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Melanin Production by *Rhizobium meliloti* GR4 Is Linked to Nonsymbiotic Plasmid pRmeGR4b: Cloning, Sequencing, and Expression of the Tyrosinase Gene *mepA*

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Melanin production by *Rhizobium meliloti* GR4 is linked to nonsymbiotic plasmid pRmeGR4b (140 MDa). Transfer of this plasmid to GR4-cured derivatives or to *Agrobacterium tumefaciens* enables these bacteria to produce melanin. Sequence analysis of a 3.5-kb *Pst*I fragment of plasmid pRmeGR4b has revealed the presence of a open reading frame 1,481-bp that codes for a protein whose sequence shows strong homology to two conserved regions involved in copper binding in tyrosinases and hemocyanins. In vitro-coupled transcription-translation experiments showed that this open reading frame codes for a 55-kDa polypeptide. Melanin production in GR4 is not under the control of the RpoN-NifA regulatory system, unlike that in *R. leguminosarum* bv. phaseoli 8002. The GR4 tyrosinase gene could be expressed in *Escherichia coli* under the control of the *lacZ* promoter. For avoiding confusion with *mel* genes (for melibiose), a change of the name of the previously reported *mel* genes of *R. leguminosarum* bv. phaseoli and other organisms to *mep* genes (for melanin production) is proposed.

Melanin production is widely present in bacteria, fungi, plants, and animals. This brownish to black pigment is synthesized by the enzyme tyrosinase (monophenol, L-dopa: oxygen oxidoreductase; EC 1.14.18.1), a copper-containing monooxygenase that has both cresolase and catecholase activities (23). These enzymatic activities mediate the ortho-hydroxylation of tyrosine and other monophenols and aromatic amines to *o*-diphenols and the subsequent oxidation of *o*-diphenols to *o*-quinones; these steps are followed by several nonenzymatic oxidation and polymerization steps (25). Several important functions and properties have been associated with these pigments (2).

Many *Rhizobium* species produce melanin pigments when grown on complete media. Increasing levels of melanin production are obtained when bacteria are grown in the presence of L-tyrosine and Cu^{2+} ($CuSO_4$), suggesting that tyrosinase may be involved in this process. The range of melanin production in rhizobia has been characterized elsewhere (9), although its biological significance in the *Rhizobium*-legume symbiosis, if any, is not well understood. Loci involved in melanin production have been identified in different locations, either on symbiotic plasmids (3, 20, 21) or on cryptic plasmids (18), for several strains. Melanin biosynthesis in strain 8002 of *Rhizobium leguminosarum* bv. phaseoli is linked to the symbiotic plasmid, and several lines of evidence suggest that it is under the control of the RpoN-NifA regulatory system (5, 14). A Fix^- derivative mutant of this strain that did not produce melanin was isolated (21). Three loci have so far been associated with this characteristic in strain 8002. A *melA* gene has been proposed to be the structural gene for tyrosinase, while the previously identified class II *mel* gene (5) actually corresponds to the regulatory gene *nifA* (14). Recently, a new *mel* gene, *melC*, that bears certain phenotypic similarities to *ntrA* was found (15). These data could suggest an unknown implication of melanin production in the symbiotic nitrogen fixation process. In other cases, however, production of the pigment is not linked to the pSym plasmids, and as the present report shows, *nif*-like regulation of melanin production has not been detected for *R. meliloti* GR4.

R. meliloti GR4 harbors two very stable large plasmids in addition to the pSym plasmids. One of them, pRmeGR4b (140 MDa), is responsible for the nodule formation efficiency (*nfe*) genotype (31, 36) and has been shown to be related to the production of melanin (this study). The aim of this work was to carry out a genetic analysis of melanin biosynthesis by this strain of *R. meliloti*.

MATERIALS AND METHODS

Strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. *Rhizobium* and *Agrobacterium* strains were grown at 30°C in TY or minimal medium (26), and *Escherichia coli* strains were grown at 30°C in LB medium. For the detection of melanin production, the media were supplemented with L-tyrosine (600 μg/ml) and $CuSO_4$ (40 μg/ml) as previously described (9). Antibiotics were added at the following concentrations (micrograms milliliter^{-1}) when required: tetracycline, 10; ampicillin, 200; kanamycin, 180; and rifampin, 20.

DNA manipulations. Standard procedures were used for electrophoresis, transformation, or purification of DNA fragments from agarose gels onto membranes of DEAE-cellulose (29). Electrophoresis to visualize plasmid profiles was done as described by Eckhardt (12). Small-scale isolation of recombinant plasmids was done by alkaline lysis as described by Birnboim and Doly (4). DNA restriction and ligation were conducted with enzymes purchased from Boehringer GmbH (Mannheim, Germany) and used in accordance with the manufacturer's instructions. Hybridization was done with a nonradioactive detection kit from Boehringer,

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TABLE 1. Strains and plasmids

Strain or plasmid	Characteristics	Reference or source
Strains		
R. meliloti		
GR4	Wild-type; Nod$^+$ Fix$^+$ Mep$^+$	7
GRM10	pRmeGR4a-cured derivative of GR4; Mep$^+$	Our laboratory
GRM8SR	pRmeGR4a- and pRmeGR4b-cured derivative of GR4; Mep$^-$ Sm^r Rif^r	Our laboratory
2011	Wild type; Nod$^+$ Fix$^+$ Mep$^-$	J. Denarié
41	Wild type; Nod$^+$ Fix$^+$ Mep$^-$	A. Kondorosi
L5.30	Wild type; Nod$^+$ Fix$^+$ Mep$^+$	M. Kowalski
102F34	Wild type; Nod$^+$ Fix$^+$ Mep$^+$	G. Ditta
1354	*nifA*::Tn*5* Fix$^-$ 2011 derivative; Sm^r Km^r	35
1681	*ntrA2*::Tn*5* Fix$^-$ 2011 derivative; Sm^r Km^r	27
5002	*ntrC*::Tn*5* 2011 derivative; Sm^r Km^r	35
GMI5704	*fixJ*::Tn*5* 2011 derivative; Sm^r Nm^r Bleo^r	10
GMI5705	*fixL*::Tn*5* 2011 derivative; Sm^r Nm^r Bleo^r	10
GMI5630	*recA*::Tn*5-233 fixK*::Tn*5* 2011 derivative; Rif^r Sm^r Nm^r Gm^r Spc^r Bleo^r	1
R. leguminosarum bv. viceae VF39	Wild type; Nod$^+$ Fix$^+$ Mep$^+$	18
A. tumefaciens GMI9023	Plasmidless strain; Rif^r Sm^r Mep$^-$	28
E. coli		
S17-1	*thi pro recA hsdR hsdM* RP4-2-Tc::Mu-Km::Tn7 Tp^r Sm^r	33
HB101	*pro leu thi lacY endA recA hsdR hsdM* Sm^r	6
DH5α	*recA1 endA1 φ80d lacZ dam-15*	BRL
5K	*res thr leu thi tonA supE*	R. L. Robson
Plasmids		
pRmeGR4b	*R. meliloti* GR4 resident plasmid; 140 MDa; *nfe*	36
pSUP5011	pBR325::Tn*5-mob* Km^r Cm^r	32
pUC18	Sequencing and cloning vector; Ap^r	39
pCK1	pKT230 with *nifA* of *K. pneumoniae*; Sm^r Km^r	C. Kennedy
pRmW54-10	pACYC177-C with *nifA* of *R. meliloti*; Ap^r	37
pIJ1578	pLAFR1 with 24 kb of pRP2J1 including *melA*; Tet^r	5
pRmNT111	pRK290 with 28 kb of pRmeGR4b; Mep$^+$ Tet^r	Our laboratory
pME100	pUC18 with a 3.5-kb *Pst*I fragment containing the *mepA* gene	This study
pFG105	pUC18 with a 3.5-kb *Pst*I fragment cloned in the opposite orientation relative to that in pME100	This study

and the chemiluminescence method was used to detect hybridization bands.

Matings. Triparental matings were conducted with pRK2013 as a helper plasmid (13). Plasmid pRmeGR4b was transferred by use of mobilizer plasmid pRmeGR4a (24). GR4 Mep$^-$ mutants were isolated following random mutagenesis with Tn*5-mob* by use of suicide plasmid pSUP5011 (32). Kanamycin-resistant colonies were screened for the ability to produce melanin by use of TY and minimal media supplemented with L-tyrosine and $CuSO_4$ and then lysis with sodium dodecyl sulfate (SDS).

DNA sequencing. The 3.5-kb *Pst*I fragment from plasmid pRmeGR4b was sequenced by the chain termination method of Sanger et al. (30) on both strands by use of Sequenase version 2.0 T7 polymerase (U.S. Biochemicals, Cleveland, Ohio) in accordance with the manufacturer's instructions. Templates were generated by making progressive unidirectional deletions in the 3.5-kb *Pst*I fragment containing the *mep* locus cloned in pUC18 by use of the Erase-a-Base system from Promega Biotec (Madison, Wis.). Plasmid DNA was purified by use of a Magic Miniprep kit from Promega. Denatured double-stranded plasmid DNA (2 μg) was labelled with 5 μCi of [α-^{35}S]dATP (1,000 Ci/mmol; Amersham). Sequencing reactions were run on 6% sequencing gels.

DNA and protein sequence analyses. Homology search and sequence analyses were performed by use of Genepro version 4.0 (Riverside Scientific Enterprises) and Genetics Computer Group software packages (University Research Park, Madison, Wis.). FASTA, PILEUP, and MOTIFS programs from the University of Wisconsin were used. The data banks used were EMBL, Swiss-Prot, and PIR.

Protein analysis. Protein synthesis by in vitro-coupled transcription-translation with an *E. coli* S-30 linear system (Promega) was carried out in accordance with the manufacturer's instructions. Plasmid pME100 and pFG105 DNAs (2 μg) were labelled with 10 μCi of [^{35}S]methionine (1,000 Ci/mmol; Amersham), and incubation with S-30 extracts was conducted at 37°C for 60 min. Protein samples were electrophoresed on SDS–15% polyacrylamide gels as described by Laemmli (19). Gels were fluorographed with Amplify (Amersham), dried, and autoradiographed.

Determination of melanin production. Tyrosinase activity in native gels was determined by the method described by Held and Kutzner (16), modified as follows. *E. coli* DH5α cells containing both plasmids pME100 and pFG105 were

grown in 50 ml of LB medium with L-tyrosine and $CuSO_4$ for 16 h at 28°C. Cells were harvested by centrifugation at 7,500 × *g* and 4°C, washed in ice-cold phosphate buffer (0.1 N Na_2HPO_4-NaH_2PO_4 [pH 6.8]), and suspended in 9.5 ml of the same buffer. Proteins were extracted by cell permeabilization with 0.5 ml of mixed trimethyl-alkyl ammonium bromide (Sigma) (4 mg/ml) and incubation at room temperature for 30 min. Cells were pelleted by centrifugation for 10 min at 9,000 × *g*. The supernatant, with the protein in solution, was concentrated by ultrafiltration through a 30-kDa-pore-size membrane with an N_2 flow rate of 45 lb/in^2 (Amicon). The final volume was 1.5 ml. Protein (4 μg) was electrophoresed on a 15% native polyacrylamide gel (19). Tyrosinase activity was detected by gel incubation in 50 ml of 0.5 M phosphate buffer (pH 6.8) with 500 μl of L-tyrosine (600 μg/ml) and 50 μl of $CuSO_4$ (40 μg/ml) at 28°C in the dark with continuous shaking. The activity band became visible in a few minutes.

Nucleotide sequence accession number. The sequence determined in this study has been deposited in the EMBL and GenBank data libraries under accession number X69526.

RESULTS

Physical location of the *mep* locus. Studies carried out in our laboratory with cured derivative strains of *R. meliloti* GR4 led to the observation that plasmid pRmeGR4b was responsible for melanin synthesis in aged colonies of this strain. Melanin synthesis was also demonstrated for *Agrobacterium tumefaciens* when this plasmid was cotransferred with the other nonsymbiotic plasmid harbored by GR4 (pRmeGR4a). Analysis of clones from a previously constructed gene library of plasmid pRmeGR4b (36) revealed the presence of one recombinant plasmid, pRmNT111, that carries all the genetic information for pigment synthesis in the plasmidless GR4 derivative GRM8 and in plasmidless *A. tumefaciens* GMI9023 but not in *E. coli*. Using as a model the results obtained with *R. leguminosarum*, we investigated by random mutagenesis experiments with Tn*5* the possible presence of other regions of the GR4 genome involved in this phenotype. Screening of more than 6,000 insertions led to the isolation of 12 Mep$^-$ mutants, which were further analyzed for the presence of Tn*5* insertions. Nine of these mutants had lost the pRmeGR4b plasmid, as visualized by Eckhardt analysis. The other three mutants showed insertions in pRmeGR4b. By use of total-DNA Southern hybridization with pSUP5011 as a probe, two closely related or identical insertions were identified, and another one was located 500 bp away. All these Tn*5* insertions were located in a 3.5-kb *Pst*I fragment of plasmid pRmeGR4b (Fig. 1). To determine whether these mutants were also affected in their symbiotic properties, nodulation and nitrogen fixation assays were performed. All these mutant strains were able to induce effective symbiosis with alfalfa and showed a Fix$^+$ phenotype.

Expression of the *mep* locus. Melanin synthesis could be observed in aged colonies on TY medium, but the process could be intensified by the addition of L-tyrosine and $CuSO_4$ and accelerated by lysis with SDS as described previously (5). Data on the transfer of plasmid pRmeGR4b or pRmNT111 to different hosts support the observations that the information carried by the latter plasmid is able to induce synthesis in a Mep$^-$ background and that no other regions are necessary to produce melanin. *nifA*, *ntrC*, or *ntrA* mutants of *R. meliloti* 2011 (a non-melanin producer) did not show elimination of the production of melanin when pRmNT111 was transferred to them. These experiments were extended to other mutants defective in nitrogen fixation (*fixJ*, *fixK*, and *fixL*), and the same results were obtained. Attempts to induce melanin synthesis in *E. coli* by the introduction of plasmid pRmNT111 into strains harboring plasmid pCK1 or pRmW54-10 failed. These results suggest that the production of melanin in GR4 is not under the control of the RpoN-NifA regulatory system; this suggestion was confirmed by DNA sequencing, since no appropriate −12 and −24 consensus sequences or upstream activator sequence was found. On the other hand, pigment production was induced in *E. coli* when the *mep* locus was cloned under the control of the *lacZp* promoter in pUC18. The transfer of plasmid pME100 but not of pFG105 made strain DH5α a melanin producer after 3 to 4 days of incubation with the appropriate medium. Melanin production in these cases was detected both on solid and in liquid media and was increased by the addition of SDS (Fig. 2).

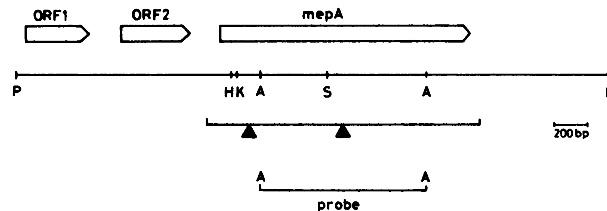

FIG. 1. Physical map of the 3.5-kb *Pst*I fragment of pRmeGR4b. Above the map are shown the three ORFs detected by computer analysis. ORF1 and ORF2 did not have any influence on melanin production, as demonstrated by a deletion of 1,095 bp affecting both ORFs (data not shown). The line under the map represents the 1,737-bp sequence presented in this paper. Solid triangles indicate the positions of the two different closely related transposon insertions obtained. The lower line defines the *Ava*I fragment used as a probe in hybridization experiments. Abbreviations: P, *Pst*I; H, *Hin*dIII; K, *Kpn*I; A, *Ava*I; S, *Sal*I.

Sequencing and analysis of the *mep* locus. The DNA sequence of the 3.5-kb *Pst*I fragment revealed the presence of a 1,481-bp open reading frame (ORF) with a high coding probability (Fig. 3). The predicted amino acid sequence is also shown. A search for homology in the data banks revealed the presence of good alignments at both the DNA and the protein levels with tyrosinase genes. DNA homologies were detected for discrete segments of the coding regions of tyrosinases and hemocyanins that form the protein domains containing the copper binding motifs proposed for these proteins. Thus, it was possible to detect 76.4% identity with the mRNA for the functional units Ode and Odf of *Octopus dofleini* hemocyanin, which represents most of the coding region for the CuA binding site. Good alignments were also achieved for *Streptomyces* DNA sequences. An identity of 53.5% was found with 375 nucleotides (positions 730 to 1105 in Fig. 3) of DNA coding for tyrosinase of *Streptomyces antibioticus*; 52.7% identity was found with 395 nucleotides (positions 106 to 500 in Fig. 3) of DNA coding for the N-terminal portion of tyrosinase of *S. glaucescens*. These two regions represent CuB and CuA binding sites, respectively. With regard to homologies detected for protein sequences, it must be noted that the alignments were located in the same regions, those involved in copper binding. The protein deduced from the GR4 tyrosinase DNA sequence is a polypeptide of 494 amino acids with a molecular mass of 54,109 Da. This protein showed homology with certain domains of tyrosinases of

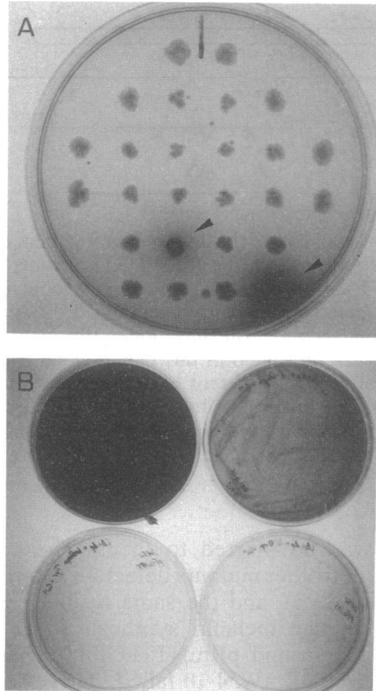

FIG. 2. (A) Detection of melanin production in two DH5α transformants (arrowheads) grown in LB with L-tyrosine and $CuSO_4$. Colonies were 1 week old, and there was no need to lyse them with SDS to show pigmentation in the surrounding medium. (B) Comparison of melanin production with L-tyrosine (left column) or L-DOPA (right column) as a substrate. The upper row shows plates with DH5α(pME100), and the lower row shows plates with DH5α (pFG105). Cells were lysed with 10% SDS after 3 days of incubation at 28°C.

Streptomyces spp., *Neurospora crassa*, humans, and mice as well as human catalase B, a tyrosinase-related protein from mice (Trp-1), and hemocyanins from *O. dofleini* and *Helix pomatia*. The best alignments were achieved with the CuB binding site of *Streptomyces* tyrosinase (46.7% identity in 60 amino acids). However, the alignments with eukaryotic tyrosinases were found at the CuA binding site (50% identity in 32 amino acids for mouse tyrosinase and 43.8% identity in 32 amino acids for human tyrosinase). The alignment with *N. crassa* tyrosinase spanned 247 amino acids, including the two copper binding sites. The deduced protein sequence also showed good homologies with hemocyanins. Positions 120 to 325 (Fig. 3) showed 25% identity with hemocyanin of *O. dofleini* and 22.90% identity with that of *H. pomatia*. Both alignments included the copper binding sites (Fig. 4). On the basis of these data, it is clear that the protein from GR4 contains the CuA and CuB binding domains. The CuA binding site is probably located between positions 38 and 80 (Fig. 3), and the CuB binding site is probably located between positions 223 and 267. A theoretical prediction of the potential role of these residues in binding copper is made possible by a comparison with similar residues that were previously proven to have this role (11, 17, 22). Thus, in the CuA binding site, the His-44 and His-53 positions are conserved; very likely the His-38 position is, too. His-224, His-228, and His-256 may be the potential functional residues for the CuB binding site. However, it remains to be demonstrated experimentally that these domains actually constitute the Cu binding sites.

Protein production and tyrosinase activity detection. Utilization of a coupled transcription-translation system from *E. coli* S-30 extracts allowed the expression and production from plasmid pME100 of a polypeptide with an approximate molecular mass of 55 kDa, a size in good agreement with the predicted size deduced from the sequence (Fig. 5, lane 2). There was no specific polypeptides synthesis when plasmid pFG105 was used, suggesting that transcription actually occurred from the *lacZp* promoter present in the construction. These results supported the observations made in incubation experiments with strain DH5α harboring these plasmids on LB plates with L-tyrosine and $CuSO_4$. It was not possible to detect melanin production with plasmid pFG105. Furthermore, the sequence revealed the absence of the −35 and −10 conserved sequences resembling *E. coli* promoters as well as typical promoters with RpoN-NifA operators.

Nondenaturing polyacrylamide gel electrophoresis and further incubation with L-tyrosine as a substrate were used to analyze tyrosinase activity. In contrast to the isoelectric point calculated for the tyrosinase of *S. michiganesis* (pI 9.0), the computer-predicted pI for GR4 tyrosinase was 4.5. Therefore, electrophoresis in native polyacrylamide gels was conducted under basic conditions with the introduction of the necessary modifications developed for the detection of the tyrosinase activity of *Streptomyces* enzymes (16). The results obtained from this experiment are presented in Fig. 5, lane 5, showing the presence of a melanin precipitation band with a calculated molecular mass that corresponded to the size of the polypeptide synthesized in vitro. This band was visible a few minutes after incubation with L-tyrosine, which is a better substrate than L-3,4-dihydroxyphenylalanine (L-DOPA) (Fig. 2). The appearance of a lower-molecular-weight faint band of activity could indicate nonspecific proteolytic activity, although the presence of an additional translational start codon was not ruled out. Nevertheless, changes in the intensity of the bands with time after the extraction (the lower-molecular-weight band became more intense) support the first hypothesis.

Detection of homology in rhizobium strains. Melanin production is widespread among *Rhizobium* species. For testing the degree of DNA conservation in GR4 tyrosinase, hybridization experiments were carried out with an internal *Ava*I fragment of 968 bp as a probe. Strong hybridization of GR4 with other melanin-producing *R. meliloti* strains, such as 102F34 and L5.30, was observed. No hybridization was detected with melanin-producing strain VF39 or between the *mepA* gene of GR4 and the *melA* gene of strain 8002 cloned in pIJ1578. The tyrosinase gene may be well conserved among *R. meliloti* melanin-producing strains, but the *R. meliloti* tyrosinase gene may be more divergent from the tyrosinase gene present in strains of *R. leguminosarum* despite their phylogenetic proximity.

DISCUSSION

The observations that aged colonies of *R. meliloti* GR4 produced a brownish to black pigment on TY medium and that young colonies lysed with SDS also produced the pigment on TY medium supplemented with L-tyrosine and Cu suggested that this strain is a melanin producer. The loss of this characteristic in plasmid pRmeGR4b-cured GR4 demonstrated the relationship between melanin production and the presence of this large plasmid. This conclusion was further confirmed by transfer experiments and the corresponding genetic analysis.

Plasmid pRmeGR4b is one of the nonsymbiotic plasmids

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1      CCCTTGCCGTAGCTGCAAGACATTCTGTCTAACACCTGTTCAGGGGATAATCTCGATGACACGCACGCGATTTCTGACGGTTTCACGCCGCTCTTTCGTC

                                                                                          M  T  S  A  D  G  Q
101    AAAGGCGCAGCGCTGCCGCTGGCACAGTGCTCTTCGCACCGAGCATTCTGCGGGCGGCGACCAAGCATAGGCGCAAGAACATGACCAGCGCCGATGGGCA
                                                                                                       *
        K  D  L  Q  S  Y  M  D  A  V  T  A  M  L  K  L  P  P  S  D  R  R  N  W  Y  R  N  G  F  I  H  L  M
201    GAAGGACTTGCAAAGTTATATGGATGCCGTTACCGCGATGCTGAAGCTTCCGCCTTCGGATCGGCGCAACTGGTACCGCAATGGCTTCATCCACCTGATG
                *                          *
       D  C  P  H  G  D  W  W  F  T  S  W  H  R  G  Y  L  G  Y  F  E  E  T  C  R  E  L  S  G  N  P  D  F  A
301    GACTGCCCCCATGGCGACTGGTGGTTCACCAGCTGGCACCGCGGCTATCTTGGCTATTTCGAAGAGACTTGCCGCGAACTGAGCGGCAATCCGGATTTCG

        L  P  Y  W  D  W  T  A  N  P  E  V  L  P  P  L  F  G  T  I  L  D  P  V  N  S  S  A  Y  I  P  D  H
401    CCCTCCCATATTGGGACTGGACGGCCAATCCCGAGGTCCTGCCGCCGCTGTTCGGCACGATTCTCGATCCCGTCAACAGCTCCGCCTACATTCCCGACCA

       N  R  F  Q  D  I  M  Q  E  P  I  K  A  Y  W  D  S  L  S  P  A  Q  L  Q  Q  Q  N  L  R  G  Y  P  D
501    CAACCGCTTCCAGGACATCATGCAGGAGCCGATCAAGGCCTATTGGGACAGTCTCAGCCCCGCCCAATTGCAGCAGCAGAACCTGCGCGGCTATCCGGAT

       F  D  A  L  W  S  D  A  M  A  S  F  A  N  Q  P  N  A  R  F  L  T  A  Q  N  P  K  L  N  P  A  T  Q  T
601    TTTGATGCGCTATGGAGCGACGCAATGGCGAGCTTCGCCAACCAGCCGAACGCCCGCTTCCTGACGGCGCAGAATCCGAAACTCAATCCCGCCACCCAAA

        A  V  D  I  D  T  I  K  A  S  L  A  P  T  T  F  A  N  D  A  G  A  P  G  L  A  F  N  S  P  V  S  S
701    CCGCAGTCGACATCGACACCATCAAGGCATCGCTGGCGCCAACAACCTTCGCCAACGACGCGGGCGCTCCGGGTCTCGCTTTCAACAGTCCGGTATCGTC
                                                         *              *
       S  H  Q  V  A  P  V  G  F  S  I  L  E  G  Q  P  H  N  R  V  H  M  S  V  G  G  Q  S  A  P  Y  G  L
801    CAGCCACCAGGTGGCACCGGTCGGCTTCTCCATTCTCGAAGGCCAGCCGCATAACCGCGTCCATATGAGCGTCGGCGGCCAGAGCGCTCCCTATGGGCTG
                                                      *
       M  S  Q  N  L  S  P  L  D  P  I  F  F  L  H  H  C  N  I  D  R  L  W  D  V  W  T  R  K  Q  Q  A  M  G
901    ATGTCACAGAACCTGTCACCGCTCGACCCGATCTTCTTTCTGCATCATTGCAACATCGATCGGCTGTGGGATGTCTGGACCCGCAAGCAGCAGGCGATGG

        L  P  V  G  P  T  A  D  Q  Q  T  Q  Y  D  P  E  P  Y  L  F  Y  V  N  A  D  G  S  P  V  S  D  K  T
1001   GCCTGCCCGTCGGGCCAACGGCTGACCAACAGACGCAGTACGATCCGGAACCCTATCTCTTTTATGTCAACGCTGACGGCAGCCCGGTCAGCGACAAGAC

       R  A  A  D  Y  L  E  I  G  D  F  D  Y  D  Y  D  P  G  S  G  E  E  V  I  P  V  A  T  A  G  R  S  A
1101   CAGGGCCGCCGACTATCTCGAAATCGGCGACTTTGACTATGATTATGACCCTGGCAGCGGCGAAGAGGTGATCCCGGTTGCAACCGCCGGCCGCTCGGCC

       P  I  P  A  L  E  A  A  V  S  A  S  A  A  V  A  I  N  K  P  A  T  A  K  L  T  V  S  Q  E  L  V  D  V
1201   CCCATTCCGGCATTGGAAGCAGCCGTGTCTGCGTCCGCGGCCGTGGCCATAAACAAACCGGCGACTGCCAAGCTCACCGTTTCGCAGGAGCTCGTGGATG

        A  A  K  P  S  E  Q  S  R  Q  F  A  K  V  S  I  A  P  P  M  D  V  G  G  L  N  F  L  V  F  I  S  P
1301   TTGCCGCGAAGCCTTCGGAACAGTCGCGTCAATTCGCGAAGGTCAGCATCGCGCCGCCGATGGACGTTGGCGGACTGAATTTCCTCGTTTTCATTTCCCC

       E  G  T  T  P  D  L  N  P  D  G  P  D  F  A  G  S  F  E  F  F  G  V  R  H  H  H  T  D  T  V  S  F
1401   CGAGGGAACGACGCCTGATCTCAACCCGGACGGACCGGATTTCGCCGGCAGTTTCGAGTTCTTCGGTGTTCGTCATCATCATACCGACACGGTCAGCTTC

       T  I  P  I  D  K  A  L  D  R  L  I  D  D  G  R  L  K  A  G  E  P  I  D  F  A  V  V  V  A  Q  E  G  K
1501   ACCATACCGATCGACAAGGCGCTCGACAGGCTGATCGACGATGGTAGGCTCAAAGCAGGCGAACCGATCGACTTCGCCGTTGTGGTCGCGCAAGAGGGCA

        R  V  E  G  S  M  P  A  K  A  Q  L  T  D  I  Q  V  G  S  F  *
1601   AGCGTGTTGAAGGCAGCATGCCGGCGAAGGCACAGCTGACCGACATTCAGGTGGGCTCGTTCTGAACCGCGCGAGGCACAGAAATCCGGGGGTTATGTTT

1701   TCGGTGATGCGCTGGTCGCAAAACATGCTCGCGGTTT
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FIG. 3. DNA sequence of the coding region for the *mepA* gene. The potential ribosome binding site is underlined. Potential protein domains involved in copper binding are set in boldface type. CuA is at positions 38 to 80, and CuB is at positions 223 to 267. Histidine residues believed to be important in copper binding are marked with an asterisk.

harbored by *R. meliloti* GR4. Although its dispensability for symbiosis is well known, its presence increases the efficiency of nodulation (36). Prior to these findings, *nfe* was the only genotype known to be associated with pRmeGR4b (31, 34).

The results presented here are different from those reported for the *mel* genes of *R. leguminosarum* bv. phaseoli (5, 14, 15). Hybridization experiments showed that there is no homology between an internal probe of the *mepA* gene of *R. meliloti* GR4 and the total DNA of melanin-producing strain VF39 of *R. leguminosarum* bv. viceae (18), nor is there homology with plasmid pIJ1578 carrying the proposed *melA* gene of *R. leguminosarum* bv. phaseoli 8002. The same hybridization experiments suggested that the structural gene for melanin synthesis does appear to be conserved in the three Mep$^+$ *R. meliloti* strains tested. Strains L5.30 and 102F34 can be added to the list of melanin producers previously reported by Cubo et al. (9). It was possible to determine that the Mep phenotype in strain L5.30 was not associated with nonsymbiotic plasmid pRmeL5.30a, since a plasmid-cured derivative that had lost this plasmid also produced melanin (data not shown).

Random mutagenesis of GR4 revealed that only one locus located within a 3.5-kb *Pst*I fragment of plasmid pRmeGR4b was involved in melanin synthesis. The Mep$^-$ mutants obtained always contained insertions in this fragment, and all of them were Fix$^+$. Several lines of evidence indicate that there is no relationship between melanin production and symbiotic nitrogen fixation in GR4, unlike the system described for strain 8002 of *Rhizobium leguminosarum* bv. phaseoli. Thus, when plasmid pRmNT111 was transferred to *nifA*, *ntrC*, or *rpoN* derivatives of non-melanin-producing strain 2011, it was always possible to observe pigment production in the corresponding transconjugants. Furthermore, complementation of *E. coli* cells harboring pRmNT111 with plasmid pRmW54-10 or pCK1 did not induce such a characteristic. Additionally, the behavior of *A. tumefaciens* GMI9023 was different, since transconjugants containing plasmid pRmNT111 made melanin in the appropriate medium and those containing pIJ1578 did not. Finally, the production of melanin in *E. coli* harboring plasmid pME100 suggested transcription from *lacZp* and the subsequent production of tyrosinase. The fact that plasmid pFG105 did not lead to melanin production in *E. coli* indicated the presence of a promoter that was not functional in this host.

Sequence analysis of the cloned fragment and further comparison with information in the data banks revealed the presence of an ORF with a high coding probability and indicated that tyrosinase motifs were involved in copper binding. This ORF has been found so far in all tyrosinases sequenced and also in the hemocyanins present in some molluscan species. The conserved residues responsible for the copper binding activity (Fig. 5) seem to be present in the GR4 tyrosinase, although more detailed investigations will be necessary to prove whether the role of such residues is the same as that reported for other, homologous proteins of *S. glaucescens* (17), *N. crassa* (11), or *O. dofleini* (22). The molecular size predicted for GR4 tyrosinase (54,109 Da) is closer to that of eukaryotic enzymes than to that reported for

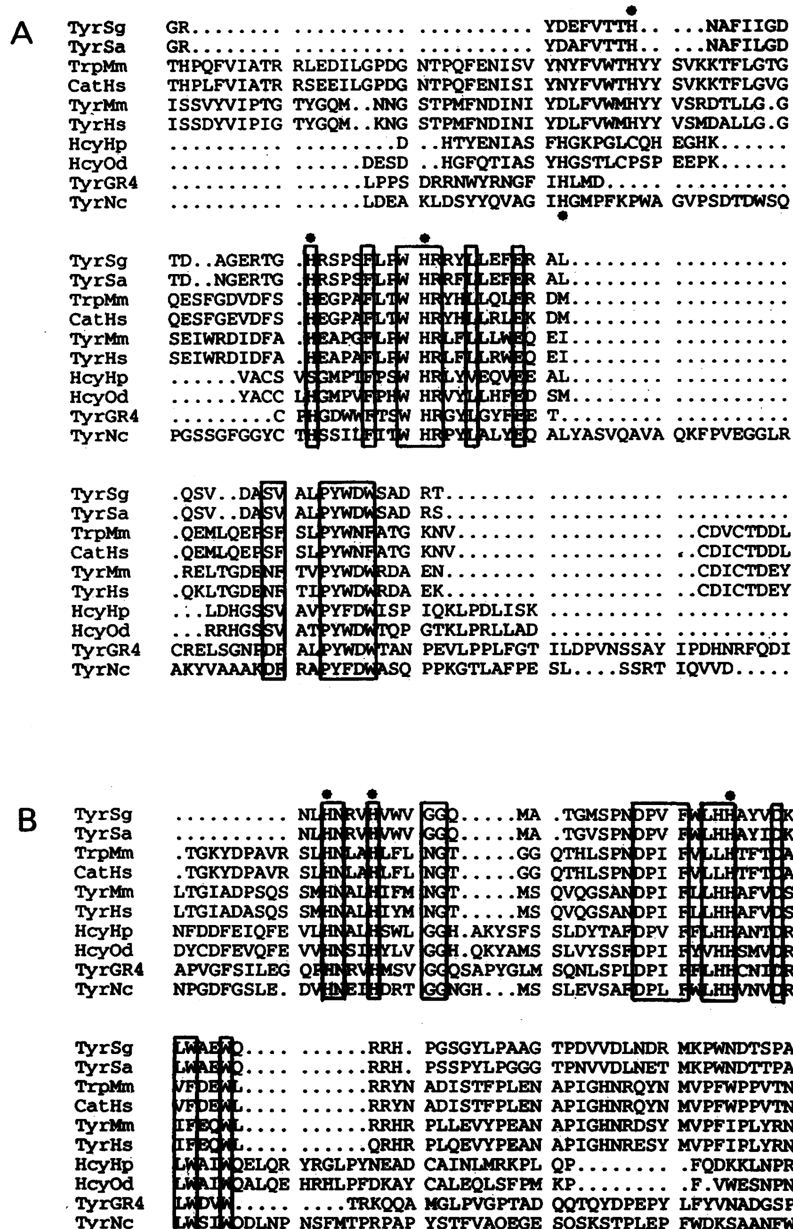

FIG. 4. Alignments of the CuA (A) and CuB (B) binding sites for several tyrosinases, tyrosinase-related proteins, and hemocyanins with proven homology to the tyrosinase of *R. meliloti* GR4. Identical residues or highly conservative substitutions are boxed. Histidine residues potentially serving as copper ligands are marked with an asterisk. Code: TyrSg, *S. glaucescens* tyrosinase; TyrSa, *S. antibioticus* tyrosinase; TrpMm, *Mus musculus* tyrosinase-related protein (brown locus); CatHs, *Homo sapiens* catalase B; TyrMm, *M. musculus* tyrosinase; TyrHs, *H. sapiens* tyrosinase; HcyHp, *H. pomatia* hemocyanin; HcyOd, *O. dofleini* hemocyanin; TyrGR4, *R. meliloti* GR4 tyrosinase; TyrNc, *N. crassa* tyrosinase.

Streptomyces enzymes. Nevertheless, a better alignment of the two copper binding domains was obtained for tyrosinases from *S. glaucescens* and *S. antibioticus*. The coupled transcription-translation system used in this work revealed the presence of a polypeptide with a molecular mass of 55 kDa, a size in good agreement with the size predicted from the sequence. It was only possible to detect this product with plasmid pME100, suggesting transcription from the *lacZp* promoter. Attention should be called here to the development of an easy method for the detection of tyrosinase activity in nondenaturing gels. Some modifications of the technique used to detect the tyrosinase activity of *Streptomyces* spp. following gel electrophoresis (16) produced a rapid and reliable system for use with *Rhizobium* tyrosinase. The results obtained could be improved by incubation with L-tyrosine instead of the L-DOPA used in the original technique. L-DOPA was less efficient than L-tyrosine, as also was observed in plate assays.

The possible role of melanin synthesis in rhizobia is poorly understood. A role for melanin in detoxifying phenolic compounds has been proposed for *Rhizobium leguminosarum* bv. phaseoli, on the basis of the accumulation of these

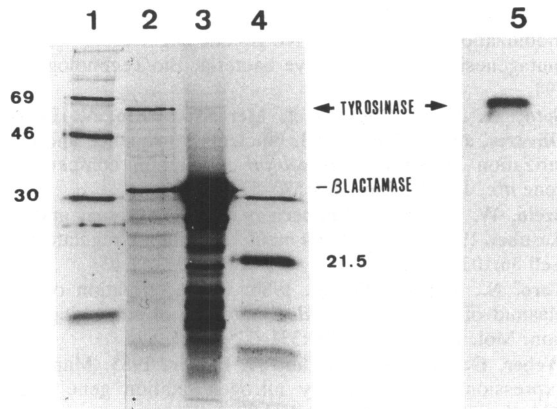

FIG. 5. (Lanes 1 to 4) Autoradiograph showing the results obtained from in vitro-coupled transcription-translation production of proteins from the 3.5-kb *Pst*I fragment cloned in pUC18. Molecular masses are given in kilodaltons. Lanes: 1 and 4, molecular mass markers; 2, pME100; 3, pUC18. (Lane 5) Detection in native polyacrylamide gels of tyrosinase activity in extracts of DH5α (pME100).

compounds in nodules and roots of senescent bean plants (5). One may assume that melanin plays a role on the basis of its chemical characteristics, such as the prevention of cellular dehydration through sequestration of compatible solutes from the environment because of its cation exchange properties (38). This activity for melanogenesis has been proposed to explain the survival of *Vibrio cholerae* in estuarine environments during the summer months, when water temperature rises and salinity increases because of evaporation (8). A similar behavior may occur in the melanogenesis of *R. meliloti*, on the basis of the soil and climatic conditions under which GR4 was isolated. With regard to this idea, it was possible to detect a decrease in cell viability for plasmid-cured GR4 derivatives under soil dessication conditions (data not shown).

The gene analyzed here is the first *mep* gene to be characterized for *Rhizobium* spp. We propose the name change from *mel* to *mep* to avoid confusion with genes involved in melibiose metabolism. This work could serve as an initial step in the elucidation of the biological implications of melanin production in these bacteria.

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