT9 and Rm1021 are able to degrade 5MT and 5FT to yield what are probably 5-methyl-IAA and 5-fluoro-IAA as well as other compounds. Neither wild type nor any of the mutants degrades αMT, indicating that altered sensitivity to Trp analogs is not caused by differential detoxification.

In transduction, mutations *αMT8, αMT9, αMT16* and *5MT60* are all linked to chromosomal insert Ω516::Tn*5-233* (Fig. 4). When any of these strains is transduced to the wild-type phenotype (αMT^s or 5MT^r), it regains the ability to form Fix⁺ nodules. Similarly, when mutation *αMT8* is introduced into Rm 1021 by linked transduction it is accompanied by the Fix⁻ phenotype. Each *MT* mutation is therefore sufficient for the Fix⁻ phenotype. Other markers in this region of the chromosome (19) include *cys-11, pyr-49, str-21*, and *rif-100*.

R. meliloti genes for all the Trp biosynthetic steps have been characterized and mapped to three chromosomal loci (G. Barsomian, personal communication), none of which corresponds to the *MT* region. When Tn*5* insertions in genes at two of these loci, *trpB* and *trpC*, are transduced into Rm1021-αMT8, the resulting auxotrophs are still viable and Fix⁻, indicating that the symbiotic phenotype does not result from abnormal production of an intermediate in the Trp pathway after *trpC* (indole glycerol phosphate synthase). Tn*3*-HoKm *lacZ* fusions in *trpD* or *trpE* (G. Barsomian, personal communication) transduced into Rm1021-αMT8 and Rm1021-5MT60 showed no dramatic alteration in *lacZ* expression as determined by blue color on X-Gal indicator agar, suggesting that the *MT* mutations do not strongly affect *trp* expression in free living cells.

Strain Rm1021-5MT60 has a characteristic slow growth phenotype on LB agar (Table I). Among plasmid clones that complement the *5MT60* mutation (Fig. 5), pMW10 also complements αMT^r strains to sensitivity, but pMW11 and pMW12 do not; unexpectedly, however, pMW11 and pMW12 do slow the growth of these strains even further on both rich and minimal media (Table II). Thus mutations *5MT60, αMT8, αMT9*, and *αMT16* are all recessive, and mutation *5MT60*, although closely linked, is not in the same complementation group as the *αMT* mutations.

Tn*5* mutagenesis of pMW10 suggests regions that correspond to genes encoding αMT^r and 5MT^s (Fig. 5). However, extensive attempts to exchange inserts in these putative genes into the chromosome with the incompatible plasmids pPH1JI or R751 were unsuccessful, suggesting that null mutations cannot be made in them.

Plasmid pMW10 also confers a high level of Sm resistance (>2 mg/mL) on Sm^s *R. meliloti* and *A. tumefaciens*, indicating that it also carries the *str-21* locus, already known to be linked by transduction (Fig. 4). pMW10 does not confer Sm^r on *Escherichia coli*. In *E. coli*, Sm^r, encoded by the ribosomal protein gene *rps*L, is recessive to the Sm^s allele in single copy phage vectors. It is not clear whether the *R. meliloti str* locus is equivalent to *E. coli rps*L, or whether the multicopy vector pLAFR1 is responsible for the dominant phenotype.

In summary, *R. meliloti* Rm1021 produces IAA. Mutations altering response to Trp analogs also confer a Fix⁻ phenotype, apparently due to a block in bacteriod differentiation. The mutations appear to be in essential genes, but these genes do not appear either to be involved in Trp biosynthesis or strongly to affect production of IAA or related compounds.

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