Rhizobium meliloti Mutants with Decreased DAHP Synthase Activity are Sensitive to Exogenous Tryptophan and Phenylalanine and Form Ineffective Nodules

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Received 6 June 1992. Accepted 2 October 1992.

We isolated two Tn*5*-generated mutants of *Rhizobium meliloti* whose growth was inhibited by rich medium or by exogenous tryptophan or phenylalanine. These mutants, Rm7479 and Rm7480, belonged to the same genetic complementation group. The mutant locus could not be found on either indigenous megaplasmid but was localized on the chromosome. The mutants formed ineffective nodules on alfalfa plants. They invaded nodules within infection threads and were released into plant cells enclosed within peribacteroid membranes, but once released into the plant cells they failed to differentiate into mature bacteroids. The mutants demonstrated a decrease in total 2-keto-3-deoxy-D-arabino-heptonic acid 7-phosphate synthase (DAHP synthase) activity, which is the first committed step in aromatic biosynthesis. Wild-type genes were isolated that complemented in one case or suppressed in another case, all three mutant phenotypes: growth on rich medium, symbiotic effectiveness, and DAHP synthase activity. Each mutant strain gave rise to linked second-site suppressor mutations that restored growth on rich medium. The suppressor mutants showed restoration of near wild-type DAHP synthase levels. One of the suppressor strains restored effective symbiosis while the other did not. Genetic complementation experiments showed that growth on rich medium, DAHP synthase activity, and effective symbiosis were all affected by the same genetic lesion. These results suggest that normal flux of metabolites through the aromatic biosynthesis pathway is essential for bacteroid development.

The gram-negative soil bacterium *Rhizobium meliloti* enters into a symbiotic relationship with alfalfa, *Medicago sativa*. This is a complex relationship whereby free-living bacteria induce root cortex tissue to divide and differentiate into a novel organ called a root nodule. As the nodule is forming, the bacteria enter the nodule through a symbiotic structure called an infection thread, which forms in an infected root hair. The bacteria proliferate within the elongating infection threads and are eventually released into the cytoplasm of newly formed nodule cells. As intracellular symbionts, the bacteria are compartmentalized within a host-derived peribacteroid membrane. They differentiate into bacteroids that characteristically show novel club and "Y" shaped morphologies as well as the ability to fix atmospheric N_2 to NH_3. The NH_3 is excreted by the bacteroids and assimilated by the plant. Numerous genes affecting nodule formation (*nod*) (Fisher and Long 1992), nodule invasion (*exo, ndv*) (Charles *et al.* 1991; Dylan *et al.* 1986; Geremia *et al.* 1987; Leigh and Coplin 1992), and nitrogen fixation (*nif, fix*) (Batut *et al.* 1989; David *et al.* 1988; Earl *et al.* 1987; Long *et al.* 1988; Putnoky *et al.* 1988) have been identified and characterized.

Nutritional conditions in the nodule or in the developing bacteroid are thought to affect bacteroid development and nitrogen fixation. Bacteroid utilization of C_4-dicarboxylic acids, derived from plant photosynthate, is essential for symbiotic nitrogen fixation (Engelke *et al.* 1987; Jiang *et al.* 1989; Waston *et al.* 1988; Yarosh *et al.* 1989). Less clear is the role of factors such as amino acids. Glutamate, serine, glycine, glutamine, and most tryptophan auxotrophs yielded effective nodules (Barsomian *et al.* 1992; de Bruijn *et al.* 1989; Kerpolla and Kahn 1985; Kraus and Kahn 1988), suggesting that these amino acids are either available to the bacteria *in planta*, or are not needed for bacteroid development. However, strains that were auxotrophic for asparagine, arginine, leucine, methionine, tyrosine, or anthranilate yielded ineffective nodules (Barsomian *et al.* 1992; Kerppola and Kahn 1985, 1988; Miller 1972), suggesting that the bacteria are not exposed to these amino acids *in planta* and are therefore unable to grow or develop into mature bacteroids. A *R. meliloti ilvC* mutant (Aguilar and Grasso 1991) and *Bradyrhizobium japonicum* histidine auxotrophs (Sadowsky *et al.* 1986; So *et al.* 1987) did not form nodules on their respective host plants. In most cases, ineffective nodules formed by the above auxotrophs were not subjected to microscopy, and it is unclear if the symbiotic defect was due to lack of bacterial colonization of the nodule or due to a defect in later steps of bacteroid development.

We have characterized two transposon-generated *R. meliloti* mutants whose growth was inhibited by exogenous phenylalanine or tryptophan. These mutants formed ineffective nodules on alfalfa plants in which the bacteria invaded nodules within infection threads and were released,

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MPMI Vol. 6, No. 1, 1993, pp. 135-143

surrounded by a peribacteroid membrane, into plant cells. However, normal bacteroid development did not occur. These mutants showed a clear defect in 2-keto-3-deoxy-D-arabino-heptonic acid 7-phosphate synthase (DAHP synthase) levels, indicating that the net flux of aromatic biosynthesis had significantly decreased. Genetic complementation and suppression experiments showed that all of the mutant phenotypes were related.

RESULTS

Isolation of mutants as well as DNA that complemented or suppressed the mutant phenotypes.

Two Tn*5* insertion mutants of *R. meliloti*, Rm7479 and Rm7480, failed to grow on L agar. These mutants grew slowly on M9 succinate medium, forming mucoid colonies that fluoresced more intensely than wild type in the presence of Calcofluor, an indicator of *R. meliloti* exopolysaccharide synthesis. To determine the linkage of the Tn*5* insertions to the mutant phenotypes, the Nm resistance markers of the above mutants were transduced into Rm1021. Almost all of the transductants (79 out of 80) derived from lysates of Rm7479, and all of the 80 transductants derived from Rm7480 lysates, failed to grow on L agar. Thus, the Tn*5* insertion in each mutant was tightly linked to the mutant locus responsible for the inability to grow on a rich medium.

A cosmid clone bank of Rm1021 genomic DNA was mobilized into strain Rm7480 and plated on L agar supplemented with Calcofluor, Sm, Nm, and Tc. Two distinct Tc-resistant colony phenotypes were observed. One class was indistinguishable from wild-type Rm1021; the other class formed more slowly growing colonies that fluoresced with extra intensity with Calcofluor (but were nonmucoid). Cosmids from representative colonies of each class were transferred to *E. coli* strain HB101 and characterized by restriction enzyme analysis. Restriction fragment patterns indicated that two distinct recombinant plasmids, pJGJ51 and pJGJ52, had been isolated and that colonies resembling wild-type Rm1021 carried cosmid pJGJ51, whereas the slow-growing colonies that fluoresced with extra intensity contained cosmid pJGJ52. Both cosmids, when mobilized into Rm7479 and Rm7480, restored growth on L agar. Strains Rm7479 and Rm7480 containing pJGJ51 continued to show wild-type colony growth on L agar and normal fluorescent intensity with Calcofluor, whereas both strains carrying pJGJ52 produced colonies that were slow growing and that fluoresced with extra intensity.

Genomic DNA prepared from strains Rm1021, Rm7479, and Rm7480 was digested with *Hin*dIII and subjected to Southern blot analysis using a nick translated 10-kb internal *Hin*dIII fragment from pJGJ51 DNA as a probe (Fig. 1). Rm1021 genomic DNA showed a 10-kb fragment that hybridized to the probe. Rm7479 and Rm7480 DNA were missing this 10-kb *Hin*dIII fragment but yielded two unique fragments with sizes consistent with a Tn*5* insertion in the 10-kb *Hin*dIII fragment. Plasmid pJGJ52 cut with *Hin*dIII did not hybridize to the probe. Therefore, pJGJ51 contained complementing DNA, whereas pJGJ52 contained DNA sequences capable of suppressing the growth defect on L agar. The Tn*5* mutant alleles of Rm7479 and Rm7480 were transferred onto pJGJ51 by homologous recombination, yielding plasmids pJGJ53 and pJGJ54, respectively. Plasmids pJGJ53 and pJGJ54 were mobilized into strains Rm7479 and Rm7480 and streaked onto L agar and M9 plates. Strains Rm7479 and Rm7480 harboring either pJGJ53 or pJGJ54 grew on M9 medium but failed to grow on L agar medium. Therefore, the two mutations belonged to the same genetic complementation group, although they may not represent identical genotypes if other genes are present in the same transcription unit.

Chromosomal localization of the mutant locus.

Genomic DNA from *Agrobacterium tumefaciens* strains with (At125, At128) or without (At123) the *R. meliloti* megaplasmids pRmeSU47a or pRmeSU47b (Finan *et al.* 1986b) were subjected to Southern blot analysis to determine if the 10-kb *Hin*dIII fragment containing the 7480 locus was located on either megaplasmid. With the 10-kb fragment from pJGJ51 as a probe, no hybridization was detected, while *nodDABC* and *dctA* probes specific to each megaplasmid hybridized to the appropriate bands in the appropriate strains. Therefore, the mutated locus in strains Rm7479 and Rm7480 did not reside on either of the *R. meliloti* megaplasmids. To map its chromosomal location, a Tn*5-233* insertion linked to Ω7480::Tn*5* (see Materials and Methods) was used in conjunction with previously described Tn*5*mob insertions in defined positions on the *R. meliloti* chromosome (Klein *et al.* 1992). The Gm-Sp

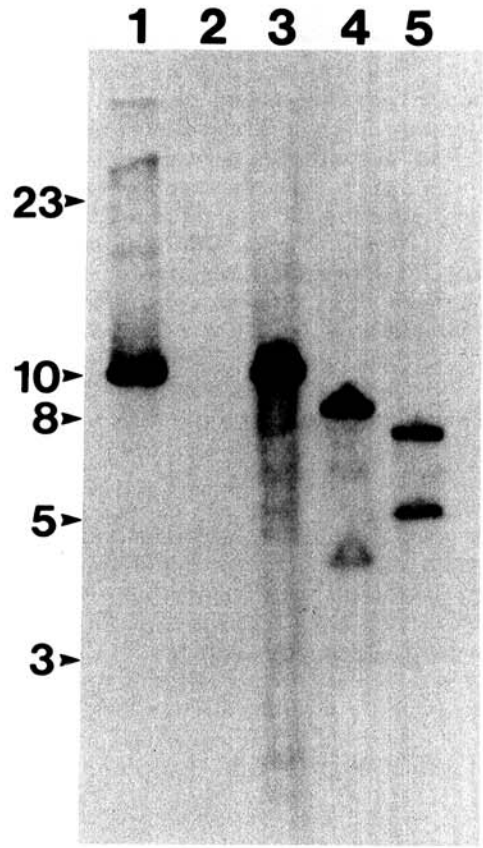

Fig. 1. Southern hybridization. *Hin*dIII digests of DNA probed with pJGJ51. Lane 1, pJGJ51; lane 2, pJGJ52; lane 3, Rm1021; lane 4, Rm7479; lane 5, Rm7480.

resistance marker of the Tn*5-233* insertion was mobilized by Ω615::Tn*5*mob at a frequency of 1×10^{-3} and by Ω611::Tn*5*mob at a frequency of 2×10^{-6}. The other four Tn*5*mob constructs showed mobilization frequencies less than 3×10^{-7}. Therefore, the marker was closely linked to Ω615::Tn*5*mob and lay between Ω615::Tn*5*mob and Ω611::Tn*5*mob on the *R. meliloti* chromosome (Klein *et al.* 1992).

Isolation of second-site suppressor mutants.

Rm7479 or Rm7480 streaked on L agar plates formed rare single colonies, which fluoresced with extra intensity with Calcofluor. Two such strains, Rm7481 and Rm7482, derived from Rm7479 and Rm7480, respectively, were isolated and further characterized. φM12 lysates were prepared from Rm7481 and Rm7482 and used to transduce the Nm resistance marker of Tn*5* into Rm1021 selecting for growth on M9 plates supplemented with Nm. Most of the Nm-resistant transductants (38 out of 58) derived from lysates of Rm7481 retained the ability to grow on L agar. Similarly, 69 out of 80 Nm resistant transductants derived from strain Rm7482 retained the ability to grow on L agar. Therefore, the mutations that restored the ability to grow on L agar were transductionally linked to the Tn*5* insertions but at lower frequencies relative to the parental mutant phenotypes. This observation indicated that the mutations in Rm7481 and Rm7482 were second-site suppressor mutations closely linked to the original Tn*5* mutations.

Two aromatic amino acids inhibit growth of mutants.

L agar medium contains NaCl, yeast extract, Bactotryptone (a tryptic digest of casein), and Bacto agar. To identify the growth-inhibiting substance in L agar, each of the above components were added to the permissive M9 succinate medium (Table 1). Yeast extract, Bactotryptone, and Casamino Acids (an acid hydrolysate of casein) all severely inhibited the growth of Rm7479 and Rm7480. Thus, one or more amino acids was sufficient to inhibit the growth of the mutant strains. The absence of NaCl from L agar did not relieve the growth inhibition of the mutants, nor did the addition of NaCl to M9 succinate medium cause any inhibition of growth. To determine which amino acid(s) were responsible for the growth inhibition, M9 succinate media were prepared that contained combinations of up to five amino acids. Each amino acid was present in two different "pools." This matrix approach has been used for identifying amino acids that restore growth to amino acid auxotrophic strains (Davis *et al.* 1980), but in this case was used to identify inhibitory amino acids. Only pool #8, containing proline, threonine, tyrosine, tryptophan, and phenylalanine showed marked growth inhibition of strains Rm7479 and Rm7480 (Table 1). When these amino acids were tested individually, neither proline, threonine, nor tyrosine alone had any inhibitory effect on the mutants. In contrast, either phenylalanine or tryptophan alone was sufficient to inhibit the growth of strains Rm7479 and Rm7480. Suppressor strain Rm7481 showed a partial restoration of growth, and suppressor strain Rm7482 showed near complete restoration of growth in the presence of these amino acids.

DAHP synthase activity.

The first step in aromatic biosynthesis is the condensation of phosphoenol pyruvate and erythrose-4-phosphate, forming DAHP. This reaction is catalyzed by DAHP synthase. *E. coli* has three isozymes of DAHP synthase, and each is inhibited allosterically by one of the three aromatic amino acids (Prittard 1987). Because growth of certain *E. coli* DAHP synthase mutants is inhibited in the presence of one or more aromatic amino acids (Prittard 1987), we were interested in determining the DAHP synthase activity in extracts of the above *R. meliloti* strains. The strains were grown without added amino acids, and sonic extracts were assayed for DAHP synthase activity with and without aromatic amino acids (Table 2). Rm1021 showed high levels of DAHP synthase activity, and this activity was subject to allosteric inhibition by tryptophan but not phenylalanine or tyrosine. Both Rm7479 and Rm7480 showed significantly decreased

Table 1. Growth of *Rhizobium meliloti* mutants

	Strain[a]				
Medium	**Rm1021**	**Rm7479**	**Rm7480**	**Rm7481**	**Rm7482**
L agar	+++	−	−	+++	+++
L agar without NaCl	+++	−	−	ND	ND
M9 succinate with					
No supplement	+++	++	++	+++	+++
NaCl	++	++	++	ND	ND
Yeast extract	++	+	−	ND	ND
Bacto tryptone	++	−	−	ND	ND
Casamino Acids	+++	−	−	+++	+++
pool #8	++	−	−	+	+
1.5 mM phenylalanine	+++	+	+	+	+++
0.5 mM tyrosine	+++	++	++	++	+++
0.5 mM tryptophan	+++	+	+	++	+++
1.5 mM threonine	+++	++	++	++	+++
10.0 mM proline	+++	++	++	+++	+++

[a] ND, not determined. +++, wild type growth on primary and secondary streak. ++, good growth on primary streak but poor growth on secondary streak. +, growth only on primary streak. −, no growth. Pool #8, contained the same concentrations of phenylalanine, tryptophan, tyrosine, threonine, and proline that were used in the single amino acid supplments.

DAHP synthase activity ($P < 0.005$); and the presence of tryptophan in the assay further lowered the activity in these extracts ($P < 0.025$ and $P < 0.005$, respectively). In contrast, the suppressor strains Rm7481 and Rm7482 regained near wild-type DAHP synthase activity levels and allosteric inhibition profiles. Rm7479 harboring either the complementing pJGJ51 or the suppressing pJGJ52 showed increases in DAHP synthase activity relative to the same strain harboring the cloning vector pLAFR1. Results with Rm7479 harboring pJGJ53 (pJGJ51Ω7479::Tn*5*) compared to Rm7479 harboring pJGJ51 showed that the 7479 locus is required for full complementation, and that pJGJ51 may contain additional DAHP synthase determinants. Thus, the original mutants had a statistically significant decrease in the rate of catalysis in the first committed step of aromatic biosynthesis, and this biochemical defect could be compensated for by either complementing or suppressing DNA.

Symbiotic phenotypes.

Plants inoculated with either Rm7479 or Rm7480 yielded symbiotically ineffective plants (Fix^-). These plants had short chlorotic shoots, roots with white nodules that varied from small and round to elongate, and average rates of acetylene reduction that were markedly less than plants inoculated with wild-type Rm1021 (Table 3). To determine if the nodules contained bacteria with the appropriate growth and antibiotic resistance phenotypes, several Rm7480 derived Fix^- nodules were harvested, surface sterilized, crushed, and the released material plated on: L agar with Sm, L agar with Nm, M9 succinate agar with Sm, and M9 succinate agar with Nm. No viable colonies were observed on either L agar medium, whereas comparable numbers of colonies were observed on both M9 media. Therefore, strain Rm7480 entered nodules, and the bacteria recovered from these nodules retained the phenotypes of Rm7480. Likewise, Rm7480 containing the vector pLAFR1 or pJGJ53 (pJGJ51Ω7479::Tn*5*) formed ineffective nodules as evidenced by stunted chlorotic plants with white nodules. In contrast, plants inoculated with Rm7480 containing either complementing or suppressing plasmids (pJGJ51 and pJGJ52, respectively) formed healthy plants with effective pink nodules.

The suppressor strain Rm7482 yielded symbiotically effective plants that showed healthy green shoots and roots with elongate pink nodules. Acetylene reduction rates were not significantly different from wild type (Table 3). The suppressor strain Rm7481, however, yielded symbiotically ineffective plants with chlorotic shoots and roots with white ineffective nodules that failed to reduce acetylene.

Microscopy of nodules induced by Rm7479 and Rm7480.

To determine at what stage of symbiotic development strains Rm7479 and Rm7480 were blocked, five nodules from each strain were examined by light and electron microscopy. We adopted the nomenclature established by Vasse *et al.* (1990) describing nodule structure and bacteroid development. This nomenclature recognizes four distinct nodule zones and five stages of bacteroid development in nodules infected with wild-type *R. meliloti*. Zone I, the meristematic region (M), was most distal and contained uninfected dividing plant cells (Fig. 2A). Zone II, the infection zone (I), consisted of bacteria released from infection threads into plant cells. There the bacteria were classified as type 1 bacteroids characterized by rod-shaped bacteroids (often with a central nucleoid) surrounded by an irregularly shaped peribacteroid membrane (Fig. 3A). In the proximal region of Zone II, type 2 bacteroids had stopped dividing, continued to elongate, and demonstrated a heterogeneous cytoplasm and a more regularly shaped peribacteroid membrane. At the Zone II–III interface, type 3 bacteroids stopped elongating, developed a more heterogeneous cytoplasm, and showed a smooth, tightly associated peribacteroid membrane (Fig. 3B). Zone III, the nitrogen fixation zone (N), was characterized by type 4 bacteroids showing distinct cytoplasmic heterogeneity between electron dense and translucent regions (Fig. 3C). Type 4 bacteroids were identical in size to type 3 bacteroids, but unlike type 3 bacteroids were found in many plant cell layers. Type 4 bacteroids were only observed in nitrogen fixing nodules. Zone IV, the senescent zone, showed type 5 bacteroids characterized by a loss of cytoplasmic heterogeneity and an increase in electron translucence. Often breakage of either bacteroid or peribacteroid membranes was observed (data not shown).

At the light microscope level, nodules induced by Rm7479 had a gross morphology that closely resembled that of wild-type nodules, with the exception that the infection zone appeared to be more extensive (Fig. 2B). Bac-

Table 2. DAHP synthase activities[a]

Strain	Amino acid in assay			
	None	Tryptophan	Phenylalanine	Tyrosine
Rm1021	8.4 ± 1.1	2.5 ± 0.3	9.7 ± 0.5	11.0 ± 0.7
Rm7479	3.0 ± 2.4	1.2 ± 1.0	3.6 ± 2.5	3.5 ± 3.0
Rm7480	1.9 ± 1.1	0.8 ± 0.4	2.4 ± 1.3	2.7 ± 1.4
Rm7481	9.0 ± 2.7	2.4 ± 0.2	10.0± 3.2	11.1 ± 3.1
Rm7482	9.5 ± 2.3	2.3 ± 0.4	10.6 ± 2.8	11.9 ± 3.1
Rm7479/pLAFR1	2.8 ± 0.3	1.7 ± 0.3	3.8 ± 0.4	3.5 ± 2.5
Rm7479/pJGJ51	18.3 ± 1.9	5.1 ± 0.3	20.4 ± 2.4	22.3 ± 2.5
Rm7479/pJGJ52	10.2 ± 2.3	4.6 ± 1.5	10.4 ± 2.6	11.2 ± 3.0
Rm7479/pJGJ53	9.8 ± 0.6	4.9 ± 0.5	11.4 ± 1.1	11.7 ± 1.0

DAHP synthase activity is expressed in μmoles DAHP min^{-1} mg^{-1} protein. Measurements for each strain and condition were taken with three separate extracts.

Table 3. Whole plant acetylene reduction assays

Strain	C_2H_4 rate[a]	SD[b]	n[c]	P value
Experiment 1				
Rm1021	235.7	157.3	50	ND
Rm7479	44.6	51.5	22	0.00005
Rm7480	23.8	45.6	42	0.00005
Experiment 2				
Rm1021	712.0	496.2	5	NA
Rm7481	37.8	57.9	5	0.02
Rm7482	488.6	283.3	5	0.02

[a] Average rate of C_2H_4 produced (μmoles C_2H_4 min^{-1} $plant^{-1}$).
[b] Standard deviation.
[c] Number of plants assayed; P value, probability that the average rate of C_2H_4 produced is identical to that of Rm1021 in that experiment.

teroid development of Rm7479 proceeded normally up to type 2 bacteroids as suggested by bacteria enclosed in a peribacteroid membrane that closely paralleled the bacteroid surface, and the granular appearance of the cytoplasm (Fig. 3D). The DNA had a fibrillar appearance and ribosomes were evident. However, unlike Rm1021 where bacteroid development proceeded through two additional cytological stages (types 3 and 4) prior to senescence (type 5), Rm7479 bacteroids present in plant cells several cells proximal showed evidence of senescence. The bacteroids were large, and the cytoplasm had lost its fine structure (Fig. 3E). In contrast, nodules induced by Rm7480 (Fig. 2C) had a narrow, poorly developed infection zone and lacked evidence of characteristic nitrogen fixation and senescent zones. Although light microscopic examination of nodules induced by Rm7480 exhibited an aberrant morphology, ultrastructual analysis indicated the formation of infection threads containing bacteria (Fig. 3F), invaded plant cells, and bacteroids reminiscent of type 2 bacteroids (Fig. 3G). A few plant cells proximal, bacteroids underwent demonstrable degradation without passing through stages 3 and 4. Most notable was an enlarged, amorphous shape, a significant loss of cytoplasmic fine structure including ribosomes and fibrillar organization of DNA, and pulling away and breakage of the peribacteroid membrane (Fig. 3H).

DISCUSSION

We have identified a new genetic locus in *R. meliloti* that affects three phenotypic characters: Growth in the presence of phenylalanine or tryptophan or on rich medium, bacteroid development, and the activity of DAHP synthase which catalyzes the first step in aromatic biosynthesis. The genetic relatedness of the three phenotypes is clear from studies of complementation and suppression. A cosmid that restored growth on L agar by complementation, pJGJ51, also restored effective symbiosis and increased DAHP synthase levels. In contrast, the same cosmid containing the original mutation Ω7479::Tn*5*, pJGJ53, did not restore either growth or effective symbiosis, and increased DAHP synthase to a lower level than pJGJ51. These results showed that the three phenotypes were caused by the same genetic lesion. An unrelated cosmid, pJGJ52, suppressed all three mutant phenotypes, strengthening the case for their causal relatedness. In addition, two second-site suppressor mutants grew on L agar, had increased DAHP synthase levels, and in one case restored effective symbiosis. The only exception to this correlation was the inability of one of the suppressor mutants, Rm7481, to develop an effective symbiosis, despite near wild-type DAHP synthase levels. It is interesting that the free-living growth phenotype of this suppressor strain was also in-

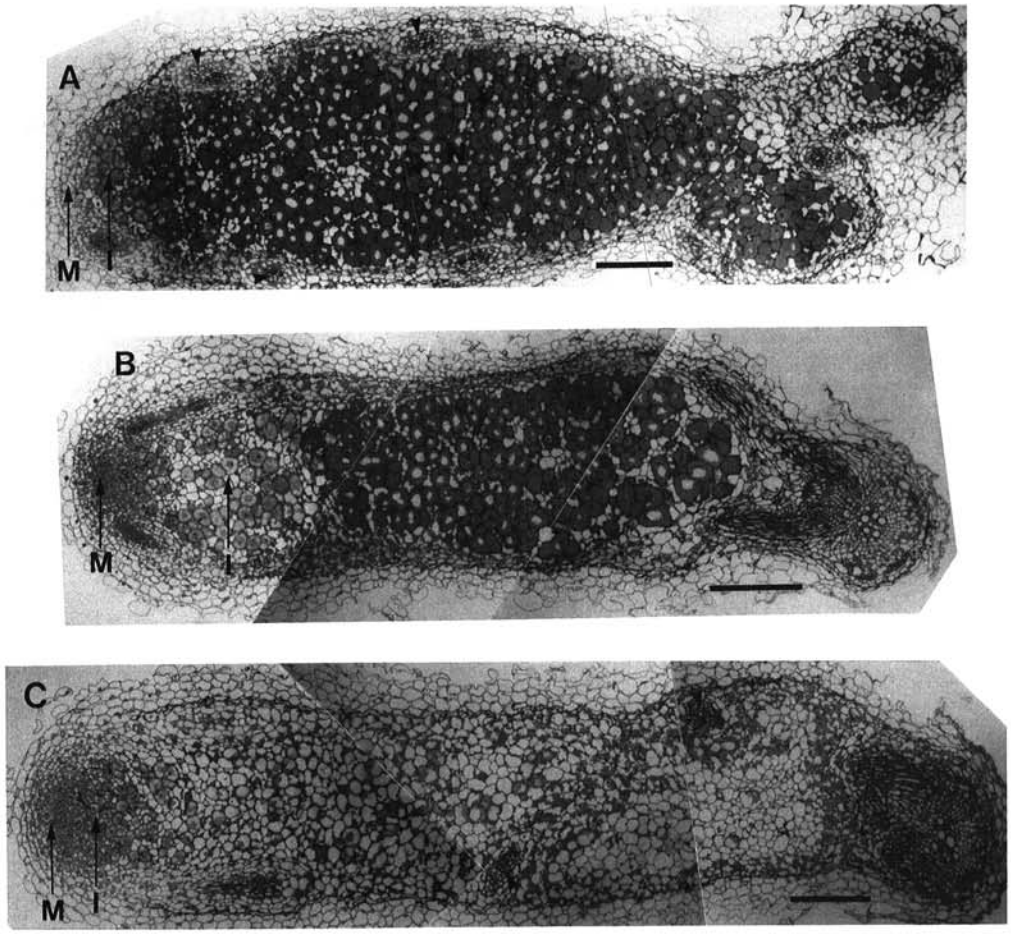

Fig. 2. Light micrographs of nodule thick sections. **A,** Rm1021; **B,** Rm7479; **C,** Rm7480. M, meristematic zone; I, infection zone; N, nitrogen fixation zone. Arrowheads indicate vascular bundles. Bar equals 0.2 mm.

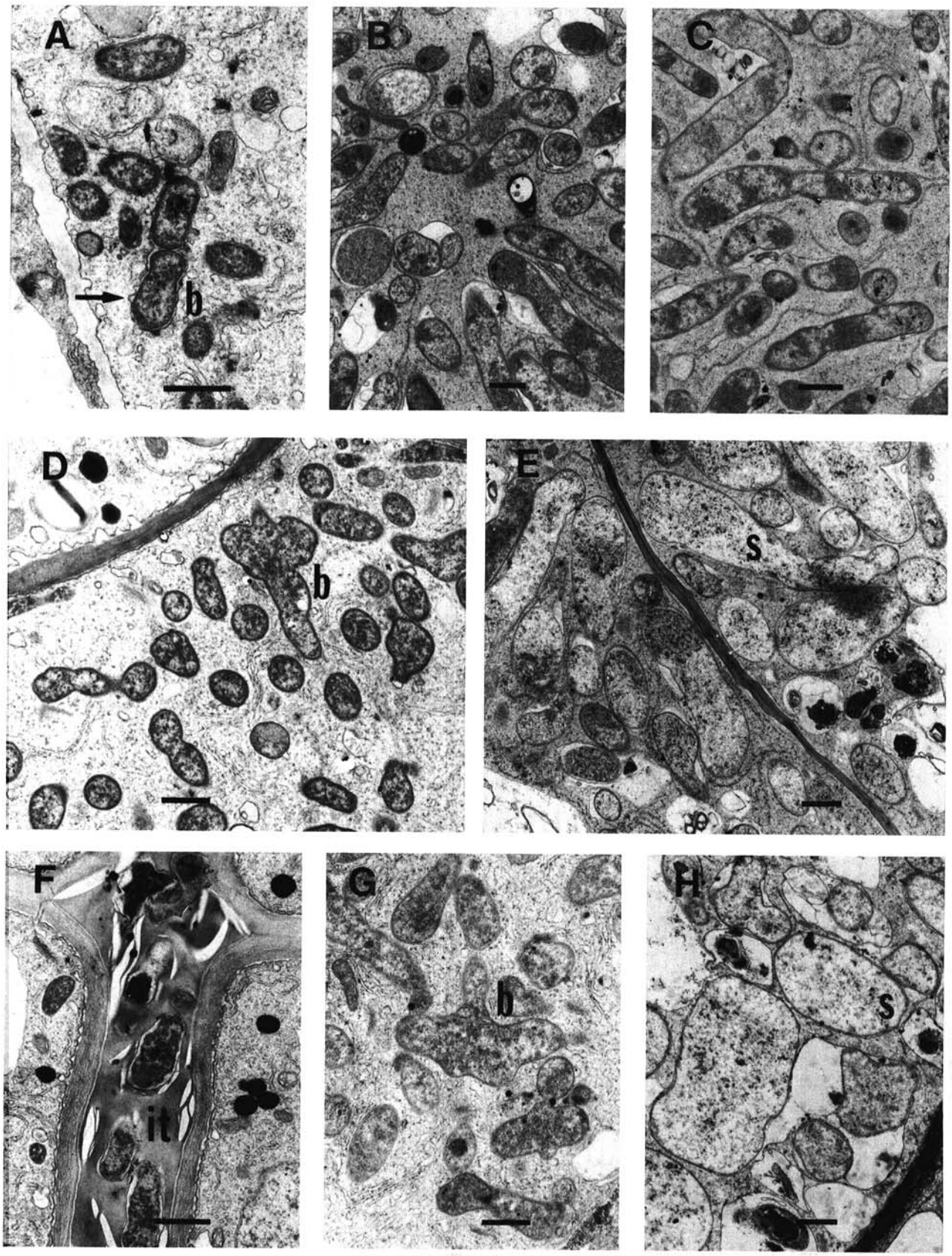

Fig. 3. Transmission electron micrographs of nodule thin sections. **A, B, C,** Rm1021; **D, E,** Rm7479; **F, G, H,** Rm7480. Arrow, peribacteroid membrane; b, bacteroid; it, infection thread; s, senescent bacteroid. Bar equals 1 μm.

completely restored; while it regained the capacity to grow on complex medium, it remained more sensitive to phenylalanine or tryptophan than either the wild type or the suppressor strain Rm7482. In summary, the defects in free-living growth and in bacteroid development were fully correlated, and also correlated in nearly all instances with changes in the first step in aromatic biosynthesis.

DAHP synthase catalyzes the first committed step in the synthesis of aromatic metabolites including tryptophan, phenylalanine, tyrosine, folate, quinones, and enterochelin. In *E. coli*, certain DAHP synthase mutants can be inhibited for growth by aromatic amino acids, presumably because the combined effects of mutations in the gene(s) for one or more isosymes and allosteric inhibition of the remaining isosyme(s) leave insufficient flux through the aromatic pathways for synthesis of the remaining aromatic metabolites (Prittard 1987). We attempted unsuccessfully to rescue growth of our *R. meliloti* mutant Rm7480 on complex medium by supplementing L agar with various aromatic intermediates (shikimate, para-hydroxybenzoate, chorismate) or end products (folate, quinones, menaquinones, tyrosine). This negative result should be viewed with caution because *R. meliloti* may not efficiently transport many aromatic metabolites into cells. Rm1021 extracts demonstrated that DAHP synthase activity was subject to allosteric inhibition by tryptophan, but not by phenylalanine or tyrosine. This contrasts with the situation in *E. coli*, where any of the three aromatic amino acids inhibit DAHP synthase activity (Prittard 1987). In the mutants Rm7479 and Rm7480, the residual DAHP synthase activity retained allosteric inhibition by tryptophan, and this could explain their growth inhibition by tryptophan. However, such a mechanism could not account for growth inhibition by phenylalanine because this amino acid did not allosterically inhibit DAHP synthase levels. Phenylalanine may instead repress the expression of DAHP synthase genes or may affect some other step in aromatic biosynthesis, thereby exacerbating the lack of DAHP synthase activity.

The mutants Rm7479 and Rm7480 were clearly reduced in DAHP synthase activity, and their growth and symbiotic defects could be overcome by genetic changes that restored DAHP synthase activity. However, lack of DAHP synthase may not be the only biochemical defect explaining the growth and symbiotic phenotypes. Rm7479 harboring either pJGJ52 or pJGJ53 showed near wild-type levels of DAHP synthase activity, but the latter did not grow on L agar or form an effective symbiosis. Apparently, a wild-type allele of the 7479 locus (as in pJGJ51) was required for growth and effective symbiosis. Likewise, suppressing strains Rm7481 and Rm7482 showed near wild-type levels of DAHP synthase activity, yet demonstrated different sensitivities to phenylalanine and tryptophan and different symbiotic phenotypes. These data suggest that although DAHP synthase levels played a major role in growth and symbiosis, other changes in aromatic pathways could be involved as well. Alternatively, some instances of suppression of the DAHP synthase defects observed in free-living cultures may not occur to the same degree during symbiosis.

Rm7479 and Rm7480 proliferated within infection threads, but failed to differentiate past type 2 bacteroids. In this respect they resembled previously characterized *nifA* (Hirsch and Smith 1987) or *sdh* (succinate dehydrogenase) mutants (Gardiol 1987) which show bacteroid development blocked immediately after formation of the peribacteroid membrane, yielding undifferentiated bacteroids that quickly become senescent. It is unclear whether the block in symbiotic development was due simply to decreased levels of aromatic metabolism within the bacteroids, or the problem was exacerbated by tryptophan or phenylalanine present in the plant cells after release from the infection thread. In either case, a normal flow of aromatic metabolites appears to be important during bacteroid development.

MATERIALS AND METHODS

Strains and plasmids.

All relevant bacterial strains and plasmids are shown in Table 4.

Media and supplements.

Unless otherwise noted, *E. coli* and *R. meliloti* strains were grown in L broth (Miller 1972) supplemented with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$. Mutant *R. meliloti* strains which were unable to grow in L broth were cultured in M9 (Miller 1972) or M9G medium (identical to M9 but NH_4Cl replaced with 0.2% Na-glutamate, pH 7.0) utilizing 0.2% Na succinate (pH 7.0) as the carbon source. L agar was used as a solid rich medium. Solid M9 or M9G medium was prepared as above with the addition of Difco Noble Agar to a final concentration of 2%. Exopolysaccharide production was detected by adding the fluorochrome Calcofluor to a final concentration of 0.2% (Leigh *et al.* 1985). "Pools" of amino acids were prepared and added to M9 succinate solid medium essentially as described, except that the final concentrations were fivefold greater than those previously used (Davis *et al.* 1980). Casamino Acids were used at a final concentration equivalent to Bacto-tryptone in L agar, 10 g/L. Antibiotic concentrations were 500 μg/ml of streptomycin sulfate (Sm), 200 μg/ml of neomycin sulfate (Nm), 10 μg/ml of tetracycline HCl (Tc), 25 μg/ml of chloramphenicol (Cm), and 60 μg/ml of naldixic acid (Nal).

Genetic manipulations.

Tn*5* mutagenesis of *R. meliloti* was performed using pRK602 as described (Leigh *et al.* 1985). Transduction of specific transposon alleles into other *R. meliloti* backgrounds was performed using φM12 lysates (Finan *et al.* 1986a), selecting for the appropriate drug resistance markers in the transductants. To make φM12 lysates of strains Rm7479 and Rm7480, plasmid pJGJ51 was first introduced into these backgrounds, imparting the ability to grow on L broth. All pLAFR1 plasmids as well as the pLAFR1 cosmid clone bank of Rm1021 DNA (Friedman *et al.* 1982) were mobilized between strains of *R. meliloti* and *E. coli* using the triparental mating technique (Ditta *et al.* 1980; Friedman *et al.* 1982) employing pRK600 (Finan *et al.* 1986b) as the helper plasmid. Plasmid isolation and Southern blot and filter hybridization techniques were performed as described (Ausubel *et al.* 1990). Rescue of

genomic Tn*5* insertions onto pJGJ1 was carried out by mating pJGJ51 out of each *R. meliloti* mutant strain into *E. coli* strain HB101 selecting for colonies which grew overnight on L agar with Sm, Km, and Tc at 37° C. These plasmids were then mobilized into *E. coli* strain C2110, selecting for colonies resistant to Nal, Tc, and Km. A Tn*5-233* (De Vos *et al.* 1986) insertion linked to Ω7480::Tn*5* was isolated using a Tn*5-233* random insertion bank lysate to infect Rm7480, selecting for transductants resistant to Gm and Sp and screening for those sensitive to Nm. Lysates of individual transductants were prepared and used to determine the transductional linkage to the Nm resistance marker of Ω7480::Tn*5*. Ω7509::Tn*5-233*, 40% linked to Ω7480::Tn*5*, was selected. Ω7509::Tn*5-233* was transduced into the previously described Tn*5*mob constructs (Klein *et al.* 1992). These constructs were then used to determine the mobilization frequency of the Gm-Sp resistance marker into Rm5000. Restriction enzymes were used according to manufacturer's specifications (Boehringer Mannheim Biochemicals, Indianapolis, IN).

Enzymes and enzyme assays.

Assays of DAHP synthase activity were performed colorimetrically as described by Srinivasan and Sprinson (1959) with the following modifications. Strains grown in M9 medium to mid log phase were diluted to OD_{600nm} of approximately 0.05 in M9 medium and allowed to grow to an OD_{600nm} of 0.4 to 0.6. Cells were pelleted and frozen at −20° C and stored for several days before sonic extracts were prepared (Srinivasan and Sprinson 1959). The sonic extracts were dialyzed against 0.04 M KPO_4 (pH 7.4) to remove metabolites. The DAHP synthase reactions were performed in 50 mM KPO_4 pH 6.4, 1.5 mM phosphoenol pyruvate, 1.5 mM erythrose-4-phosphate, 1 mg/ml BSA, with or without 0.25 mM aromatic amino acid. Reactions were run at 30° C for 12 min and were started with the addition of sonic extracts and terminated with the addition of 0.2 ml of 10% TCA. The phosphoenol pyruvate and erythrose-4-phosphate dependent DAHP synthase activity was determined by subtracting the activity of reactions that contained sonic extracts but that lacked phosphoenol pyruvate and erythrose-4-phosphate. Measurements were taken from the linear portion of the curve with respect to time and enzyme concentration. Activities were normalized to total protein, determined by Bio-Rad (Richmond, CA) protein assay utilizing bovine serum albumin as a standard.

Table 4. Strains, plasmids, and phage used in this study

Strain	Genotype	Source or reference
Escherichia coli		
HB101	*rpsL*20, *proA*2, *recA*13, *hsdS*20	G. Walker[a]
C2110	*polA*, Nal resistant	E. Nester[b]
Plasmids		
pRK600	pRK2013 *npt*::Tn*9*	Finan *et al.* 1986
pRK602	pRK600 ΩTn*5*	Leigh *et al.* 1985
pRK607	pRK2013 ΩTn*5-233*	De Vos *et al.* 1986
pJGJ51	Complementing cosmid	This work
pJGJ52	Suppressing cosmid	This work
pJGJ53	pJGJ51 Ω7479::Tn*5*	This work
pJGJ54	pJGJ51 Ω7478::Tn*5*	This work
Rhizobium meliloti, Agrobacterium tumefaciens phage		
ϕM12	*R. meliloti* transducing phage	Finan *et al.* 1986a
Rm1021	Sm^r, derivative of SU47	Leigh *et al.* 1985
Rm5000	Rf^r, derivative of SU47	Leigh *et al.* 1985
Rm7479	Rm1021 Ω7479:Tn*5*	This work
Rm7480	Rm1021 Ω7480::Tn*5*	This work
Rm7481	Rm7479 with suppressor mutation	This work
Rm7482	Rm 7480 with suppressor mutation	This work
Rm7509	Ω7509::Tn*5*-233 tag	This work
Rm7510	Ω601::Tn*5*mob, Ω7509::Tn*5*-233	This work
Rm7511	Ω602::Tn*5*mob, Ω7509::Tn*5*-233	This work
Rm7512	Ω611::Tn*5*mob, Ω7509::Tn*5*-233	This work
Rm7513	Ω612::Tn*5*mob, Ω7509::Tn*5*-233	This work
Rm7514	Ω614::Tn*5*mob, Ω7509::Tn*5*-233	This work
Rm7515	Ω615::Tn*5*mob, Ω7509::Tn*5*-233	This work
At123	Sm^r *A. tumefaciens*	T. Finan[c]
At125	At123 pRmeSU47bΩTn*5*-oriT	T. Finan
At128	At123 pRmeSU47aΩTn*5*-11	T. Finan

[a] MIT, Cambridge, MA.
[b] University of Washington, Seattle.
[c] McMaster University, Hamilton, Ontario.

Plant assays.

Sterile seedlings of *M. sativa* 'Iroquois' were inoculated and grown as described (Leigh *et al.* 1985). Plants were grown on Jensen's plates. Estimates of symbiotic nitrogen fixation were determined using a modified version of the whole plant assay whereby plants grown on Jensen's plates were transferred to 15- × 150-mm glass tubes, stoppered, and assayed for the rate of acetylene reduction using a Hewlett-Packard 5700a gas chromatograph equipped with a flame ionization detector; the signal was integrated using a Water's 470 data module. The average rate of C_2H_4 production was statistically compared to a wild-type control using the calculated Student's *t* value to determine the *P* value level of significance, an estimate of the probability that the averages were identical (Zuwayif 1979).

Light and electron microscopy.

Four-week-old nodules were fixed in 3.5% gluteraldehyde 1% paraformaldehyde in 0.05 M cacodylate buffer (pH 7.4) for approximately 17 hr at room temperature. After rinsing in cacodylate buffer, nodules were fixed in 1% aqueous OsO_4 at 4° C for 3 hr, and dehydrated through a graded series of ethanol. Nodules were embedded in Spurr low-viscosity embedding medium (Polysciences, Inc. Warrington PA.) using acetone as a transitional solvent. For light microscopy 1- to 2-μm sections were cut and stained with 1% toluidine. For transmission electron microscopy ultrathin sections were stained with uranyl acetate-lead citrate (Reynolds 1963) and examined in a JEOL 100B transmission electron microscope operating at 60 kV.

ACKNOWLEDGMENTS

We are grateful to E. W. Nester for the suggestion that the inability of the mutants to grow on rich media may be due to feedback inhibition of a biosynthetic pathway and to T. Finan for supplying *A. tumefaciens* strains carrying the *R. meliloti* megaplsmids. J.G.J. is especially grateful to David Ozga for the gift of the Tn*5-233* random hop bank lysate and the Tn*5*mob lysates. This work was supported by Public Health Service grant GM 39785 from the National Institutes

of Health. J.G.J. was supported by National Institutes of Health National Research Service Award 2T32HD07183.

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