Isolation and symbiotic characterization of aromatic amino acid auxotrophs of *Sinorhizobium meliloti*

C Krishna Prasad*, Vineetha K E', Raad Hassani, Ruma Gupta & Gursharn S Randhawa

Department of Biosciences and Biotechnology, University of Roorkee, Roorkee 247 667, India

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Ten aromatic amino acid auxotrophs of *Sinorhizobium meliloti* (previously called *Rhizobium meliloti*) Rmd201 were generated by random mutagenesis with transposon Tn5 and their symbiotic properties were studied. Normal symbiotic activity, as indicated by morphological features, was observed in the tryptophan synthase mutants and the lone tyrosine mutant. The *trpE* and *aro* mutants fixed trace amounts of nitrogen whereas the *phe* mutant was completely ineffective in nitrogen fixation. Histology of the nodules induced by *trpE* and *aro* mutants exhibited striking similarities. Each of these nodules contained an extended infection zone and a poorly developed nitrogen fixation zone. Transmission electron microscopic studies revealed that the bacteroids in the extended infection zone of these nodules did not show maturation tendency. A leaky mutant, which has a mutation in *trpC*, *trpD*, or *trpF* gene, was partially effective in nitrogen fixation. The histology of the nodules induced by this strain was like that of the nodules induced by the parental strain but the inoculated plants were stunted. These studies demonstrated the involvement of anthranilic acid and at least one more intermediate of tryptophan biosynthetic pathway in bacteroidal maturation and nitrogen fixation in *S. meliloti*. The alfalfa plant host seems to provide tryptophan and tyrosine but not phenylalanine to bacteroids in nodules.

Rhizobia enter into symbiotic relationship with leguminous plants. This is a complex relationship in which free living bacteria induce and invade root nodules, transform into bacteroids and fix nitrogen. Most of the genes involved in these processes have been characterized. These include genes governing nodulation and nitrogen fixation. Except for the rhizobial dependence on the host for C4-dicarboxylic acids information about the other nutrients required by bacteroids and provided by the host is very little.

Disruption of the biosynthetic pathways of some amino acids has been known to affect the symbiotic capabilities of *Rhizobium* 

In most of the cases it is unclear if the symbiotic defect is merely due to unavailability in planta of particular amino acids to the bacterium or a direct role of the intermediates of these amino acids in symbiosis. The tryptophan biosynthetic pathway has been shown to be involved in nodulation in *Bradyrhizobium japonicum* and nitrogen fixation in *Sinorhizobium meliloti*. It has also been shown that a diminished flow of metabolites through aromatic pathway affects nitrogen fixation. It is apparent from these investigations that more information on the role of the pathways of aromatic amino acids in symbiosis is required. In this paper we present the symbiotic properties of 10 aromatic amino acid auxotrophs and describe the histological aspects of the nodules induced by them.

**Materials and Methods**

**Bacterial strains and plant cultivar**

The bacterial strains used/constructed in the present study are given in Table 1. Alfalfa (*Medicago sativa* cv. T9) seeds were procured from National Seeds Corporation, New Delhi.

**Media, growth conditions and supplements to media**

Complete media TY and MSY, and minimal medium RMM were used for growing *S. meliloti* strains. E. coli strain WA803 (pGS9) was grown in LB medium. *S. meliloti* and *E. coli* cultures were incubated at 30° and 37°C, respectively. Whenever required streptomycin sulphate and kanamycin sulphate (Sigma), at concentrations of 200 and 400 μg/ml, respectively, and each amino acid/amino acid intermediate at a concentration of 50 μg/ml, were
Table 1 — Bacterial strains and plasmids used/constructed

<table>
<thead>
<tr>
<th>Strains/Plasmids</th>
<th>Relevant characteristics</th>
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<tbody>
<tr>
<td>Sinorhizobium meliloti Rmd201</td>
<td>Spontaneous Str' derivative of AK 631 (Nod', Fix')</td>
<td>Khanuja and Kumar, 1988</td>
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<td>PP631</td>
<td>AK631 (pJB3JI)</td>
<td>Peter Pupnoky</td>
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<td>ZB555</td>
<td>Rm41 cys46 phe15 rif1 str1</td>
<td>This study</td>
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<tr>
<td>VK1</td>
<td>Rmd 201 trpE::Tn5</td>
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<td>VK15</td>
<td>Rmd 201 trp::Tn5</td>
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<td>VK18</td>
<td>Rmd 201 ara::Tn5</td>
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<tr>
<td>VK28</td>
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<td>WA803 (pGS9)</td>
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<td>Selvaraj and Iyer, 1983</td>
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<td>Plasmids</td>
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<td>IncN, repP15A, Cm', Km'</td>
<td>Selvaraj and Iyer, 1983</td>
</tr>
<tr>
<td>pJB3JI</td>
<td>Km' derivative of pR68.45 Capable of mobilising genomic segments of its host, Tc', Ch', Nal'</td>
<td>Brewin et al. 1980</td>
</tr>
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</table>

added to RMM medium. Nitrogen free plant growth medium was used for carrying out plant assays.

**Tn5 mutagenesis and screening for aromatic amino acid auxotrophs**

Random mutagenesis of *S. meliloti* Rmd201 strain with transposon Tn5 was done as described. Transposon Tn5-induced kanamycin resistant transconjugants were streaked on RMM and TY media to identify auxotrophs. The nutritional requirements of auxotrophs were determined by observing their growth on RMM medium supplemented with Holliday pools.

**Location of block in biosynthetic pathway in each auxotroph**

The tryptophan auxotrophs were streaked on RMM medium supplemented with anthranilic acid, indole, or tryptophan and *aro* mutants on RMM medium supplemented with shikimic acid. The growth patterns of auxotrophs were recorded after an incubation period of 3-5 days at 30°C.

For intermediate accumulation studies, tryptophan auxotrophs were inoculated in minimally supplemented (with 2 μg/ml tryptophan) RMM liquid medium and incubated for 3 days. The supernatant obtained after centrifugation of the culture was assayed for the presence or absence of anthranilic acid and indole using p-dimethyl aminobenzaldehyde reagent as described by Snell and Snell, and indole glycerol phosphate using ferric chloride reagent according to the method of Yanofsky and Smith.

Cross feeding assays were done by streaking different combinations of *trp* auxotrophs close to each other on minimally supplemented (2 μg/ml tryptophan) RMM solid medium. The growth of the strains was followed over a period of 3-5 days at 30°C.

**Linkage of Tn5 insertion to auxotrophy**

Bacterial matings were performed according to Kondorosi et al. The plasmid pJB3JI, which has the ability to mobilize chromosomal segments, was introduced into each auxotroph by conjugation and selected by its tetracycline resistance marker. The resulting strain was mated as a donor with the recipient strain *S. meliloti* ZB555. Forty kanamycin resistant transconjugants were selected for each mating and tested for presence of the donor’s auxotrophic markers.

**Plant inoculation studies**

Alfalfa seeds were sterilized as described by Vincent and transferred onto nitrogen-free agar slants in 20×2.5 cm tubes. Two 2-days old seedlings in each tube (5 replicates) were inoculated with 10⁷ cells (suspended in sterile distilled water) of a particular rhizobial strain. The growth conditions for the plants were 2000 lux light, a photoperiod of 16 hr, a dark period of 8 hr and 25°C temperature. The morphological features of plants were recorded six weeks after inoculation. For determining the dry plant shoot weight the plant tops were collected and dried.
in an oven at 65°C for 72 hr and then weighed. Reisolation of bacteria from nodules was done to confirm the nodule occupancy by a particular strain. If nodules on a plant were found to be inhabited by prototrophic revertant cells the data on the plant was discarded.

**Histology of nodules**

Nodules from 6 weeks old plants were excised and processed for optical and transmission electron microscopic (TEM) studies. For a given strain four nodules, one from each of four different plants, were collected and each was cut into two halves. One half was used for determining the nodule occupancy and the other half was immediately dipped in fixative. To facilitate penetration of the fixative 0.1% acrolein was added to it. The fixation was carried out at 4°C for 2 to 24 hr. Following fixation the nodules were washed twice in 0.1M phosphate buffer and postfixed in 1% OsO₄ at 4°C for 2 hr. The nodules were dehydrated in graded acetone series (30-100%) and cleared off acetone by placing them in toluene at room temperature for 60 min. Embedding was done in araldite medium using beam capsules. An ultramicrotome was used to cut thin sections (0.5-2.0 μm) which were stained with 1% toluidine blue (prepared in 1% borax) for 30 sec and observed under optical microscope after washing with distilled water. Ultrathin sections (60-90 nm) stained with lead citrate and uranyl acetate were used for TEM studies.

**Results**

**Tn5 mutagenesis and isolation of aromatic amino acid auxotrophs**

Random mutagenesis with transposon Tn5 yielded 18,100 kanamycin resistant transconjugants of S. meliloti Rmd201. Auxotrophs among these Tn5 derivatives were identified on the basis of their inability to grow on minimal medium RMM. By streaking these auxotrophs on RMM medium supplemented with Holliday pools and subsequently observing growth after incubation 10 aromatic amino acid auxotrophic mutants were obtained. Out of these mutants six (VK1, RH4, RH6, VK15, VK28 and VK30) were auxotrophic for tryptophan, one (RG3) for tyrosine, one (RH38) for phenylalanine and two (VK18 and RH5) for all these three amino acids.

**Location of biochemical block in each auxotroph**

Out of six tryptophan (trp) auxotrophs three (VK1, RH4 and RH6) grew on anthranilic acid supplementation and did not accumulate any tryptophan intermediate; these three were hence *trpE* mutants. The *trp* mutant VK15, which is a leaky mutant, grew on indole supplementation and

![Pathways of synthesis of aromatic amino acids showing positions of mutations in the auxotrophs obtained](image)

**Fig. 1** — Pathways of synthesis of aromatic amino acids showing positions of mutations in the auxotrophs obtained. Abbreviations: DAHP, 3 hydroxy- D-arabino-heptulosonate-7-phosphate; PRA, Phosphoribosyl anthranilate; CDRP, 1-(o-carboxyphenylamino)-1-deoxyribulose-5-phosphate; IGP, Indole glycerol phosphate; HPP, 4-Hydroxy phenylpyruvate; PP, Phenylpyruvate.
accumulated anthranilic acid. The two trp auxotrophs, VK28 and VK30, were tryptophan synthase mutants since they grew only on tryptophan supplementation and accumulated anthranilic acid and indole glycerol phosphate. All trpE mutants were cross-fed by mutant VK15 as well as tryptophan synthase mutants, and VK15 mutant was cross-fed by tryptophan synthase mutants. The mutants VK18 and RH5 required the supplementation of all three aromatic amino acids for their growth on minimal medium. The mutation in each of these two mutants was possibly in the common aromatic pathway and hence these were called aro mutants. These mutants did not grow on shikimic acid supplementation. The possible location of biochemical block in each auxotroph has been given in Fig. 1.

Symbiotic properties

The nodules induced by the tryptophan synthase mutants and the tyrosine mutant were pink and cylindrical like those induced by the parental strain Rmd201. The symbiotic effectiveness (as indicated by the mean dry shoot weight of the inoculated plants) of these strains was exactly like that of the parental strain. On the other hand, the plants inoculated with the aro and trpE mutants became chlorotic six weeks after inoculation. The mean dry shoot weight of the plants inoculated with an aro or trpE mutant was significantly less than that of the Rmd201 inoculated plants but significantly more than that of the un inoculated plants. The nodules induced by the aro and trpE mutants were white and cylindrical. The plants inoculated with VK15 auxotroph showed no shoot elongation four weeks after inoculation but chlorosis did not appear. Even though the nodules on these plants were pink, the mean dry shoot weight of these plants was significantly less than that of plants inoculated with the parental strain. The mean dry shoot weight of the VK15 inoculated plants was however more than that of the aro and trpE inoculated plants. The plants inoculated with the phe mutant did not differ significantly from the uninoculated plants with respect to dry shoot weight. The nodules induced by this mutant were irregular and white (Table 2).

Light microscopic studies of the nodules

The longitudinal cross section of a nodule induced by the parental strain Rmd201 revealed four main zones: An apical meristem, a short infection zone, an extensive nitrogen fixation zone and a senescent zone (Fig. 2A). The histology of nodules induced by the tryptophan synthase mutants and the tyrosine mutant resembled that of the nodules induced by Rmd201 strain in all respects (data not shown). The nodules induced by strain VK15 also exhibited the same gross histological features (Fig. 2B). The nodules induced by trpE or aro mutants had histological features quite different from that of the parental strain induced nodules. Unlike in the parental strain induced nodule, where the infection zone was small, the nodule induced by a trpE or aro mutant had an infection zone that occupied most part of the nodule. A distinct nitrogen fixation zone was not seen but it was represented near to the base of the nodule by a few nodule cells filled with mature bacteroids (Fig. 2C and 2D). In the same way a distinct interzone between the infection zone and the nitrogen fixation zone, like that of the Rmd201 induced nodule, was also missing. The nodules induced by the phe mutant were altogether different; these were poorly developed and the histology of the entire nodule resembled the histology of the infection zone of the Rmd201 induced nodule. A network of infection threads was seen throughout the nodule (Fig. 2E).

Higher magnifications of the infection zone of the parental strain Rmd201 induced nodules showed prominent nuclei and many bacteroids in the infected cells (Fig. 3A). In comparison to the parental strain induced nodules the infected cells in the infection zone of the nodules induced by trpE and aro mutants contained less prominent nuclei and less number of bacteroids (Fig. 3B and 3C). Each nodule cell containing bacteroids in the so called nitrogen fixation

| Table 2 — Nodule characteristics and mean dry shoot weights of plants inoculated with aromatic amino acid auxotrophs of Sinorhizobium meliloti Rmd201 strain |
|---|---|---|---|
| Strain | Nodule characteristics | Mean dry shoot weight (mg) |
| Uninoculated control | — | 6.2 ± 0.9 |
| Rmd201 | Cylindrical | 33.6 ± 2.5 |
| VK28 | — | 29.5 ± 3.2 |
| VK30 | — | 31.0 ± 2.7 |
| RG3 | — | 28.7 ± 4.0 |
| VK1 | — | 19.8 ± 2.0* |
| RH4 | — | 12.9 ± 1.6* |
| RH6 | — | 12.0 ± 1.4* |
| VK15 | — | 20.8 ± 1.2* |
| H5 | — | 11.2 ± 2.0* |
| VK18 | — | 12.9 ± 1.9* |
| RH38 | Irregular | 8.1 ± 1.6* |

*Significantly less than that of the parental strain Rmd201 (P < 0.05)  
†No significant difference from uninoculated control  
‡Each mean dry shoot weight value is a mean of dry shoot weights of eight plants.
zone was similar to that of the parental strain induced nodules in having a centrally located vacuole and radially arranged bacteroids; however a few such cells were present (Fig. 3D-3F).

**TEM studies of the nodules**

The TEM studies showed that the cytoplasm of released bacteria in the infection zone of the nodules induced by the parental strain Rmd201 was somewhat heterogeneous (Fig. 4A). It has been reported that this change in bacteroidal cytoplasm is due to the condensation of the nuclear material. The bacteroidal development in different zones of the nodules induced by the tyrosine mutant, tryptophan synthase mutants and the VK15 mutant was similar to that of the parental strain induced nodules (photographs not shown). The bacteroids in the infection zone in case of trpE and aro mutants did not show maturation. The size of the bacteroids remained small and the bacteroidal cytoplasm appeared homogeneous indicating that the

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*Fig. 2—Light microscopic studies of longitudinal-semithin sections of the nodules induced by *Sinorhizobium meliloti* Rmd201 and its aromatic amino acid auxotrophs. A. Rmd201 induced nodule showing apical meristematic, infection, nitrogen fixation and senescence zones, and peripheral vascular bundle. B. VK15 induced nodule showing histological structure like that of Rmd201 induced nodule. C. VK1 (trpE mutant) induced nodule showing extended infection zone and poorly developed nitrogen fixation zone. D. VK18 (aro mutant) induced nodule showing structure similar to that of VK1 induced nodule. E. RH38 (phe mutant) induced nodule containing an extended infection zone. Bar, 100 μm (x100). Abbreviations: Apical meristem (M), infection zone (I), nitrogen fixation zone (NZ), senescence zone (S) and peripheral vascular bundle (VB).*
condensation of nuclear material did not occur. An electron dense material (likely to be leghæmoglobin) present in the infected cells of the parental strain induced nodules was also missing in the infected cells of the nodules induced by trpE and aro mutants (Fig. 4B and 4C). Lysing bacteroids were not seen in the trpE mutant induced nodules but were visible in the nodules induced by the aro mutant. Some of the nodule cells near to the nitrogen fixation zone of trpE or aro mutant induced nodules had bacteroids with slightly condensed nuclear material and the number of bacteroids in each of these cells was certainly more than that in each of the infection zone cells. The bacteroids in the nitrogen fixation zone in trpE or aro induced nodules were similar to the mature bacteroids of the parental strain in all respects except for the decreased heterogeneity of the cytoplasm (photographs not shown). In the phe mutant induced nodules the released bacteria were in a degenerating condition immediately after their release (Fig. 4D).

**Linkage of Tn5 insertion to auxotrophy and symbiotic defect**

Plasmid pJB3JI has the ability to mobilize the

![Fig. 3 — Light microscopic studies of longitudinal-semithin sections of nodules induced by Sinorhizobium meliloti Rmd201 and its trpE and aro mutants. A, B and C. Infection zones of nodules induced by Rmd201, VK1 (trpE mutant) and VK18 (aro mutant), respectively; number of bacteroids per nodule cell is less in VK1 and VK18 induced nodules as compared to that in Rmd201 induced nodules. D, E and F. Nitrogen fixation zones of nodules induced by Rmd201, VK1 and VK18, respectively; only a few nodule cells contain bacteroids in the latter two cases as compared to many in Rmd201 induced nodules. Bar, 25 µm (×400). Abbreviations: Bacteroid (bc), infection thread (it), vacuole (v), nucleus (n) and empty cell (ec).
segments of rhizobial genome from donor cells to suitable recipient cells; integration of the mobilized donor DNA occurs in the recipient cell. A donor strain of each auxotroph was constructed by the introduction of plasmid pJB3JI. Transposon Tn5 encoded kanamycin resistance was transferred to the S. meliloti ZB555 recipient strain. In these crosses all kanamycin resistant transconjugants had respective donor’s auxotrophy which showed 100% linkage of Tn5 insertion to auxotrophy in each auxotroph. The revertants of auxotrophs showed normal symbiotic properties indicating that in each mutant a single Tn5 insertion was responsible for auxotrophy as well as the symbiotic defect.

**Discussion**

Symbiotic properties of 10 aromatic amino acid auxotrophs of S. meliloti Rmd201 strain were studied to determine the role of biosynthetic pathways of aromatic amino acids towards symbiotic nitrogen fixation process. Anthranilate synthase (trpE) mutants were almost ineffective in nitrogen fixation and each nodule induced by these mutants had an extended infection zone and a poorly developed nitrogen fixation zone located at the base. Electron microscopic studies showed that the bacteroids in the infection zone as well as in the interzone between the infection zone and the nitrogen fixation zone did not show any tendency towards maturation. It seems that

Fig. 4 — TEM studies of longitudinal-ultrathin sections of nodules induced by Sinorhizobium meliloti Rmd201 and its aromatic amino acid auxotrophs. A, B and C: Bacteroids in the nodule cells from the infection zones of nodules induced by Rmd201, VK1 (trpE mutant) and VK18 (aro mutant), respectively; cytoplasm of Rmd201 bacteroids is slightly heterogeneous whereas homogeneous cytoplasm is present in VK1 and VK18 bacteroids. D. RH38 (phe mutant) induced nodule showing degenerating bacteroids in the nodule cells from infection zone. Bar, A. 1 μm (x4,100), B. 1 μm (x8,400), C. 1 μm (x10,800), and D. 1 μm (x5,800). Abbreviations: Bacteroid (bc), peribacteroid membrane (pbm), vacuole (v) and senescent bacteroid(s).
The absence of some factor(s) halted the bacterial growth and maturation processes. Similar results for the anthranilate synthase mutants of S. meliloti were obtained by Barsomian et al. who suggested that anthranilic acid may act as in planta siderophore, promoting iron uptake which is essential for heme and nitrogenase synthesis and thereby the maturation of bacteroids.

Tyrosine seems to be available to the aro mutants in the nodules from the host plant since the tyr auxotroph was symbiotically effective. Anthranilic acid unavailability may have resulted in extended infection zones in the nodules induced by these mutants as similar to that of the nodules induced by the aro mutants as the histology of the nodules induced by these mutants was similar to that of the nodules induced by trpE mutants. It is unknown why the nodules induced by the aro mutants did not show the histological aspects similar to that of the phe mutant induced nodules in which the defect was severe and the bacteria lysed immediately after their release without proceeding to any of the committed transformation stages. It may be possible that the plant host provided one or more intermediates (most probably prephenate) of phenylalanine from which the aro mutant could synthesize phenylalanine whereas the phe mutant could not do so due to the position of mutation in the biosynthetic pathway.

The plants inoculated with VK15 auxotroph, which could synthesize anthranilic acid, were stunted. The histological studies revealed no abnormalities in bacteroid development. It seems that the mature bacteroids need, apart from anthranilic acid, some other tryptophan intermediate(s) also for normal nitrogen fixation. The partial efficiency of this mutant was probably due to its leakiness, allowing the production of trace amounts of intermediates. Normal nodule development and nitrogen fixation in the mutants blocked at the last step of tryptophan biosynthesis indicates that tryptophan is available to bacteroids from the plant host. Thus we conclude that the alfalfa plant supplies tryptophan, tyrosine and at least one intermediate of phenylalanine to the S. meliloti microsymbiont. Normal supply of anthranilic acid and at least one more intermediate (unspecified) of tryptophan biosynthesis in these bacteria appears to be necessary for normal symbiotic activity with alfalfa plants.

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References