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Melanin Production by *Rhizobium* Strains

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Different *Rhizobium* and *Bradyrhizobium* strains were screened for their ability to produce melanin. Pigment producers (Mel$^+$) were found among strains of *R. leguminosarum* biovars *viceae*, *trifolii*, and *phaseoli*, *R. meliloti*, and *R. fredii*; none of 19 *Bradyrhizobium* strains examined gave a positive response. Melanin production and *nod* genes were plasmid borne in *R. leguminosarum* biovar *trifolii* RS24. In *R. leguminosarum* biovar *phaseoli* CFN42 and *R. meliloti* GR015, *mel* genes were located in the respective symbiotic plasmids. In *R. fredii* USDA205, melanin production correlated with the presence of its smallest indigenous plasmid.

Many *Rhizobium leguminosarum* biovar *phaseoli* strains produce a dark-brown pigment when grown on complete (TY) medium (6). This pigment seems to be a type of melanin because the addition of L-tyrosine to the medium increases its production and also because the pigment bleaches when hydrogen peroxide is added to colonies growing on agar in petri dishes.

Genetic studies of melanin production by rhizobia have been restricted to *R. leguminosarum* biovar *phaseoli* strains, in which it has been shown that *mel* genes are located in the symbiotic plasmid (6, 23, 32). Lamb et al. (32) demonstrated that melanin production is not required for symbiotic nitrogen fixation because several Mel$^-$ mutants obtained by transposon Tn*5* mutagenesis of the *R. leguminosarum* biovar *phaseoli* symbiotic plasmid pRP2JI were able to nodulate and fix nitrogen on *Phaseolus* beans. However, one Mel$^-$ mutant (strain JW169) was also Fix$^-$, suggesting that at least one *mel* gene is required for symbiotic nitrogen fixation. Borthakur et al. (7) demonstrated that the symbiotic plasmid pRP2JI contains two separate regions (I and II) required for melanin synthesis. Cloned region II (containing class II *mel* genes) complemented the Mel$^-$ Fix$^-$ mutant strain JW169 for melanin synthesis and effective nodulation on *Phaseolus* beans. Interestingly, the cloned DNA fragment containing class II *mel* genes hybridized to a *nifA*-like gene of *R. leguminosarum* biovar *viceae*. Although it is not known whether melanin production by rhizobia plays any role in the *Rhizobium*-legume symbiosis, the results suggest that both processes could be linked.

The aim of this work was to investigate whether melanin production is restricted to *R. leguminosarum* biovar *phaseoli* strains or whether it can be found in other *Rhizobium* species, because it is generally believed that only *R. leguminosarum* biovar *phaseoli* strains produce melanin (28, 32). We also wished to determine whether *mel* genes are always located in the symbiotic plasmid of melanin-producing rhizobia.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids are shown in Table 1.

Culture conditions and media. For growth of *Rhizobium* strains, minimal (SY) and complete (TY) media as described by Beringer (4) were used. *Agrobacterium tumefaciens* and *Escherichia coli* strains were grown on TY or LB (33).

Liquid cultures of *Rhizobium* and *Agrobacterium* strains were incubated at 28°C on an orbital shaker at 120 rpm. *E. coli* strains were grown at 37°C. For plasmid-curing experiments, YM medium (48) was used.

Plasmids were transferred by conjugation on membranes as described by Buchanan-Wollaston et al. (12) or by patch matings as described by Simon (44).

Mobilization of indigenous rhizobial plasmids. To mobilize indigenous plasmids, the Tn*5*-Mob carrier pSUP5011 (44) was transferred from *E. coli* S17-1 to *R. leguminosarum* biovar *trifolii* RS24 and *R. meliloti* GR015. Kanamycin-resistant transconjugants of strain RS24 were patch-mated in triparental crosses with *A. tumefaciens* GMI9023 (the recipient) and *E. coli* HB101 containing the helper plasmid pRK2013.

To mobilize the symbiotic plasmid of *R. meliloti* GR015, kanamycin-resistant transconjugants were randomly selected, purified by single-colony isolation, and conjugated with *E. coli* 1843, selection being made for inheritance of the helper plasmid pJB3JI. Transconjugants carrying the helper plasmid were assayed for their ability to donate kanamycin resistance in membrane crosses with *A. tumefaciens* AB274.

Plasmid. Plasmid visualization was done by agarose electrophoresis as described by Buendía-Clavería and Ruiz-Sainz (13). For better visualization of the plasmid profiles of *R. leguminosarum* biovar *trifolii* RS24 and its derivatives, electrophoresis was longer (5 instead of 3 h) and at higher voltage (120 instead of 100 V). The transfer of plasmid DNA from agarose gels to nitrocellulose paper and high-stringency hybridizations to nick-translated DNA probes were done by standard techniques (33).

Plasmid curing. Test tubes containing 5 ml of YM medium (48) supplemented with acridine orange, at final concentrations of 2, 5, and 10 μg of dye per ml of medium, were inoculated with *R. leguminosarum* biovar *trifolii* RS2400 containing Tn*5*-Mob (ca. 10^4 cells per tube). Cultures were incubated in the dark at 28°C for 1 week and then plated on TY medium for single-colony isolation. Colonies were individually screened for resistance to kanamycin (60 μg/ml). Colonies that showed a kanamycin-sensitive phenotype

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TABLE 1. Bacterial strains and plasmids used in this study

Strain	Relevant characteristics[a]	Source or reference
Rhizobium leguminosarum biovar *phaseoli*		
RCR3644	Wild type, Mel^+†	17
CFN42	Wild type, Mel^+†	37
CFN2001	pSym-cured derivative of CFN42, Mel^-	37
Rhizobium leguminosarum biovar *trifolii*		
RS24	Wild type, Mel^+*	R. Orive
RS240	Spontaneous Rif^r derivative of RS24, Mel^+	This work
RS2400	Km^r derivative of RS240 by insertion of transposon Tn*5*-Mob, Mel^+	This work
RS2400a	Kanamycin-sensitive derivative of RS2400 after treatment with acridine orange, Mel^-	This work
RS2400b	Kanamycin-sensitive derivative of RS2400 after treatment with acridine orange, Mel^+	This work
RS2400c	Kanamycin-sensitive derivative of RS2400 after treatment with acridine orange, Mel^+	This work
IST11	Wild type, Mel^+*	R. Orive
RS169	Wild type, Mel^-	42
RS169-NA545	Nonnodulating derivative of RS169 after treatment with acridine orange, Mel^-	3
RS169-NA4	Spontaneous Str^r derivative of RS169-NA545, Mel^-	R. Bellogín
NA400	RS169-NA4(pJERS1) (from cross RS2400 × RS169-NA4), Mel^+*	This work
Rhizobium leguminosarum biovar *viceae*		
JI241	Wild type, Mel^+	29
B151	128C53 *str-279* (ΔpRL6JI), Mel^-	10
B1511	Spontaneous Rif^r derivative of B151, Mel^-	43
TC285	B1511(p42d) (from cross *A. tumefaciens* C58C1 × B1511), Mel^+	This work
TC286	B1511(p42d) (from cross *A. tumefaciens* C58C1 × B1511), Mel	This work
Rhizobium meliloti		
RCR2012	Wild type, Mel^+	17
GRO15	Wild type, Mel^+	F. Temprano
TC281	Spontaneous Rif^r derivative of strain GRO15, Mel^+*	This work
TC282, TC283, and TC284	Km^r derivatives of TC281 by insertion of transposon Tn*5*-Mob into pRmeGRO15, pJB3JI, Mel^+	This work
GRO19	Wild type, Mel^+	F. Temprano
M3	Wild type, Mel^+	F. Temprano
Rhizobium fredii		
USDA205	Wild type, Mel^+*	30
USDA205-M1	Spontaneous Rif^r derivative of strain USDA205, Mel^-	This work
USDA205-M2	Spontaneous Rif^r derivative of strain USDA205, Mel^-	This work
AB55	Spontaneous Str^r derivative of strain USDA205, Mel^+*	13
TC290	AB55(R68.45) (from cross *E. coli* 1230 × AB55), Mel^+	This work
AB034	Spontaneous Rif^r derivative of strain USDA205, Mel^+*	This work
AB034M1 to AB034M19	Km^r transconjugants of AB034 by transposon Tn*5*-Mob mutagenesis, Mel^-	This work
HH003	Wild type, Mel^+*	15
HH102	Wild type, Mel^+*	15
HH103	Wild type, Mel^+*	15
Agrobacterium tumefaciens		
GMI9023	Rif^r Str^r Mel^-	40
AB274	Spontaneous CM^r derivative of strain GMI9023, Mel^-	This work
G-2400	AB274(pJERS1) (from cross RS2400 × AB274), Mel^+*	This work
G-1503	AB274(pTC1) (from cross TC282 × AB274), Mel^+	This work
G-1520	AB274(pTC2) (from cross TC283 × AB274), Mel^+	This work
G-1528	AB274(pTC3) (from cross TC284 × AB274), Mel^+	This work
C58C1	Ery^r Cml^r pTi-cured derivative of *A. tumefaciens* C58, Mel^-	22
AS-1987	C58C1(p42d), Mel^+†	M. Megías
Escherichia coli		
S17-1	294 Rec^-, chromosomally integrated RP4 derivative, Tp^r Str^r	45
1230	Pro^- Met^- Nal^r (R68.45)	5
1843	Pro^- Met^- Nal^r (pJB3JI)	5
HB101	*hsdS hsdM pro leu thi gal lacY recA* Str^r	8
Plasmids		
pSUP5011	pBR325::Tn*5*-Mob	45
pRK2013	Used for mobilizing plasmids, Km^r	18
R68.45	P1 group R-plasmids: Ap^r Km^r Tc^r	19
pJB3JI	R68.45 Km^s, used for mobilizing plasmids	9
pRtRS24g	220-MDa indigenous plasmid of *R. leguminosarum* biovar *trifolii* RS24; Mel^+	This work

Continued on following page

TABLE 1—*Continued*

Strain	Relevant characteristics[a]	Source or reference
pJERS1	Tn*5*-Mob derivative of pRtRS24g; Mel$^+$	This work
pCFN42d	Sym plasmid of *R. leguminosarum* biovar *phaseoli* CFN42; Mel$^+$	38
p42d	Tn*5*-Mob derivative of pCFN42d; Mel$^+$	37
pRmeGRO15	Sym plasmid of *R. meliloti* GRO15, Mel$^+$	This work
pTC1, pTC2, and pTC3	Tn*5*-Mob derivatives of pRmeGRO15; Mel$^+$	This work
pAT153::Tn*5*	Used as Tn*5* probe	G. Matassi
pID1	pBR322::*nifHDK* of *R. meliloti* 41	2

[a] Mel$^+$, Melanin production was detected in TY medium supplemented with L-tyrosine and $CuSO_4$. Melanin production was (*) or was not (†) detected in SY medium supplemented with L-tyrosine and $CuSO_4$. The following bacterial strains were Mel$^-$ in TY medium supplemented with L-tyrosine and $CuSO_4$: *R. leguminosarum* biovar *phaseoli* C57 (J. Day) and CIAT899 (34); *R. leguminosarum* biovar *trifolii* RCR5, RCR221, RCR227, RCR0420 (17), IST3, IST8, IST9, IST10, IST14, IST15, IST17, and IST22 (R. Orive); *R. leguminosarum* biovar *viceae* 300 (25), T3 (21), RCR1007, RCR1055, and RCR1045 (17); *R. meliloti* RCR2001 (17); *R. fredii* strains USDA192, USDA193, and USDA194 (30); *R. loti* strains U226, NZP2238, NZP2034 (F. Temprano), and RCR3011 (17); *Rhizobium* (*Macroptilium*) sp. strain MPIK3030 (47); *Rhizobium* (*Ornithopus*) sp. strain L13-3 (F. Temprano); *Rhizobium* (*Cicer*) sp. strains RCR3827 (17), IC122, and IC2073 (E. S. P. Bromfield); *Rhizobium* (*Cajanus*) sp. strains IHP307 and IHP509 (E. S. P. Bromfield); *Rhizobium* (*Sesbania*) sp. strain ORS571 (16); *Bradyrhizobium japonicum* RCR3402, RCR3407, RCR3410, RCR3442 (17), USDA110 (20), and 845 (41); *Bradyrhizobium* (*Cajanus*) sp. strains IHP38 and IHP53 (E. S. P. Bromfield); *Bradyrhizobium* (*Cicer*) sp. strains IC72 and IC94 (E. S. P. Bromfield); *Bradyrhizobium* (*Vigna*) sp. strain 32H1 (31); *Bradyrhizobium* (*Lupinus*) sp. strains RCR3211 (17), L13-4 (F. Temprano), and ORI-4 (R. Orive); *Bradyrhizobium* (*Onobrychis*) sp. strains On16, On17, On17.1, and On18 (T. Mozo).

were further purified, and their plasmid profiles were analyzed by electrophoresis.

Nodulation tests. Nodulation tests on *Glycine max* cv. Malayan, *Trifolium pratense* cv. Britta, *Trifolium subterraneum* cv. Clare, *Trifolium repens* cv. Huia, *Trifolium alexandrinum*, and *Medicago sativa* cv. Aragón were done as described by Buendía-Clavería et al. (14). Nodulation tests on *Phaseolus vulgaris* cv. Rocquencourt were done as described by Beringer (4). Nitrogen fixation by nodules was assessed by acetylene reduction as described previously (14).

Detection of melanin production. Bacteria were grown on TY plates supplemented with L-tyrosine (600 μg/ml) and $CuSO_4$ (40 μg/ml). Fully grown colonies (3 to 4 days for fast growers and 7 to 10 days for slow growers) were treated with 0.05 ml of 10% (wt/vol) sodium dodecyl sulfate (SDS) in TBE (33) (pH 8.3). After a maximum of 24 h of incubation at room temperature, those colonies that had produced a diffusible dark brown pigment were scored as melanin producers (Mel$^+$). For detection of melanin production on SY minimal medium, the same procedure was followed. Melanin production by nodules was monitored as follows. Mature nodules were sliced in half and placed face down on top of a drop of SDS solution on the surface of a TY plate supplemented with L-tyrosine (600 μg/ml) and $CuSO_4$ (40 μg/ml). Melanin production around the pieces of nodule was scored in the first 3 h of incubation.

RESULTS

Developing a method to identify melanin-producing strains. The results of a screening for melanin production are shown in Table 1. These showed that strains of *R. leguminosarum* biovars *viceae*, *trifolii*, and *phaseoli*, *R. meliloti*, and *R. fredii* produced melanin (Mel$^+$) and that none of the slow-growing rhizobia were Mel$^+$ under the growth conditions described. The intensity of pigmentation, size of the dark areas formed around the colonies (or patch), and time necessary for detection of melanin production varied by strain. Many strains (e.g., *R. fredii* USDA205) produced melanin within the first hour of SDS treatment. In contrast, *R. leguminosarum* biovar *viceae* JI241 needed higher concentrations of L-tyrosine (1.5 mg/ml) and copper sulfate (80 μg/ml) and 24 h of incubation in SDS for melanin to be detected reliably. A few strains were also assayed for melanin production on minimal medium containing copper and tyrosine (Table 1); most of the Mel$^+$ strains produced melanin on this medium.

Melanin production from nodules. *Phaseolus* nodules induced by *R. leguminosarum* biovar *phaseoli* CFN42, RCR3644, and 899 were assayed for melanin production. Nodules formed by the Mel$^+$ strains CFN42 and RCR3644 produced the pigment within the first 3 h of SDS treatment, whereas nodules induced by the Mel$^-$ control (strain 899) remained white after SDS treatment.

Melanin production by *R. leguminosarum* biovar *phaseoli* CFN42. The symbiotic-plasmid-cured derivative of *R. leguminosarum* biovar *phaseoli* CFN42, strain CFN2001, failed to produce pigment (Mel$^-$). In addition, *A. tumefaciens* C58C1 is Mel$^-$, but its derivative (AS-1987), which carries the symbiotic plasmid p42d, a Tn*5*-Mob derivative of pCFN42d, was Mel$^+$.

These findings suggest that *mel* genes are located in the symbiotic plasmid pCFN42d. To confirm this, a Tn*5*-Mob derivative (p42d) was transferred to the nonnodulating *R. leguminosarum* biovar *viceae* strain B1511. Transconjugants (strains TC285 and TC286) inherited the ability to form nitrogen-fixing nodules on *Phaseolus* beans and became melanin producers (Table 1). Isolates from *Phaseolus* nodules induced by strains TC285 and TC286 retained their Mel$^+$ phenotype.

Melanin production by *R. leguminosarum* biovar *trifolii* RS24. Strain RS240 (RS24, Rif^r) was chosen to investigate whether *mel* genes are plasmid borne by using Tn*5*-Mob mutagenesis. Kanamycin-resistant RS240 transconjugants were obtained at a frequency of approximately 10^{-5}. Of 100 kanamycin-resistant RS240 transconjugants examined, 12 (strains RS2400 to RS2411) were able to transfer kanamycin resistance to *A. tumefaciens* GMI9023. One *A. tumefaciens* transconjugant from each cross (strains G-2400 to G-2411) was purified further and assayed for melanin production: one *A. tumefaciens* transconjugant (strain G-2400) coinherited Km^r and Mel$^+$. Plasmid electrophoresis (Fig. 1) showed that strain G-2400 had inherited a plasmid (ca. 220 megadaltons [MDa]) that was similar to the third largest plasmid of strain RS24 (pRtrRS24g). To investigate whether the putative melaninogenic plasmid also contained symbiotic genes, strain RS2400 was mated with the nonnodulating strain RS169-NA4, which has been cured of its symbiotic plasmid. Kanamycin-resistant transconjugants of strain RS169-NA4

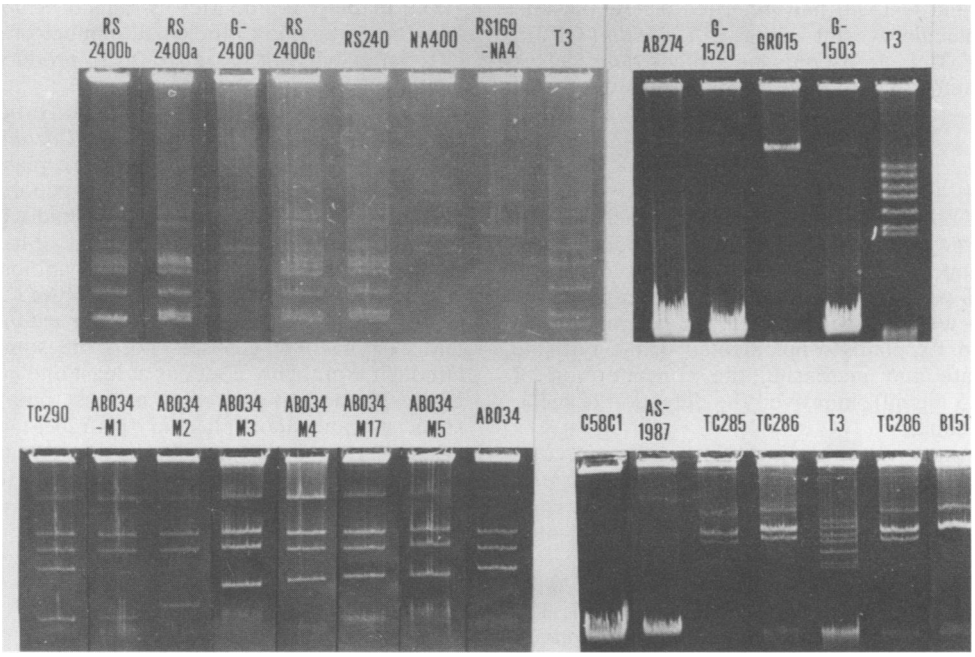

FIG. 1. Plasmid profiles of *Rhizobium* and *Agrobacterium* strains used in this study. See Table 1 for strain designations.

became Mel$^+$ (Table 1). One of the transconjugants (NA400) was selected for further analysis.

Plasmid electrophoresis showed that strain NA400 had inherited a plasmid with the same molecular weight as that shown by *A. tumefaciens* G-2400. This plasmid, carrying transposon Tn*5*-Mob and *mel* genes, was termed pJERS1. Strain NA400 was assayed for its ability to nodulate clover. It was an extremely poor symbiont on *Trifolium repens*, *T. alexandrinum*, and *T. subterraneum*, inducing less than five nodules per nodulated plant. None of the *T. pratense* plants were nodulated. Further confirmation that plasmid pJERS1 harbored *mel* genes was obtained from plasmid-curing experiments with acridine orange used to treat strain RS2400. Of 400 colonies tested, 3 were kanamycin sensitive. One of these (RS2400a) failed to produce melanin and had lost a plasmid similar in molecular weight to pJERS1 (Fig. 1). The other two kanamycin-sensitive derivatives (RS2400b and RS2400c) were Mel$^+$ and retained the plasmid that was lost in strain RS2400a, although plasmid pRtrRS24b was lost or deleted in RS2400b (Fig. 1). All of them formed nitrogen-fixing nodules on *T. repens*, *T. subterraneum*, *T. alexandrinum*, and *T. pratense*.

Melanin production by *R. fredii* USDA205. *R. fredii* USDA205, one of the most efficient melanin-producing rhizobia we have observed, was used to screen for spontaneous Mel$^-$ mutants on TY medium containing copper and tyrosine. Of 16,147 colonies studied, only 1 Mel$^-$ spontaneous mutant was identified.

Two independent Mel$^-$ derivatives of USDA205 (strains USDA205-M1 and USDA205-M2) were isolated when USDA205 was plated on TY supplemented with rifampin to isolate rifampin-resistant mutants. Plasmid electrophoresis showed that both strains had small deletions in the smallest plasmid (pRfr205a) (data not shown).

To obtain Mel$^-$ mutants of USDA205, random Tn*5*-Mob mutagenesis was carried out on strain AB034 (a spontaneous rifampin-resistant derivative of strain USDA205) by using the suicide plasmid pSUP5011. Nineteen Mel$^-$ mutants (AB034M1 to AB034M19) were identified in a screening of 1,194 kanamycin-resistant transconjugants. Plasmid electrophoresis of the Mel$^-$ mutants showed different-sized deletions in the smallest resident plasmid (pRfr205a) (Fig. 1).

Plasmid DNA from mutants AB034M2 to AB034M9 was transferred to nitrocellulose and hybridized to a Tn*5* probe (pAT153::Tn*5*). No deleted derivatives of plasmid pRfr205a hybridized to the Tn*5* probe. Only the indigenous plasmid pRfr205c of mutant strain AB034M9 showed strong hybridization to the Tn*5* probe (data not shown). Plasmids of strain USDA205 were also hybridized to a probe (pID1) containing the *R. meliloti nifHDK* genes. It only hybridized to plasmid pRfr205b.

The symbiotic properties of five Mel$^-$ mutants (AB034M1, AB034M2, AB034M3, AB034M5, and AB034M17) showing deletions of different sizes were assayed on *Glycine max* cv. Malayan. All the Mel$^-$ mutants formed nitrogen-fixing nodules.

Melanin production by *R. meliloti* GR015. *R. meliloti* strains GR015 and GR019 produce melanin, and electrophoresis of their DNA revealed one and four plasmid bands, respectively. Consequently, *R. meliloti* TC281 (a spontaneous rifampin-resistant derivative of strain GR015) was chosen to investigate whether the *mel* genes were located in the single plasmid. Random Tn*5*-Mob mutagenesis was carried out by using the suicide plasmid pSUP5011. Neonmycin-resistant transconjugants arose at a frequency of 10^{-5} per recipient. Twenty-five neomycin-resistant transconjugants were mated individually with *E. coli* 1843 (which contains the helper plasmid pJB3JI). One transconjugant clone from each cross was purified by selecting for the presence of both Tn*5*-Mob (Nm^r) and pJB3JI (Tc^r). The 25 Nm^r Tc^r clones were individually mated with *A. tumefaciens* AB274, and Nm^r transconjugants were selected. Eight Nm^r Tc^r *R. meliloti* clones transferred neomycin resistance, and three of them (TC282, TC283, and TC284) cotransferred the ability to produce melanin. Plasmid electrophoresis showed that Nm^r Mel$^+$ transconjugants of *A. tumefaciens* AB274 (clones

G-1503, G-1520, and G-1528) had inherited a new plasmid with the same molecular weight as that is *R. meliloti* GR015 (Fig. 1). The Nm^r Mel^+ transconjugants of *A. tumefaciens* AB274 induced ineffective nodules on alfalfa plants.

DISCUSSION

Johnston et al. (27) modified TY medium by adding L-tyrosine (30 μg/ml) and $CuSO_4$ (10 μg/ml) to facilitate the detection of melanin production by rhizobia. Although these supplements enhance melanin production, cultures often take longer than a week to be scored as melanin producers. The modifications we described in this paper, adding SDS to cultures grown on TY plates supplemented with L-tyrosine and copper sulfate and increasing the concentration of tyrosine (up to 1.5 mg/ml), improved the detection of melanin-producing rhizobia.

The results demonstrated that melanin production commonly occurs in *R. leguminosarum* biovars *viceae*, *trifolii*, and *phaseoli*, *R. meliloti*, and *R. fredii* and is not restricted to *R. leguminosarum* biovar *phaseoli*, as has been indicated (28, 32).

The *R. leguminosarum* biovar *phaseoli* plasmid pCFN42d is a symbiotic plasmid carrying *nod* and *fix* genes (37, 38). Transfer of this plasmid to the nonnodulating *R. leguminosarum* biovar *viceae* strain B1511 produced transconjugants which nodulated *Phaseolus* beans effectively and produced melanin. These results are in accordance with those in which *mel* was shown to be located on the symbiotic plasmid of Mel^+ *R. leguminosarum* biovar *phaseoli* strains (6, 23, 32).

Transfer of the third largest resident plasmid (pJERS1) of *R. leguminosarum* biovar *trifolii* RS24 to *A. tumefaciens* AB274 and to *R. leguminosarum* biovar *trifolii* RS169-NA4 produced Mel^+ transconjugants (G-2400 and NA400, respectively), demonstrating that *mel* genes are plasmid borne in *R. leguminosarum* biovar *trifolii* RS24. This plasmid also contains at least one *nod* gene, because transconjugants of the Nod^- strain RS169-NA4 carrying pJERS1 were able to induce a few Fix^- nodules on some *T. repens*, *T. alexandrinum*, and *T. subterraneum* plants. These results suggest that *R. leguminosarum* biovar *trifolii* RS24 has more than one plasmid with *nod* genes, because transfer of pJERS1 alone was insufficient to restore wild-type nodulation properties to strain RS169-NA4.

The observation that all 21 Mel^- mutants isolated in two different ways had deletions in the smallest resident plasmid (pRfr205a) indicates that melanin production by *R. fredii* USDA205 is also plasmid specified. However, the reason that 1.5% of the kanamycin-resistant transconjugants arising from transposon mutagenesis were Mel^- mutants because of deletions in their smallest resident plasmid is not known. The lack of hybridization between the Tn*5* probe and the plasmid with deleted DNA in all eight Mel^- Km^r transconjugants assayed suggests that the deletions might not have been caused by Tn*5* insertions or that insertion led to deletion of DNA carrying the transposon. There might be a correlation between the presence of rifampin in the medium used to select transconjugants and the appearance of deletions in plasmid pRfr205a. This would explain how Mel^- mutants (USDA205-M1 and USDA205-M2) were obtained when USDA205 was plated on TY medium containing rifampin to select spontaneously resistant mutants. Because Mel^- mutants of USDA205 formed nitrogen-fixing nodules on soybean plants, it is clear that in this strain melanin production is not essential for either nodulation or nitrogen fixation.

Our inability to find Mel^+ strains of *R. loti* or *Bradyrhizobium* could be due to the small number of strains examined or to an inability of those strains to produce melanin on TY medium.

Genes for nodulation and nitrogen fixation are carried on large plasmids in most fast-growing *Rhizobium* species (2, 6, 24, 26, 36). Similarly, *mel* genes are plasmid borne in all *Rhizobium* species tested. Previous reports have indicated that *mel* genes are situated in a plasmid which also harbors symbiotic genes (6, 7, 23, 32). Here, we have demonstrated that *mel* genes are located in the symbiotic plasmid of *R. leguminosarum* biovar *phaseoli CFN42 and R. meliloti* GR015. In *R. leguminosarum* biovar *trifolii* RS24, the plasmid carrying *mel* genes is not "the symbiotic plasmid," although it probably contains at least one *nod* gene. *R. fredii* USDA205 appears to carry at least one *mel* gene on its smallest indigenous plasmid (pRfr205a). As reported by others (11, 36), we have found that this plasmid does not hybridize to *R. meliloti nifHDK* genes. However, it is not clear whether pRfr205a carries *nod* genes or not (1, 35).

It is clear that further investigations are needed to ascertain whether melanin production by *Rhizobium* plays any role in the symbiotic process.

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