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The Product of the *Rhizobium meliloti ilvC* Gene Is Required for Isoleucine and Valine Synthesis and Nodulation of Alfalfa

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Tn*5*-induced mutants of *Rhizobium meliloti* that require the amino acids isoleucine and valine for growth on minimal medium were studied. In one mutant, 1028, the defect is associated with an inability to induce nodules on alfalfa. The Tn*5* mutation in 1028 is located in a chromosomal 5.5-kb *Eco*RI fragment. Complementation analysis with cloned DNA indicated that 2.0 kb of DNA from the 5.5-kb *Eco*RI fragment restored the wild-type phenotype in the Ilv⁻ Nod⁻ mutant. This region was further characterized by DNA sequence analysis and was shown to contain a coding sequence homologous to those for *Escherichia coli* IlvC and *Saccharomyces cerevisiae* Ilv5. Genes *ilvC* and *ilv5* code for the enzyme acetohydroxy acid isomeroreductase (isomeroreductase), the second enzyme in the parallel pathways for the biosynthesis of isoleucine and valine. Enzymatic assays confirmed that strain 1028 was a mutant defective in isomeroreductase activity. In addition, it was shown that the *ilvC* genes of *Rhizobium meliloti* and *E. coli* are functionally equivalent. We demonstrated that in *ilvC* mutant 1028 the common nodulation genes *nodABC* are not activated by the inducer luteolin. *E. coli ilvC* complemented both defective properties (Ilv⁻ and Nod⁻) found in mutant 1028. These findings demonstrate that *R. meliloti* requires an active isomeroreductase enzyme for successful nodulation of alfalfa.

Rhizobium meliloti induces nitrogen (N_2)-fixing nodules on the roots of alfalfa (*Medicago sativa*). This symbiotic relationship begins on the root surface, proceeds with the invasion of root hairs, and culminates in the development of an effective N_2-fixing nodule. *R. meliloti* nodulation (*nod*) genes are located on a large symbiotic plasmid termed pSym (6, 37) and have been cloned and characterized. The common nodulation genes (*nodABC*) are structurally and functionally conserved among several *Rhizobium* and *Bradyrhizobium* species (23, 27). Other nodulation genes, such as *nodFEGHPQ*, are believed to confer specificity for nodulation (12, 21). It has been proposed that the common genes *nodABC* and the specific genes *nodHPQ* produce alfalfa-specific signals that trigger a host response (16, 26, 38). The *nodD* gene codes for a protein that activates the expression of the other *nod* genes in the presence of inducing compounds exuded by alfalfa seeds or roots (20, 30). Flavonoids have been identified as inducers of *R. meliloti nod* genes (32, 33). Other genes, located at several places in the genome and required under particular growth conditions in the free-living state of *R. meliloti*, are also required for successful symbiosis. Mutants defective in exopolysaccharide synthesis (24, 25) or synthesis and export of β-(1,2)-glucan (18, 41) induce small bacterium-free nodules. Mutations affecting defined metabolic functions can cause a Fix phenotype (15, 36, 48). In addition, uncharacterized auxotrophic mutants with altered symbiotic properties have been described (14). In this study we have examined the symbiotic properties of two Ilv⁻ mutants of *R. meliloti* and found that in only one strain the same mutation (*ilvC*) causes the mutant to be symbiotically defective. We have demonstrated that the mutation in the Ilv⁻ Nod⁻ mutant maps in the gene coding for the enzyme acetohydroxy acid isomeroreductase (isomeroreductase) (E.C. 1.1.1.89) (*ilvC*). We have determined the primary sequence of the *R. meliloti ilvC* gene and have shown that the *Escherichia coli ilvC* gene complements both defective phenotypes in the Ilv⁻ Nod⁻ mutant of *R. meliloti*.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The strains and plasmids used in this study are listed in Table 1. *E. coli* strains were maintained on LB agar. *R. meliloti* was grown on either TY medium or minimal medium (28).

Amino acids and precursors were purchased from Sigma Chemical Co. unless stated otherwise and were used as necessary in the following concentrations (milligrams per liter): isoleucine, 50; valine, 50; α-ketobutyrate, 125; α-ketoisovalerate, 90; calcium D-pantothenate, 8.4; racemic acetolactate (Aldrich Chemical Co.), 400; and racemic α,β-dihydroxyisovalerate (a generous gift from H. M. Steinman, Albert Einstein College of Medicine, New York, N.Y.), 270.

Cloning of the Tn*5*-containing *Eco*RI DNA fragment from strain 1028 (plasmid pQB1). Total DNA from strain 1028 was prepared and digested with *Eco*RI. Digested DNA was separated by agarose gel electrophoresis. A piece of gel containing 6 to 12 kb was cut out, and the DNA was eluted by the freeze-dry procedure (43). An aliquot of this DNA preparation was re-run in a gel and subjected to Southern analysis to confirm the presence of Tn*5* DNA in the sample with a radioactive Tn*5* probe. The DNA was ligated to *Eco*RI-cut and dephosphorylated plasmid pACYC184. After transformation of *E. coli* HB101, kanamycin-resistant (Km^r) colonies were selected. A plasmid purified from one Km^r Tc^r colony was subjected to restriction analysis and found to carry an 11-kb *Eco*RI DNA fragment containing the Tn*5* sequence flanked by *R. meliloti* DNA in addition to vector plasmid DNA. This plasmid was designated pQB1.

Cloning of the wild-type 5.5-kb *Eco*RI DNA fragment from strain 1021 (plasmid pQB3). The strategy for cloning of the wild-type 5.5-kb *Eco*RI fragment containing the Ilv Nod

TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristic(s)	Source or reference
Bacterial strains		
E. coli		
HB101	*hsdR hsdM recA13*	9
S17-1	RP4-2 (Tc::Mu) integrated into the chromosome	40
CU424	*ilvC464 galTR* λ^+	H. E. Umbarger (Purdue University)
R. meliloti		
1021	Wild type, Str^r Nod^+ Fix^+ on alfalfa	28
1028	Ilv^- Nod^- Tn*5* mutant derivative of 1021	F. Ausubel (28)
MA6-B	Ilv^- Nod^+ Tn*5* mutant derivative of 1021	This work
Plasmids		
pACYC184	Cm^r Tc^r	11
pSUP104	Broad-host-range Cm^r Tc^r pACYC184 derivative	R. Simon (Bielefeld, Germany)
pSUP202	Tc^r Cm^r Ap^r pBR325-Mob	40
pSUP1021	Tc^r Cm^r Nm^r pSUP102::Tn*5*	40
pUC4K	Ap^r, multiple cloning site	45
pUC18	Ap^r, multiple cloning site	45
pRW1A	*E. coli ilvA ilvY ilvC*	G. W. Hatfield (49)
M13mp18	Derivative of M13	31
pRmM57	*nodABC-lacZ* fusion	S. R. Long (30)
pRmM61	*nodD1-lacZ* fusion	S. R. Long (30)
pQB1	11-kb *EcoRI* fragment from strain 1028 in pACYC184	This work
pQB2	11-kb *EcoRI* fragment from strain 1028 in pSUP202	This work
pQB3	5.5-kb *Eco*RI fragment from strain 1021 in pACYC184	This work
pQB5	5.5-kb *Eco*RI fragment from strain 1021 in pSUP104	This work
pQB7	4.0-kb *Bam*HI-*Eco*RI fragment from pQB3 in pSUP104	This work
pQB8	pQB7 with the fragment cloned in the opposite orientation	This work
pQB9	5.5-kb *Eco*RI fragment from strain 1021 in pUC18	This work
pQB10	2.0-kb *Hin*dIII-*Bam*HI fragment from pQB7 in pSUP104	This work
pQB11	2.0-kb *Hin*dIII-*Bam*HI fragment from pQB8 in pSUP104	This work
pQB12	3.2-kb *Hin*dIII-*Eco*RI fragment from pQB3 in pSUP104	This work
pQB17	2.0-kb *Hin*dIII-*Bam*HI fragment from pQB10 in pUC4K	This work
pQB19	pQB17 with 0.3-kb *Pst*I fragment deleted	This work
pQB20	Deletion of *Hin*dIII-*Bam*HI fragment from pQB19 in pSUP104	This work
pQB26	2.0-kb *Bgl*II-*Hin*dIII fragment (*ilvC*) from pRW1A in pSUP104	This work
pQB27	Small deletion in the *ilvC* gene of pQB26	This work

locus was similar to that used for cloning of the homologous Tn*5*-mutated fragment from strain 1028, except that pQB1 labeled with ^{32}P was used as a probe to screen a library in colony hybridization experiments. Plasmid DNA from one positively reacting colony was subjected to restriction analysis. Restriction sites were found to be identical to recognition sites initially detected in the *R. meliloti* DNA cloned in pQB1. The plasmid containing the *R. meliloti* 5.5-kb *Eco*RI fragment cloned into vector pACYC184 was designated pQB3.

Construction of plasmids for complementation (plasmids pQB5, pQB7, pQB8, pQB10, pQB11, pQB12, and pQB20). Plasmid pQB5 was constructed by cloning the *R. meliloti* 5.5-kb *Eco*RI fragment of pQB3 into the *Eco*RI cloning site of vector pSUP104. Plasmids pQB7, pQB8, and pQB12 were constructed in the following manner. The 5.5-kb *Eco*RI fragment was cloned into the polylinker sequence of pUC18 to produce pQB9. In this cloning step, flanking recognition sites for *Bam*HI and *Hin*dIII were added. The *R. meliloti* 4.0-kb *Bam*HI-*Eco*RI fragment was excised as a *Bam*HI fragment from pQB9 and cloned into the *Bam*HI site present in the tetracycline gene of pSUP104. The orientation of this restriction fragment in the resulting plasmid, pQB7, was such that the *tet* promoter was closer to the *Eco*RI site. Plasmid pQB8 contains the same *Bam*HI fragment in the opposite orientation. For the construction of pQB12, a 3.2-kb *Hin*dIII-*Eco*RI fragment was isolated from pQB9 as a *Hin*dIII fragment and ligated to *Hin*dIII-cut pSUP104.

Plasmids pQB10 and pQB11 were derived from plasmids pQB7 and pQB8, respectively, as follows. The *R. meliloti* sequence cloned in pQB7 and pQB8 has a single *Hin*dIII site approximately 2.3 kb from the *Eco*RI site. A second *Hin*dIII site in plasmid pQB7 is located on the *tet* gene of vector pSUP104 immediately adjacent to the *R. meliloti* DNA. The presence of these recognition sites was useful for the subsequent steps. Plasmid pQB7 was linearized with *Hin*dIII and self-ligated. Plasmid pQB10 was constructed by removal of the small *Hin*dIII fragment from plasmid pQB7. The same procedure, but with plasmid pQB8, was used to construct plasmid pQB11. For the construction of pQB20, the 2.0-kb *Hin*dIII-*Bam*HI fragment from pQB10 was blunt ended with the Klenow fragment of DNA polymerase. This fragment was ligated to *Bam*HI-digested and blunt-ended plasmid pUC4K to create plasmid pQB17. The latter plasmid was digested with *Pst*I and self-ligated to produce plasmid pQB19. This procedure removed an internal *Pst*I fragment and generated a small 0.3-kb deletion within the original 2.0-kb *Hin*dIII-*Bam*HI fragment. The remaining *R. meliloti* DNA was excised from pQB19 as an *Eco*RI fragment and

cloned into the *Eco*RI cloning site of pSUP104 to produce plasmid pQB20.

For the construction of pQB26, a 2.0-kb *Bgl*II-*Hin*dIII restriction fragment containing the *E. coli ilvC* gene in pRWIA was isolated and ligated into the *Bam*HI and *Hin*dIII sites of pSUP104. pQB27, which is a derivative of pQB26, was constructed as follows. Plasmid pQB26 has a unique *Kpn*I site in the coding region of the *ilvC* gene. pQB26 was linearized with *Kpn*I, treated with T4 DNA polymerase to remove protruding 3′ termini, and religated. After transformation and selection on an appropriate medium, restriction analysis of plasmid DNA purified from one transformant indicated the absence of a *Kpn*I recognition site. The plasmid was designated pQB27.

Construction of Tn*5* mutants. Random Tn*5* mutagenesis of *R. meliloti* 1021 with *E. coli* S17-1(pSUP1021) was performed essentially as described by Simon et al. (40). *R. meliloti* neomycin-resistant (Nm^r) transconjugants were screened on minimal medium and on minimal medium supplemented with 50 mg each of isoleucine and valine per liter. Fragment-specific Tn*5* mutagenesis of *R. meliloti* was done by marker exchange as described earlier (2).

Plasmid transfer. Plasmid DNA was introduced into *E. coli* by transformation of competent cells. For genetic complementation, mobilizing strain *E. coli* S17-1 was used to introduce plasmids into *R. meliloti* by conjugal transfer, and plating was done on TY medium containing streptomycin (400 mg/liter) and tetracycline (5 mg/liter) or chloramphenicol (100 mg/liter). Plasmid transfer was confirmed by examination of the plasmid profile in Eckardt gels modified as described by Hynes et al. (22).

Sequence analysis. The *R. meliloti* DNA fragment from plasmid pQB17 was cloned in both orientations into the appropriate restriction sites of phage vector M13mp18. Single-stranded deletion clones were prepared as described earlier (3). Sequence analysis of both strands was performed by the dideoxy chain termination procedure with Sequenase (U.S. Biochemical Corp.). In some cases, *R. meliloti*-specific synthetic primers were used to resolve ambiguous sequences. Sequence data were analyzed with Mac Vector software (IBI, New Haven, Conn.).

For mapping the location of the Tn*5* insertion in the *ilvC* region of mutant strain 1028, a clone in M13mp18 was constructed by cloning the *Hin*dIII fragment that was isolated from pQB1 and that spanned the region from the *Hin*dIII site of Tn*5* to the adjacent *Hin*dIII site present in the *R. meliloti* DNA. The DNA adjacent to Tn*5* was sequenced with a Tn*5*-specific oligonucleotide primer.

Isomeroreductase assay. *R. meliloti* cells were grown at 28°C in TY medium to a density of approximately 10^8 cells per ml, collected by centrifugation, and resuspended in an equal volume of minimal medium. After 4 h of incubation at 28°C, approximately one generation, the cells were harvested by centrifugation, washed, and resuspended in a buffer containing 10 mM Tris (pH 7.0), 5.0 mM dithiothreitol, and 5 mM $MgCl_2$. Extracts were made by sonication followed by centrifugation at 20,000 × *g* for 15 min at 4°C. Isomeroreductase activity was assayed by the method described by Szentirmai et al. (42), except that racemic acetolactate prepared from 2-acetoxy-2-methyl-3-oxobutyric acid ethyl ester (Aldrich) as described by Aulabaugh and Schloss (5) was used as the substrate. Enzymatic activity was monitored as the disappearance of $NADPH_2$ at 340 nm in a reaction mixture (1 ml) containing the following: Tris (pH 7.5), 400 μmol; $MgCl_2$, 1.5 μmol; $NADPH_2$, 0.13 μmol; substrate, 7 μmol; and crude extract, 300 to 500 μg of protein. The rate of $NADPH_2$ disappearance was corrected for the endogenous oxidase activity of the extracts. Assays of the mutant strain were run in parallel with those of the wild-type strain. The protein concentration in extracts was determined with an assay kit from Bio-Rad Laboratories, Richmond, Calif., with bovine serum albumin as the standard.

Plant test. Seeds of alfalfa (*M. sativa* cv. Iroquois or *M. sativa* cv. Don Arturo [INTA, Hilario Ascasubi, Argentina]) were aseptically grown on nitrogen-free agar in test tubes (25 by 250 mm). Inoculation was done with a suspension of approximately 10^8 rhizobia in sterile water (46).

For the supplementation experiments, amino acids and precursors from sterile stock solutions were added to Jensen's medium after the medium was autoclaved but before it was poured into the plant test tubes. Supplements were assayed at the same concentrations as those used to supplement minimal medium for the growth of mutant 1028 and also at twice these concentrations. Twenty alfalfa seedlings were tested for each experimental condition.

β-Galactosidase assay. β-Galactosidase activity was measured in sodium dodecyl sulfate (SDS)-chloroform-permeabilized cells with the *o*-nitrophenyl-β-galactoside (ONPG) cleavage assay (29). Rhizobial strains carrying *nod-lacZ* fusions were grown with continuous shaking at 30°C in minimal medium or TY medium supplemented with the appropriate antibiotic. A 0.2- to 0.4-ml sample of a logarithmic-phase culture (A_{600} = 0.6 to 0.8) was mixed in a 1.5-ml microcentrifuge test tube with 0.4 ml of Z buffer (29)–0.05 ml of chloroform–0.1 ml of 0.1% SDS; this mixture was vortexed for 1 min, and the reaction was initiated by the addition of 0.15 ml of 4-mg/ml ONPG. After 10 or 15 min at room temperature, 0.4 ml of 1 M Na_2CO_3 was added. This mixture was centrifuged, and the A_{420} of the supernatant was spectrophotometrically measured. To assay for the induction of *nod* genes, we diluted overnight cultures 1:100 with fresh medium and incubated them at 30°C. After 5 h, each culture was divided in half. Luteolin from a 2.5 mM stock solution in 50% ethanol was added at a final concentration of 10 μM to half of the culture for induction. Both halves of the culture (induced and noninduced) were incubated for 12 h, and the cells were assayed for β-galactosidase as described above. Units of β-galactosidase were calculated as described by Miller (29) and corrected for noninduced cells. The concentration of luteolin in the stock solution was determined spectrophotometrically at 350 nm with an extinction coefficient (log ε) of 4.17. Rhizobial growth was monitored by measuring the A_{600} of cultures. Reported data are the averages of five repetitions done in duplicate, except for the *nodD1-lacZ* fusion assay, which was repeated only once. Standard errors are also given.

Nucleotide sequence accession number. The sequence reported here has been submitted to GenBank and has the accession number M74076.

RESULTS

Auxotrophy to isoleucine and valine and symbiotic properties. We have compared the symbiotic properties of two independently isolated *R. meliloti* mutants, strains MA6-B and 1028, that require external supplementation of both isoleucine and valine for growth on minimal medium. Mutant MA6-B induced effective nodules on alfalfa roots, whereas mutant 1028 did not induce nodules or induced small, ineffective nodules in about 10% of inoculated plants. For convenience, we refer to the symbiotic phenotype of mutant

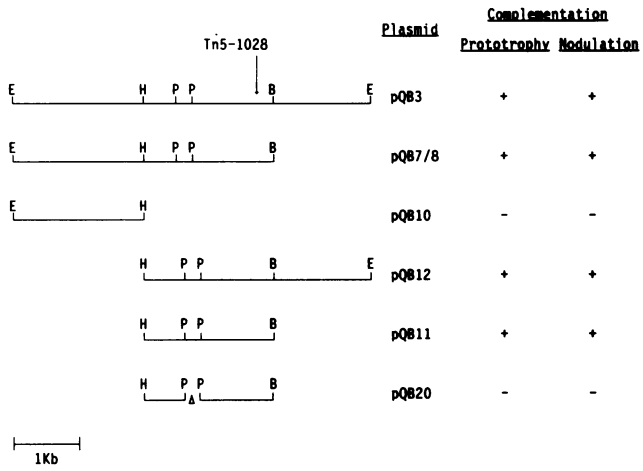

FIG. 1. Complementation of mutant 1028 by plasmids carrying the Nod-Ilv region. A partial restriction map for the chromosomal 5.5-kb *Eco*RI fragment from *R. meliloti* is given in the top line. The location of the Tn*5* insertion in mutant 1028 is indicated by a vertical arrow. The ability of plasmids to complement the Ilv$^-$ and Nod$^-$ phenotypes of mutant 1028 is shown on the right. The construction of subclones derived from pQB3 is described in Materials and Methods. Abbreviations for restriction enzymes: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I.

1028 as Nod$^-$. These observations on the symbiotic properties of mutant 1028 confirmed earlier results described by Hirsch et al. (19). Overall, these results indicated that in *R. meliloti* auxotrophy to isoleucine and valine is not always associated with defective symbiosis, as is the case with mutant MA6-B, and a particular gene product(s) of the isoleucine and valine synthetic pathway may also be involved in the process of nodulation.

Genetic analysis of mutant 1028. By using an Eckardt gel and Southern hybridization, we confirmed the previous report of Buikema et al. (10) that mutant 1028 carries transposon Tn*5* inserted into the chromosome (1). To confirm that this Tn*5* insertion correlates with the phenotype found in mutant 1028, we cloned Tn*5* flanked by chromosomal DNA from mutant 1028 as an 11.0-kb *Eco*RI fragment as described in Materials and Methods. The mutated region, the 5.5-kb *Eco*RI::Tn*5* fragment, was recombined in wild-type *R. meliloti* 1021 by marker exchange. Randomly chosen exchange mutants were assayed for growth on minimal medium and inoculated onto alfalfa seedlings. All six strains assayed required isoleucine and valine to grow on minimal medium and failed to induce nodules on alfalfa. Therefore, both phenotypes, Ilv$^-$ and Nod$^-$, are associated with the mutation present in the 5.5-kb *Eco*RI::Tn*5* fragment from mutant 1028. A limited gene bank was constructed with *Eco*RI-digested DNA from wild-type strain 1021. The gene bank was probed for homology to the cloned sequence carrying the 5.5-kb *Eco*RI::Tn*5* fragment from mutant 1028. This analysis identified one recombinant plasmid, pQB3, carrying a 5.5-kb *Eco*RI fragment homologous to the probe. A restriction map of the 5.5-kb *Eco*RI fragment is shown in Fig. 1. Restriction fragments derived from this 5.5-kb *Eco*RI fragment were subcloned into the broad-host-range vector pSUP104 for complementation analysis. The ability of these plasmids to complement both the Ilv$^-$ and the Nod$^-$ phenotypes of mutant 1028 was determined after the plasmids had been introduced by conjugation. The results of this analysis, shown in Fig. 1, indicated that a 2.0-kb *Hin*dIII-*Bam*HI fragment contained in plasmid pQB11 was sufficient to restore the wild-type phenotype in mutant 1028.

To define further the sequences important in restoring the Ilv and Nod phenotypes, we deleted 0.3 kb of DNA from the 2.0-kb *Hin*dIII-*Bam*HI fragment to produce plasmid pQB20. Mutant 1028(pQB20) required isoleucine and valine for growth on minimal medium and failed to induce nodules (Fig. 1).

Sequence analysis. To gain further insight into the organization and function of genes in the Ilv-Nod region, we performed sequence analysis. The complementation analysis indicated that information for Ilv and Nod most likely would be contained in the 2.0-kb *Hin*dIII-*Bam*HI fragment cloned in plasmid pQB11. Therefore, we sequenced the 2,019-bp *Hin*dIII-*Bam*HI fragment and found two open reading frames that start with ATG initiation codons and that are preceded by classical ribosome binding sites. These open reading frames, identified as ORF1 and ORF2, are divergently transcribed. The nucleotide sequence of ORF1 begins at position 933 in Fig. 2 and predicts a polypeptide of 34,563 daltons. ORF2, which codes for a deduced polypeptide of 192 amino acids, begins 166 bp from ORF1. In addition, the position of the Tn*5* insertion in mutant 1028 was precisely determined by sequencing of the region adjacent to the insertion. The insertion was found to map within ORF1, 730 nucleotides from initiation codon ATG (Fig. 2).

Thus, the sequence data suggested that ORF1 might encode a protein involved in the two processes affected in mutant 1028. Therefore, upon translation of ORF1, the sequence of the putative polypeptide was compared with other sequences in the National Biomedical Research Foundation protein data base. Strong homology was found between this polypeptide and the gene products of *E. coli ilvC* (49) and *Saccharomyces cerevisiae ilv5* (34) (Fig. 3). *ilvC* and *ilv5* encode the structural gene for isomeroreductase, the second enzyme in the parallel pathways for the biosynthesis of isoleucine and valine (44). The degree of homology between the putative *R. meliloti ilvC* gene product and the *E. coli* IlvC protein is greater than that between the putative *R. meliloti ilvC* gene product and *S. cerevisiae* Ilv5. Conservation is higher in the NH_2-terminal part of the protein. In particular, two domains identified in the N-terminal part and in the middle of *R. meliloti* IlvC are highly conserved among the three proteins compared in Fig. 3. The deduced molecular mass of *R. meliloti* IlvC differs significantly from those reported for *E. coli* IlvC (54.0 kDa) and *S. cerevisiae* Ilv5 (44.3 kDa).

Strain 1028 is an IlvC mutant. To provide biochemical evidence that the gene identified by DNA sequencing encodes a functional isomeroreductase, we assayed cell extracts of *R. meliloti* for isomeroreductase activity. Table 2 shows the levels of isomeroreductase determined in wild-type strain 1021 and mutants 1028 and MA6-B. Activity could be detected in extracts from the wild type and mutant MA6-B but not mutant 1028. Upon introduction of plasmid pQB11, wild-type levels of isomeroreductase were found in mutant 1028. This result clearly demonstrated that the Ilv$^-$ phenotype of mutant 1028 is due to its inability to produce a functional isomeroreductase enzyme.

A partial genetic characterization of Ilv$^-$ Nod$^+$ mutant MA6-B was performed and, by hybridization analysis, it was found that transposon Tn*5* was inserted in a chromosomal region different from that in mutant 1028. In addition, mutant MA6-B had isomeroreductase activity and was able to grow on minimal medium supplemented with α-ketoisovalerate

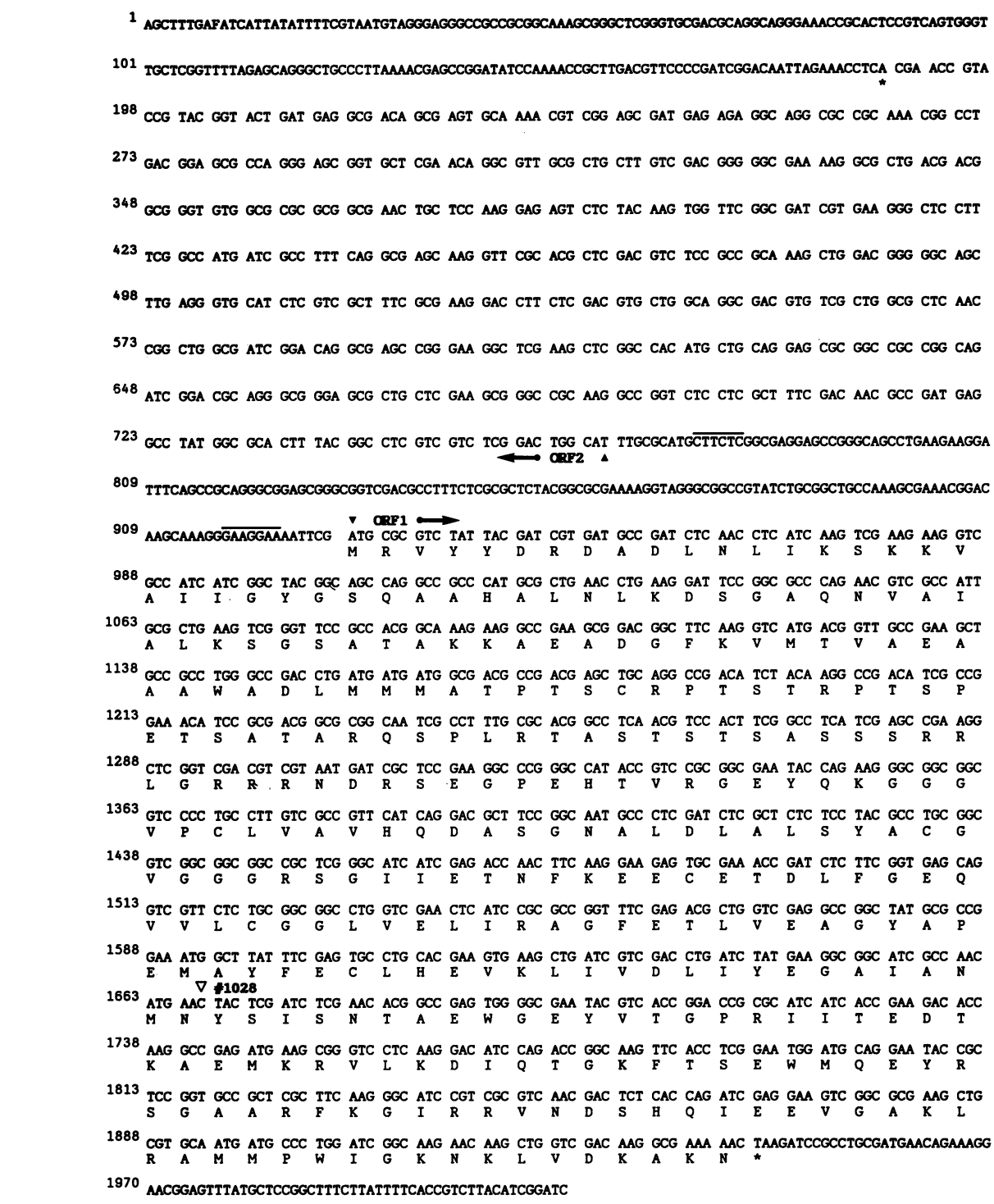

FIG. 2. Nucleotide sequence of the Ilv-Nod region of *R. meliloti*. The nucleotide sequence (2,019 bp) of the *Hin*dIII-*Bam*HI fragment (plasmid pQB11 in Fig. 1) is presented. Two open reading frames are identified: ORF1 begins at base 931 (▼), and ORF2 is divergently transcribed and begins at base 764 (▲). The corresponding amino acid sequence for ORF1 is presented. Putative ribosome binding sites are overlined. The Tn*5* insertion site in mutant 1028, mapped by sequencing, is indicated by an open inverted triangle. Stop codons are indicated by asterisks.

and isoleucine. In contrast, mutant MA6-B did not grow on minimal medium supplemented with α-ketobutyrate and valine; α-ketobutyrate is the product of the reaction catalyzed by threonine deaminase (*ilvA*). Taken together, these results indicated that the mutation in mutant MA6-B most likely maps in a gene homologous to the *ilvD* gene. *ilvD* encodes dihydroxy acid dehydrase, which converts dihydroxy acids into keto acids after the isomeroreductase

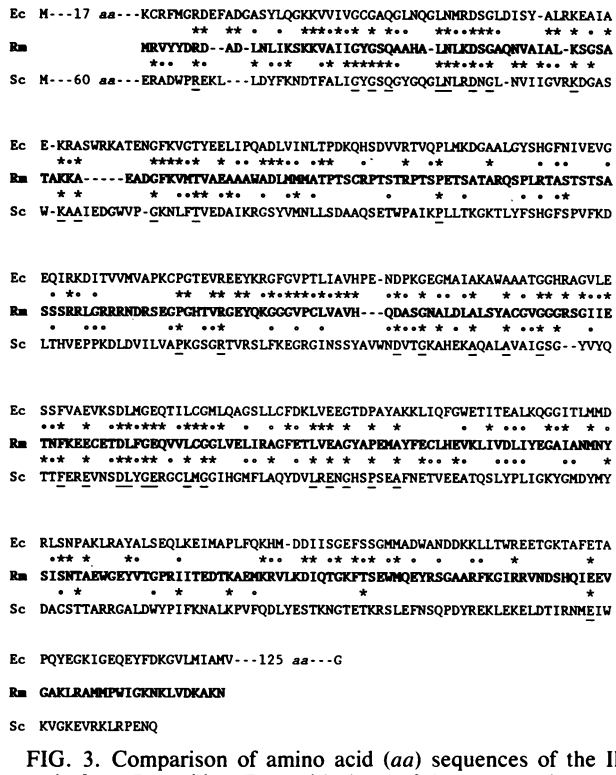

FIG. 3. Comparison of amino acid (*aa*) sequences of the IlvC protein from *R. meliloti* (Rm) with those of the gene products of *E. coli ilvC* (Ec) and *S. cerevisiae ilv5* (Sc). The asterisks indicate amino acid identities; the open circles indicate conservative substitutions. Amino acids conserved among all the polypeptides are underlined.

reaction. Furthermore, consistent with these pieces of evidence, we observed that MA6-B cross-feeds 1028.

The *ilvC* genes of *R. meliloti* and *E. coli* are functionally equivalent. The sequence data and the biochemical analysis demonstrated that Nod$^-$ mutant 1028 is defective in isomeroreductase activity. Thus, the question arose as to whether heterologous *ilvC* genes can restore a wild-type phenotype to mutant 1028. To test this possibility, we introduced plasmid pQB26 carrying the structural gene for the *E. coli* isomeroreductase into mutant 1028. The resulting strain, 1028 (pQB26), was found to grow on minimal medium, a result which correlates with the wild-type levels of isomeroreductase detected in the respective extract (Table 2). We next examined the symbiotic phenotype of strain 1028(pQB26) after inoculation onto alfalfa seedlings; it was found to induce effective nodules similar to those induced by the wild-type strain. However, this positive effect was not observed when plasmid pQB27 was introduced into mutant 1028. Plasmid pQB27 carries a mutation that alters the coding region of the *E. coli ilvC* gene cloned in pQB26. These results are clear evidence that a functional isomeroreductase is required for *R. meliloti* to establish symbiosis.

TABLE 2. Isomeroreductase activity in cell extracts of *R. meliloti*

Strain	Phenotype	Sp act (nmol/min/mg of protein)[a]
1021	Wild type	7.8
MA6-B	Ilv$^-$ Nod$^+$	8.5
1028	Ilv$^-$ Nod$^-$	0.6
1028(pQB11)	Ilv$^+$ Nod$^+$	7.3
1028(pQB26)	Ilv$^+$ Nod$^+$	6.7
1028(pQB27)	Ilv$^-$ Nod$^-$	ND

[a] Data represent the averages of two independent assays the results of which differed by no more than 15%. ND, not determined.

TABLE 3. Expression of nodulation genes in *R. meliloti* Ilv$^-$ mutants

nod-lacZ plasmid	U of β-Galactosidase in:		
	Wild-type 1021	Ilv$^-$ Nod$^-$ 1028	Ilv$^-$ Nod$^+$ MA6-B
pRmM61 (*nodD1-lacZ*)	267	231	ND[a]
pRmM57 (*nodABC-lacZ*)	183 ± 32	7 ± 4	150 ± 41
pRmM57 + supplements[b]	142 ± 89	9 ± 7	ND
pRmM57 + pQB11 (*R. meliloti ilvC*)	117 ± 36	38 ± 15	ND
pRmM57 + pQB26 (*E. coli ilvC*)	122 ± 27	35 ± 18	ND

[a] ND, not determined.

[b] Supplements were used at the concentrations listed in Materials and Methods and included L-isoleucine, L-valine, α-ketoisovalerate, racemic acetolactate, racemic α,β-dihydroxyisovalerate, and D-pantothenate.

For comparison, the ability of the *R. meliloti ilvC* gene to complement *ilvC* mutants of *E. coli* was examined. For this experiment, plasmids pQB11 (*R. meliloti ilvC*) and pQB1 (*R. meliloti ilvC*::Tn*5*) were separately introduced into *E. coli ilvC* mutant strain CU424 by transformation. After selection on medium supplemented with the appropriate antibiotic, transformants were tested for growth on minimal medium. Plasmid pQB11, but not plasmid pQB1, complemented *E. coli ilvC* mutant strain CU424. These results demonstrated that the *ilvC* genes of *R. meliloti* and *E. coli* are functionally equivalent.

Supplementation experiments. In addition to assessing mutant 1028 for isomeroreductase activity, we investigated the ability of this mutant to induce nodulation on alfalfa when the plant medium was supplemented with certain pathway intermediates. We undertook precursor feedings and found, in agreement with our earlier evidence, that mutant 1028 grew on minimal medium supplemented with α-ketoisovalerate and isoleucine. In contrast, the addition of these substrates to the plant medium did not restore effectiveness in mutant 1028. The common products of the isomeroreductase reaction, α,β-dihydroxyisovalerate and dihydroxy-β-methylvalerate, were not tested since they are not commercially available. However, we tested pantothenate because in enterobacteria the synthesis of pantothenate is linked to the synthesis of isoleucine and valine by the precursor α-ketoisovalerate. Although mutant 1028 required no exogenous supply of pantothenate for growth on minimal medium supplemented with isoleucine and valine, we assumed that low levels of pantothenate in mutant 1028 might be a limitation for nodulation. Plant experiments in which pantothenate was added to the plant medium revealed no effect on the nodulation phenotype of mutant 1028.

Expression of nodulation genes. The defective nodulation phenotype found in IlvC mutant 1028 prompted us to investigate whether nodulation genes are expressed in that particular genetic background. To address this question, we used plasmids carrying fusions of nodulation genes and the *lacZ* gene. Gene fusions *nodABC-lacZ* and *nodD1-lacZ* on plasmids pRmM57 and pRmM61, respectively, were assayed in wild-type strain 1021 and mutant 1028 (Table 3). Similar

levels of β-galactosidase activity were detected in cells of both the wild type and the mutant harboring the *nodD1-lacZ* fusion plasmid. This result indicates that the constitutive pattern of expression of at least one copy of *nodD* is not affected by the mutation present in mutant 1028. On the contrary, after incubation in the presence of the inducing compound luteolin, very low β-galactosidase activity was found in mutant 1028(pRmM57), representing about 4% the β-galactosidase activity found in the wild-type strain. This pattern of induction also was found in additional experiments in which rhizobium cells harboring plasmid pRmM57 were assayed at different times after incubation with luteolin or at different stages of growth. Furthermore, no activation of *nodABC* was detected in mutant 1028 when induction experiments were performed in the presence of precursors found to restore growth on minimal medium, even with α,β-dihydroxyisovalerate, the product of the isomeroreductase reaction.

Mutant 1028 carrying the plasmid containing the *ilvC* gene from either wild-type *R. meliloti* or *E. coli* was found to induce nodulation on alfalfa; however, in the present in vitro assay, the levels of β-galactosidase in 1028(pRmM57, pQB11) and 1028(pRmM57, pQB26) were lower than those in the wild type (Table 3). The mechanism of regulation of the *R. meliloti ilvC* gene is not known, and it is possible that the *ilvC* gene cloned in pQB11 and pQB26 in a multicopy vector does not reflect the physiological status of wild-type cells.

Nevertheless, our present results reveal that a particular step in the bacterium-plant interaction in which inducible *nod* genes are switched on in response to inducer molecules is affected by a mutation in the *ilvC* gene.

DISCUSSION

In the past few years, significant effort has been devoted to the elucidation of the molecular bases underlying the ability of rhizobia to interact symbiotically with the host legume. Thus, a set of genes specifically allocated to rhizobia, namely, the nodulation (*nod*) genes and the nitrogen fixation (*nif*) genes, have been identified and characterized. Less attention has been given to the study of genes that have a pleiotropic effect on the symbiotic properties of rhizobia and that are also found to be involved in metabolic functions in several other genera of bacteria. In this paper, the genetics of an *R. meliloti* Ilv$^-$ mutant, 1028, in which the requirement for isoleucine and valine is associated with defective symbiosis was investigated.

Microscopic examination of roots after infection with strain 1028 was described by Hirsch et al. (19). These authors classified strain 1028 as a reactive Nod$^-$ mutant, since root hair curling and penetration of host cells were observed. We observed in some plant inoculation experiments that small white nodules were induced in a few of the inoculated plants. We do not know the cause of this occasional plant response. Although we took care in the procedure and conditions that we used in the plant tests, diversity in the genetic background or in the physiological state of the population of seeds assayed may have generated conditions leading to this unusual response.

The association between auxotrophy to isoleucine and valine and altered symbiotic properties was found in only one of the two Ilv$^-$ mutants assayed in this work. This result agrees with that previously reported by Dénarié et al. (14). These authors examined the symbiosis properties of *R. meliloti* auxotrophs chemically induced with nitrosoguanidine and found that some of the Ilv$^-$ mutants were also defective in symbiosis. However, these particular mutants were not further characterized. The fact that strain 1028 is a Tn*5*-induced mutant facilitated the identification of the locus involved in the pleiotropic mutation.

Genetic data from analyses such as mapping of the Tn*5* insertion in mutant 1028, complementation experiments, and the use of deletion plasmids provide evidence that ORF1 codes for a protein. Furthermore, our biochemical analysis confirmed the identification of the ORF1 gene product as the enzyme isomeroreductase. We found that plasmids containing the *ilvC* gene from either *R. meliloti* or *E. coli* complemented to prototrophy *ilvC* mutants of *R. meliloti* or *E. coli*. This result showed that the IlvC proteins from these two unrelated bacteria are functionally equivalent, although conservation was not very extensive between the two IlvC proteins. Furthermore, since *E. coli ilvC* complemented both the Ilv$^-$ and the Nod$^-$ phenotypes found in mutant 1028, we concluded that IlvC is directly involved in the interaction between *R. meliloti* and alfalfa. The structure and regulation of expression of *ilv* genes have been extensively studied in enteric bacteria (4, 8, 47, 49). It was proposed that the expression of the *ilvC* gene is induced by the isomeroreductase substrate acetohydroxy butyrate or acetolactate and that this induction requires the positive activator encoded by the *ilvY* gene. *ilvC* is linked to *ilvY*, but their transcription is divergent. In the DNA analysis of the *R. meliloti ilvC* region, we identified a short open reading frame, ORF2, adjacent to the *ilvC* coding region and transcribed in a direction opposite that of the *ilvC* gene. However, we found no homology between ORF2 and *E. coli ilvY*. Nevertheless, it seems that conservation of the *ilvC* gene products in *R. meliloti* and *E. coli* is sufficient for them to be considered functionally equivalent.

We do not yet know the role of IlvC in the nodulation process or even whether the *ilvC* gene has any significance in other rhizobial species. Our finding that another *R. meliloti* Ilv$^-$ mutant, MA6-B, induced effective nodules on alfalfa excluded the possibility that the end products isoleucine and valine are directly involved. This is also the case for the precursor 2-ketoisovalerate, which lies after the isomeroreduction step at the branch point in the synthesis of leucine, valine, and pantothenate of enteric bacteria (7). With mutant 1028 we found requirements for only isoleucine and valine and were unable to restore efficient nodulation by the addition of 2-ketoisovalerate or pantothenate to the alfalfa-*R. meliloti* 1028 system. Pantothenate is particularly significant, since sequence analysis of the *nodF* genes of *R. meliloti* and *R. leguminosarum* reported, respectively, by Debellé and Sharma (13) and Shearman et al. (39) identified a conserved pantothenate binding site in the deduced sequence of the NodF protein. Recently, it was demonstrated that the NodF protein of *R. leguminosarum* carries 4′-phosphopanteine as a prosthetic group (17). Our supplementation studies do not provide evidence to indicate that the defective nodulation phenotype is due to a shortage of the particular precursors studied.

We found that the promoter of the common nodulation genes (*nodABC*) is not activated by luteolin in Ilv$^-$ mutant 1028. The *nodABC* gene products are required for early host response to infection. Thus, the step of induction of *nod* genes to initiate morphogenesis in the host plant may be a possible target for *R. meliloti ilvC* involvement. Although we demonstrated that the presence of the isomeroreductase precursor acetolactate has no effect on the induction in vitro of *nodABC* by luteolin, it is possible that the cellular

concentration of acetolactate in the *ilvC* mutant reaches levels that inhibit the expression of nodulation genes or antagonize the inducing effect of luteolin. A further alternative is that, as has been shown for enterobacteria (35), the enzyme isomeroreductase in *R. meliloti* is able to recognize a substrate other than those in the isoleucine-valine pathway and that the product of its conversion is required for the expression of nodulation genes. We are currently exploring this possibility. Overall, the present results represent another example in which *R. meliloti* utilizes the general metabolic pathways that are common to a larger number of bacteria for the active expression of specific symbiotic functions (15, 25, 36, 38, 48).

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