A *Rhizobium leguminosarum* gene required for symbiotic nitrogen fixation, melanin synthesis and normal growth on certain growth media

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The gene *nfrX* in *Azotobacter vinelandii* activates transcription of other *nif* genes in that species. A cosmid containing cloned *Rhizobium leguminosarum* DNA that corrected the Nif⁻ defect of an *nfrX* mutant of *A. vinelandii* was isolated. Following Tn5 transposon mutagenesis of the cosmid in *Escherichia coli*, mutant derivatives unable to correct the *A. vinelandii nfrX* mutants were obtained in two separate regions of DNA. In addition, mutations close to one of the *nfrX* regions conferred a complex phenotype when introduced into the *Rhizobium* genome by marker exchange. These mutants induced non-fixing nodules on peas, were slow-growing on media with succinate as C source or nitrate as N source and, when present in *R. leguminosarum* biovar *phaseoli*, they failed to make melanin, a pigment that is normally synthesized by *R. l. bv. phaseoli*. The mutated gene, termed *melC*, was fused to *uidA* (which encodes β -glucuronidase); it was found that transcription of *melC-uidA* was enhanced in microaerobic conditions and that it was expressed at high levels in infection threads in pea nodules.

Introduction

Gram-negative soil bacteria known collectively as the 'rhizobia' have the ability to induce and occupy nodules on the roots (and sometimes shoots) of leguminous plants within which the bacteria fix atmospheric N₂. The interaction is complex and precise, involving many defined morphological and biochemical changes in both partners. As might be expected, many genes in both participants in the interaction are required for the development of a mature, N₂-fixing nodule, and with the bacterial partner, in particular, many of these symbiotic genes have been identified and analysed in some detail (see Long, 1989, for a review).

In the fast-growing genus *Rhizobium* many but by no means all of the symbiosis-related genes are on large Sym plasmids. In the species *R. leguminosarum* there are three biovars, namely *phaseoli*, *trifolii* and *viciae*. These are defined on the basis of their host-ranges which are, respectively, represented by *Phaseolus* beans, clover and peas, the host-range determinants being a cluster of genes on the particular Sym plasmid contained in each of these three biovars. Apart from host-range specificity

there are very few features that distinguish these three biovars. One characteristic, though, is that nearly all strains of *bv. phaseoli* but very few of the other two make the pigment melanin (Johnston *et al.*, 1978; Beynon *et al.*, 1980). It has been shown that at least two genes required for its synthesis are located on the Sym plasmid of *R. l. bv. phaseoli*. Mutations in one of these abolish symbiotic N₂ fixation on *Phaseolus* beans and it was shown that this 'mel' gene was in fact the regulatory *nifA* gene which is also required for the activation of transcription of many *nif* genes (Borthakur *et al.*, 1987; Hawkins & Johnston, 1988). A second gene, *melA*, which is also on the Sym plasmid, is required for melanin synthesis but mutations in *melA* had no apparent effect on symbiotic N₂ fixation (Borthakur *et al.*, 1987). Transcription of *melA* was activated, under conditions of low oxygen tension, by the regulatory *nifA* gene of *R. l. bv. phaseoli* (Borthakur *et al.*, 1987; Hawkins & Johnston, 1988); indeed when the cloned *melA* gene was transferred to an *Escherichia coli* strain containing the cloned *nifA* gene of the diazotroph *Klebsiella pneumoniae*, the colonies of *E. coli* were brown (Hawkins & Johnston, 1988). Though not formally proven, the fact that transfer of *melA* to *E. coli* resulted in melanin production (provided that *nifA* was present to activate transcription of *melA*) suggested that *melA* was the structural gene for tyrosinase, the enzyme that catalyses the conversion of tyrosine to the polymer melanin.

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Given that mutations in *melA* have no discernible effects on symbiotic N₂ fixation under the conditions that we employ in the laboratory, it is clear that melanin synthesis *per se* is not absolutely required for N₂ fixation. However, the fact that the putative gene for tyrosinase is on the Sym plasmid and, more strikingly, that *melA* is activated by *nifA*, implies that there is a selective advantage to strains of *R. l. bv. phaseoli* which possess the ability to make melanin. One possible role is that the 'tyrosinase' in fact acts as a polyphenol oxidase; potentially harmful phenolics, made by the plant as the nodule senesces, may be detoxified due to polymerization mediated by the product of *melA*.

Having shown that two genes on the Sym plasmid of *R. l. bv. phaseoli* were required (and apparently sufficient) for melanin synthesis, we report here the presence of another gene, *melC*, which is not on the Sym plasmid and which is required, not only for melanin synthesis but also for symbiotic N₂ fixation. Further, mutations in *melC* cause a reduction in growth rate of *Rhizobium* in certain growth media.

Methods

Strains and plasmids. Strains of *Rhizobium*, *E. coli*, *Azotobacter vinelandii* and plasmids that were used are shown in Table 1.

Media and general microbiology. Growth media and general culture conditions for growing *Rhizobium* and *E. coli* were as in Beringer (1974). *A. vinelandii* was grown on Burk's sucrose medium with (BSN) or without (BS) fixed nitrogen (Newton *et al.*, 1953).

Plasmids were transferred by conjugation as described by Johnston *et al.* (1978); in some cases the 'helper' plasmid pRK2013 was also present in triparental matings (Figurski & Helinski, 1979). For conjugational matings between *E. coli* and *A. vinelandii* the latter was present in 10-fold excess.

Melanin production was determined by the method of Johnston *et al.* (1982). (1987). Cells were grown on TY medium supplemented with L-tyrosine and CuSO₄; after 3 d at 28 °C, cells were lysed by the addition of a drop of 1% (w/v) SDS.

Peas were inoculated and grown, nodules excised and bacteria isolated as described by Beynon *et al.* (1980).

β -Galactosidase activity was assayed according to the method of Miller (1972) as modified by Rossen *et al.* (1985). β -Glucuronidase was measured according to Jefferson *et al.* (1986). Bacteria were grown under controlled O₂ tension as described by Hawkins & Johnston (1988). The units of β -glucuronidase were calculated in the same way as the Miller units of β -galactosidase; however, the units obtained were two orders of magnitude less than for β -galactosidase and therefore the units are presented as $\times 100$.

The transposons Tn5 and Tn3 *gus* (*uidA*) were used as mutagens. The latter transposon contains a promoterless *uidA* gene close to one end and retains the ampicillin resistance of the parental Tn3. *E. coli* strains harbouring a chromosomally located copy of either transposon, and containing pIJ1651, were used as donors in triparental crosses with a recipient (either the *polA* *E. coli* strain C2110 or *Rhizobium*) which cannot support the replication of the helper plasmid pRK2013. Selection was made for the co-transfer of the resistance marker (Tc) of the target plasmid and that (Km or Ap) of the transposon. Marker exchange into the *Rhizobium* genome was as described by Ruvkun &

Table 1. *Strains and plasmids used in this study*

Strain or plasmid	Characteristics	Source
<i>E. coli</i>		
803	Used for transformation	Wood (1966)
C2110	Nal ^r ; used as recipient for transposon mutagenesis	Stachel <i>et al.</i> (1985)
UNF510	Contains chromosomal Tn5	Merrick <i>et al.</i> (1978)
<i>Rhizobium leguminosarum</i>		
4292	<i>bv. phaseoli</i> wild-type; also <i>nif</i>	Lamb <i>et al.</i> (1982)
8401pRL1JI	<i>bv. viciae</i> wild-type; also <i>str</i>	Downie <i>et al.</i> (1983)
<i>Azotobacter vinelandii</i>		
MV16	<i>nfrX</i> , Nif ⁻	Santero <i>et al.</i> (1988)
MV17	<i>nfrX</i> , Nif ⁻	Santero <i>et al.</i> (1988)
MV700	<i>nfrX</i> , Nif ⁻	Toukdarian & Kennedy (1986)
Plasmids		
pRP2JI	<i>R. l. bv. phaseoli</i> Sym plasmid	Lamb <i>et al.</i> (1982)
pRL1JI	<i>R. l. bv. viciae</i> Sym plasmid	Johnston <i>et al.</i> (1978)
pLAFR1	P1 group cosmid; Tc ^r	Friedman <i>et al.</i> (1982)
pKT230	Q group cloning vector – Kan ^r Str ^r	Bagdasarjan <i>et al.</i> (1981)
pPH1JI	P1 group plasmid used for marker exchange – Gm ^r	Johnston <i>et al.</i> (1978)
pRK2013	Used to mobilize plasmids in triparental crosses	Figurski & Helinski (1979)
pIJ1553	<i>R. l. bv. phaseoli nifA</i> cloned in pKT230	Borthakur <i>et al.</i> (1987)
pIJ1578	<i>melA</i> in pLAFR1	Borthakur <i>et al.</i> (1987)
pIJ1608	Cloned <i>melA-lacZ</i> fusion in pLAFR1	Borthakur <i>et al.</i> (1987)
pIJ1651	Contains approx. 30 kb of DNA including <i>nfrX</i> and <i>melC</i> in pLAFR1	This work
pIJ1745	pIJ1651 <i>melC::Tn5</i>	This work
pIJ1758	pIJ1651 <i>melC::uidA</i>	This work
pIJ1759	<i>melC</i> cloned in pKT230	This work

Ausubel (1981) except that pPH1JI was used to eliminate the pLAFR1-based plasmid.

DNA manipulations. Transformation, DNA isolation and restriction enzyme digestion were done essentially as described by Maniatis *et al.* (1982).

Histochemical staining of nodules. Histochemical assays were performed on nodule sections (Jefferson *et al.*, 1987). Nodule sections were cut by hand from unfixed nodules from inoculated plants and fixed in 0.3% formaldehyde in 10 mM-MES, pH 5.6, 0.3 M-mannitol for 45 min at room temperature, followed by several washes in 50 mM-NaH₂PO₄, pH 7.0. All fixatives and substrate solution were introduced into the sections with a brief (approx. 1 min) vacuum infiltration.

Results

Isolation of a plasmid containing nfrX and melC

Santero *et al.* (1988) isolated a mutant derivative MV16 of *A. vinelandii* which failed to fix N₂. It was shown that MV16 had a mutation in a gene termed *nfrX* which

resulted in the failure of the *nifHDK* operon to be transcribed; thus *nfrX* appears to be a positively-acting regulatory gene. MV16 also grew more slowly on medium containing ammonium acetate than the wild-type parent strain VW136. A fast-growing derivative, MV17, retained a Nif⁻ phenotype and had Tn5 in its original site of insertion.

To test whether there was a functional equivalent to *nfrX* in the genome of *R. leguminosarum*, recombinant plasmids representing a clone bank of *R. l. bv. phaseoli* (Lamb *et al.*, 1985) comprising fragments of DNA approximately 30 kb in size cloned in pLAFR1 were mobilized into *A. vinelandii* strain MV17. Selection was made for Nif⁺ Tc^r transconjugants. Several such transconjugants were obtained; plasmid DNA was isolated from these, transformed into *E. coli* strain 803 and then remobilized into MV17. In one case the MV17 transconjugants were uniformly Nif⁺, showing that a plasmid, termed pIJ1651, had been isolated which corrected the *nfrX* defect (Hawkins, 1989). It was shown by hybridization experiments in which pIJ1651 was used as a probe to genomic DNA from wild-type *R. l. bv. phaseoli* strain 4292 and to DNA from a derivative strain 8401 lacking the Sym plasmid pRP2JI, that pIJ1651 did not contain DNA originating from that Sym plasmid since the patterns of hybridizing fragments were identical for the two strains. Plasmid pIJ1651 was mutagenized with Tn5 and mutant derivatives which failed to correct the Nif⁻ phenotype of MV17 were screened directly by transferring the mutant plasmids into this strain and identifying any that failed to grow on N-free media. These mutations in pIJ1651 were in two clusters, separated by approximately 25 kb, each being towards the extremities of the cloned DNA (Hawkins, 1989).

In addition to this direct screening for insertions in *nfrX*-like genes in pIJ1651, we sought to determine whether there were other genes on this plasmid that were important for symbiotic N₂ fixation. To do this, pIJ1651 was introduced into *E. coli* strain UNF510, which contains a chromosomally located Tn5, and this strain was used as a donor in a triparental mating with *R. l. bv. phaseoli* strain 4292. Selection was made for the joint transfer of Km^r (Tn5) and Tc^r (pIJ1651). Some 50 such mutant derivatives were isolated and for each, the transposon was introduced by marker exchange into the genome of strain 4292. One mutant was altered in its phenotype compared to the wild-type in that it (a) failed to make melanin on complete TY medium and (b) on minimal (Y) medium was slow-growing as judged by visual assessment, the appearance of colonies being delayed by approximately 1 d. Once grown to full size the colonies had a similar appearance to those of the wild-type, indicating that they were not defective in exopolysaccharide production.

The derivative of pIJ1651 containing this mutation, pIJ1745, was isolated, transformed into *E. coli* and then transferred to *R. l. bv. phaseoli* by conjugation. Tn5 was again introduced into the *Rhizobium* genome by marker exchange. In all cases examined, the homogenotes were unable to make melanin even after prolonged (12 d) incubation on media supplemented with L-tyrosine plus CuSO₄ and following lysis of the cells with SDS. Further, these homogenotes were again slow-growing on minimal Y medium. Both these mutant phenotypes were restored to wild-type by the introduction of pIJ1651 but not by its mutant derivative pIJ1745. Thus it was shown that the unusual phenotype was due to the Tn5 insertion in a gene located on pIJ1651. Because of the role of this gene in melanin production, it was termed *melC*.

The Tn5 insertion in pIJ1745 was mapped using *Bam*HI, *Hind*III and *Eco*RI. The transposon was located in a 6.0 kb *Bam*HI fragment. The corresponding non-mutated *Bam*HI fragment was cloned from pIJ1651 into pKT230 to form pIJ1759. Introduction of pIJ1759 into the *melC* derivative of strain 4292 restored the ability to make melanin and to grow at normal rates. The Tn5 insertion was located approximately 5 kb downstream of one of the regions of pIJ1651 which had been shown to be required to correct the *nfrX* mutant MV17 of *A. vinelandii* (Hawkins, 1989). As might be expected, pIJ1745 when introduced into *A. vinelandii* strain MV17 retained the ability to correct its Nif⁻ defect.

Possible role of melC in melanin production

It had previously been demonstrated that when the *mela* gene, which encodes tyrosinase, of *R. l. bv. phaseoli* was introduced into *E. coli*, the colonies made melanin provided that the *E. coli* strain also contained the positively-acting *nifA* gene which is required to activate transcription of *mela* (Borthakur *et al.*, 1987; Hawkins & Johnston, 1988). This had suggested that *mela* and *nifA* were sufficient for melanin production so it was surprising to us to have identified a third genetic element, not on the Sym plasmid, which was also required for the synthesis of the pigment. This indicated that *E. coli* must have a functional counterpart to *melC*. We tested two possible roles for *melC* in melanin synthesis: firstly that it might be involved in the regulation of *mela* and secondly that it might be required for the uptake of Cu²⁺, a co-factor for tyrosinase.

To test the first hypothesis, plasmid pIJ1608, which contains a *mela-lacZ* transcriptional fusion, was introduced into *R. l. bv. phaseoli* strain 4292 and into 4292 *melC::Tn5* and β -galactosidase activities were measured on cells grown microaerobically; transcription of *mela* is enhanced under such conditions (Hawkins & Johnston, 1988). In both backgrounds β -galactosidase activities

Table 2. Effect of copper concentration on the growth and melanin production of mutant *melC*, wild-type, and multicopy *melC* strains of *R. l. bv. phaseoli*

CuSO ₄ concn ($\mu\text{g ml}^{-1}$)	Rhizobium strain*:					
	4292 <i>melC</i> ::Tn5 (0)		4292 (1)		4292/pIJ1651 (5-10)	
	Mel†	Growth‡	Mel	Growth	Mel	Growth
50	—	+	+	+	+	+
100	—	+	++	+	++	+
150	—	+	+++	+	+++	+
200	—	+	+++	+	+++	+
300	—	+	+++	+	+++	+
400	—	—	—	—	—	—

* The number of copies of the *melC* gene per cell is shown in parentheses.

† Melanin production was assessed visually following lysis of cells with 1% SDS. Strains were grown for 3 d at 28 °C on TY agar plates supplemented with tyrosine (30 mg ml⁻¹) and CuSO₄ at the concentration indicated. The amount of melanization was scored from — for no melanin production, to +++ for extensive melanin production.

‡ Bacterial growth (as judged by the appearance of single colonies) on TY agar plates supplemented with tyrosine (30 mg ml⁻¹) and CuSO₄ at the concentration indicated. Bacterial growth was scored as — (no growth) or + (appearance of single colonies after 3 d incubation at 28 °C).

were approximately 500 units, indicating that *melC* does not influence the transcription of *mela*.

However, it was shown that over-production of the *Rhizobium mela* gene product could overcome the Mel⁻ defect of *melC* mutant strains. Plasmid pIJ1578 contains the *mela* gene of *R. l. bv. phaseoli* strain 4292 cloned in pLAFR1. When pIJ1578 was introduced into 4292 *melC*::Tn5 the transconjugants could make melanin at normal rates. Further, when the *nifA* gene of *R. l. bv. phaseoli* cloned in pKT230 to form pIJ1553 was present in 4292 *melC*::Tn5, melanin was again made, owing, presumably, to the hyper-expression of *mela* due to multi-copy *nifA* (see Hawkins & Johnston, 1988).

A second possible explanation for the Mel⁻ phenotype, that *melC* is involved in Cu²⁺ uptake/processing, was examined as follows. If a mutation in *melC* resulted in reduced Cu²⁺ uptake, such a mutant might be predicted to exhibit enhanced copper tolerance compared to the wild-type and, conversely, a strain with multicopy *melC* might be more sensitive. Therefore, wild-type strain 4292, the *melC* mutant and 4292/pIJ1651 were each grown on solid TY + L-tyrosine supplemented with CuSO₄ at concentrations of 50–400 $\mu\text{g ml}^{-1}$. After 4 d growth at 28 °C there was no observable difference among the three different strains with regard to their copper tolerance: in all cases, a concentration of 400 $\mu\text{g ml}^{-1}$ completely inhibited growth (Table 2).

Further, the presence of multi-copy *melC* did not enhance melanin production relative to the wild-type nor did the higher concentrations of CuSO₄ restore to the *melC* mutant the ability to make melanin. Thus it seems unlikely that the *melC* mutation affects copper uptake and the biochemical role of *melC* in melanin synthesis is unknown.

Growth characteristics of the *melC* mutant

R. l. bv. phaseoli strain 4292 *melC*::Tn5 was originally identified as being unable to produce melanin and slow-growing on minimal (Y) medium but not on complete (TY) medium. The basis of slow growth on minimal medium was investigated with different C and N sources (succinate and sodium glutamate are the routine C and N sources, respectively), added to Y medium. Strains 4292 and 4292 *melC*::Tn5 were grown in liquid TY medium and in liquid Y medium supplemented with: (a) succinate and sodium glutamate; (b) succinate and ammonium chloride; (c) glucose and sodium glutamate; or (d) glucose and ammonium chloride. The growth rates (measured as OD₆₀₀) of the strains in the various media were determined (Fig. 1). Strain 4292 *melC*::Tn5 had a slower rate of growth than the wild-type strain 4292 when succinate was the sole C source. The growth rates of the wild-type and the *melC* mutant strains were identical when glucose was the C source or when the bacteria were grown in TY medium.

Having observed a difference in growth rate of the *melC* mutant strain compared to wild-type, depending on the C source, the ability of the two strains to utilize different N sources was then examined. This was done by visual assessment of the appearance of colonies of the two strains on plates of minimal (Y) medium supplemented with either succinate or mannitol as the C source and with sodium glutamate, ammonium chloride, proline, arginine or sodium nitrate as the N source. On all media containing succinate as the C source, the growth of strain 4292 *melC*::Tn5 was delayed compared to that of wild-type strain 4292. On media containing mannitol as the C source and sodium glutamate, ammonium chloride, proline or arginine as the N source, no difference in the growth rate of the two strains was observed. However, the growth of strain 4292 *melC*::Tn5 was delayed compared to the wild-type on minimal medium supplemented with mannitol plus sodium nitrate; this was typified by a 1–2 d delay in the appearance of single colonies for the *melC* mutant strain.

melC cannot complement an *A. vinelandii ntrA* mutation

In *Rhizobium meliloti*, the *ntrA* gene product, a sigma factor of RNA polymerase, is required for succinate

Fig. 1. Growth characteristics of wild-type *R. l. bv. phaseoli* (strain 4292) and its *melC* mutant derivative (strain 4292 *melC*::Tn5). Media used: (a) Y + succinate + sodium glutamate; (b) Y + succinate + NH₄Cl; (c) Y + glucose + sodium glutamate; (d) Y + glucose + NH₄Cl; (e) TY. ●, Strain 4292; ■, strain 4292 *melC*::Tn5. Each strain was inoculated into 3 × 100 ml liquid medium, such that initial OD₆₀₀ values were equivalent and the flasks were incubated, shaking, at 25 °C. The growth rate was monitored by measuring the OD₆₀₀ at 4 h intervals. Each point represents the mean OD₆₀₀ value of the three replicates; variation between replicates was less than 5%.

transport as well as for nitrate assimilation and symbiotic N₂ fixation (Ronson *et al.*, 1987). Mutations in the *R. l. bv. phaseoli melC* gene reduced, but did not abolish, the ability to utilize succinate as sole carbon source and nitrate as the sole nitrogen source and (see below) abolished N₂ fixation. Thus, the phenotype due to a *melC* mutation was similar, but not identical, to that of an *ntrA* mutant. To test whether *melC* corresponded to *ntrA*, pIJ1651, which contains *melC*, was mobilized into the Nif⁻ *A. vinelandii ntrA* mutant strain MV700 and the resulting transconjugant was tested for its ability to fix N₂. The presence of pIJ1651 did not restore the ability of MV700 to grow on BS, indicating that *melC* is not functionally equivalent to *ntrA* of *Azotobacter*.

Expression of *melC* and its role in N₂ fixation

Transcription of *melA*, a gene required for melanin production, was shown to be *nifA*-dependent, and oxygen-sensitive in its activation (Hawkins & Johnston, 1988). To investigate the possibility that the expression of *melC* (another gene required for melanin production) was also oxygen-sensitive, a fusion between *melC* and a reporter gene was constructed. So that the activity of *melC* in the bacteria present in nodules could also be examined, a *melC-uidA* fusion was constructed. The reporter gene *uidA* codes for β-glucuronidase (GUS) (Novel & Novel, 1972), which can be assayed histochemically both in free-living bacterial cultures and in

plant tissue due to the very low background levels in both rhizobia (unpublished) and eukaryotic organisms. β -Glucuronidase is a hydrolase that catalyses the cleavage of a wide variety of β -glucuronides.

To construct the appropriate fusion, pIJ1651 was mutagenized with Tn3-*uidA*. This is a Tn3-based transposon containing the *uidA* gene for glucuronidase production which requires for its expression transcriptional read-through from the gene into which it is inserted. Selection for a *melC-uidA* fusion was made by screening for loss of the ability of the pIJ1651 derivatives to complement strain 4292 *melC::Tn5* for melanin production. One such mutant derivative, pIJ1758, was identified. The Tn3-*uidA* insertion within this plasmid mapped close to the location of Tn5 in *melC* in pIJ1745.

Strains 4292 *melC::Tn5*/pIJ1758 and 4292 were inoculated in duplicate into 10 ml liquid Y medium (supplemented with succinate and sodium glutamate) and a sample of each was incubated at 28 °C for 48 h under 20% or 3% oxygen. The samples were then assayed for β -glucuronidase activity. The β -glucuronidase activity obtained from the 4292 *melC::Tn5*/pIJ1758 culture incubated at 20% oxygen was 54 units; that obtained from an equivalent culture incubated at 3% oxygen was 125 units. The wild-type strain had 40 units of activity when grown under either condition. These results indicated that the expression of the *melC-uidA* fusion is enhanced in microaerobic conditions.

The *melC-uidA* fusion, present in pIJ1758, was introduced into *R. l. bv. viciae* strain 8401pRL1JI. This strain is isogenic to *R. l. bv. phaseoli* except that it contains the Sym plasmid pRL1JI which specifies the ability to nodulate peas, rather than pRP2JI, the Sym plasmid of *bv. phaseoli*. The fusion was not introduced into the genome of *bv. phaseoli* itself because pPH1JI, the plasmid that was used to eliminate the Tn-marked recombinant plasmid pIJ1758, is itself detrimental to nodulation and N₂ fixation on *Phaseolus* beans, the host of this biovar (F.K.L.H., unpublished) but does not delay nodulation by *bv. viciae* on peas. Following marker exchange, it was found that the 8401pRL1JI *melC::Tn3 uidA* mutant was, as expected, Mel⁻ and slow-growing compared to the wild-type strain 8401pRL1JI on Y medium supplemented with either succinate as the C source or nitrate as the N source.

When inoculated onto peas, strain 8401pRL1JI *melC::Tn3-uidA*/pPH1JI formed nodules but they were delayed in their appearance by approximately one week. Eventually some of the nodules became pink (pink nodules indicate that N₂ fixation is taking place); three weeks after inoculation, each plant contained approximately 50% green nodules and 50% pink nodules. Acetylene reduction tests on individual nodules of each type confirmed that the pink and green nodules were,

respectively, Fix⁺ and Fix⁻. Green and pink nodules were individually crushed and bacteria from within the nodules were isolated. Bacteria isolated from pink nodules were sensitive to ampicillin (specified by Tn3-*uidA*) and were no longer slow-growing on Y medium supplemented either with succinate and sodium glutamate or with glucose and sodium nitrate. Thus it is apparent that these Fix⁺ nodules contain bacteria that have lost the transposon and have reverted for the mutant phenotypes. In contrast, bacteria isolated from green nodules retained the original phenotype, i.e. they were ampicillin resistant, and slow-growing on minimal medium containing succinate as the C source or nitrate as the N source. All the bacteria isolated from both the pink and green nodules were gentamicin resistant (specified by pPH1JI) and streptomycin resistant (specified by 8401pRL1JI).

To examine the expression of *R. l. bv. viciae melC* within the nodule, pink and green nodules were sectioned and stained for glucuronidase activity, using 5-bromo-4-chloro-3-indolyl β -D-glucuronidase as the histochemical substrate. Blue staining, indicative of glucuronidase activity, of structures which may have been infection threads was observed in some sections from green nodules, indicating *melC-uidA* transcription in these areas; no staining was observed in pink nodules (not shown).

Discussion

Random mutagenesis of plasmid pIJ1651 revealed a gene (*melC*) which is required for melanin production and for the ability of the bacteria to grow normally with succinate as C source and nitrate as N source. A mutation in *melC* resulted in Fix⁻ strains when inoculated onto peas. The *melC* mutations were located approximately 5 kb downstream of *nfrX* and restriction analysis of the *melC-uidA* mutation indicates that the transcription of *melC-uidA* is in the same orientation as *nfrX* (Hawkins, 1989). Since Tn5 insertions in *nfrX* do not abolish melanin production, nor do they affect growth or symbiotic N₂ fixation, it is unlikely that *nfrX* and *melC* are in the same transcriptional unit (F.K.L.H., unpublished).

The inability of the *R. l. bv. phaseoli melC* mutant strain to produce melanin could be overcome by over-expression of *melA* either by cloning *melA* itself or by over-expressing it through introduction of multi-copy *nifA* (Hawkins & Johnston, 1988). One possible explanation for the Mel⁻ phenotype of the *R. l. bv. phaseoli melC* mutant strain is that *melC* is regulatory; this was tested by investigating the transcription of a *melA-lacZ*

fusion in a *melC* mutant background. Transcription of *melA* was unaffected in a *melC* mutant compared to that in the wild-type under the conditions tested, indicating that the Mel⁻ phenotype was not due to lack of expression of *melA*. The 'link' between the four observed phenotypes caused by *melC* mutations is unclear. To our knowledge, there is no common step that is involved in (a) N₂ fixation ability, (b) melanin production, (c) growth on succinate, and (d) growth on nitrate. It is significant, however, that *nifA*-activated genes, succinate utilization and nitrate utilization in *R. meliloti* are all affected by mutations in *ntrA*, which encodes an essential sigma factor for N₂ fixation (Ronson *et al.*, 1987). There is no direct evidence that *melC* corresponds to *ntrA*, although there are certain similarities in the phenotypes of *ntrA* and *melC* mutant strains.

The Fix⁻ phenotype of strain 8401pRL1JI *melC*::Tn3 gave a high incidence of revertants, i.e. Fix⁺ nodules, indicating that the Tn3-*uidA* insertion in *melC* may be unstable. Staining of nodules for glucuronidase activity to detect expression of the *melC*-*uidA* fusion indicated that the *melC* gene is active in the infection thread. Staining may have taken place only in the infection thread and not in the bacteroids either because *melC* is only active in the former or because the *melC* mutation prevented the differentiation of the bacteria into bacteroids. An alternative staining strategy to reveal root nodule morphology and the presence or absence of bacteroids could be used to differentiate between these possibilities.

This work on *melC* indicates that it is a gene intrinsic to many aspects of symbiotic N₂ fixation. Further detailed investigation is required to pinpoint its exact function but the indications are that it promises to be an influential and multi-faceted gene.

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References

- BAGDASARIAN, M., LURZ, R., RUCKERT, B., FRANKLIN, F. C. H., BAGDASARIAN, M. M., FREY, J. & TIMMIS, K. N. (1981). Specific-purpose plasmid cloning vectors. II. Broad host range, high copy RSF1010-derived vectors, and a host-vector system for gene cloning in *Pseudomonas*. *Gene* **16**, 237-247.
- BERINGER, J. E. (1974). R. factor transfer in *Rhizobium leguminosarum*. *Journal of General Microbiology* **84**, 188-198.
- BEYNON, J. L., BERINGER, J. E. & JOHNSTON, A. W. B. (1980). Plasmids and host range in *Rhizobium leguminosarum* and *Rhizobium phaseoli*. *Journal of General Microbiology* **120**, 421-429.
- BORTHAKUR, D., LAMB, J. W. & JOHNSTON, A. W. B. (1987). Identification of two classes of *Rhizobium phaseoli* genes required for melanin synthesis, one of which is required for nitrogen fixation and activates the transcription of the other. *Molecular and General Genetics* **207**, 156-160.
- DOWNIE, J. A., HOMBRECHER, G., MA, Q.-S., KNIGHT, C. D., WELLS, B. & JOHNSTON, A. W. B. (1983). Cloned nodulation genes of *Rhizobium leguminosarum* determine host range specificity. *Molecular and General Genetics* **190**, 359-365.
- FIGURSKI, D. H. & HELINSKI, D. R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proceedings of the National Academy of Science of the United States of America* **76**, 1648-1652.
- FRIEDMAN, A. M., LONG, S. R., BROWN, S. E., BUIKEMA, W. J. & AUSUBEL, F. M. (1982). Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. *Gene* **18**, 289-296.
- HAWKINS, F. K. L. (1989). *Studies on the nifA gene of Rhizobium leguminosarum*. PhD thesis, University of East Anglia.
- HAWKINS, F. K. L. & JOHNSTON, A. W. B. (1988). Transcription of a *Rhizobium leguminosarum* biovar *phaseoli* gene needed for melanin synthesis is activated by *nifA* of *Rhizobium* and *Klebsiella pneumoniae*. *Molecular Microbiology* **2**, 331-337.
- JEFFERSEN, R. A., BURGESS, S. M. & HIRSH, D. (1986). β -Glucuronidase from *Escherichia coli* as a gene-fusion marker. *Proceedings of the National Academy of Sciences of the United States of America* **83**, 8447-8451.
- JEFFERSEN, R. A., KAVANAGH, T. A. & BEVAN, M. W. (1987). GUS fusions: β -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO Journal* **6**, 3901-3907.
- JOHNSTON, A. W. B., BEYNON, J. L., BUCHANAN-WOLLASTON, A. V., SETCHELL, S. M., HIRSCH, P. R. & BERINGER, J. E. (1978). High frequency transfer of nodulation ability between strains and species of *Rhizobium*. *Nature, London* **276**, 634-636.
- JOHNSTON, A. W. B., HOMBRECHER, G., BREWIN, N. J. & COOPER, M. C. (1982). Two transmissible plasmids in *Rhizobium leguminosarum* strain 300. *Journal of General Microbiology* **128**, 85-93.
- LAMB, J. W., DOWNIE, J. A. & JOHNSTON, A. W. B. (1985). Cloning of the nodulation (*nod*) genes of *Rhizobium phaseoli* and their homology to *R. leguminosarum* *nod* genes. *Gene* **34**, 235-241.
- LAMB, J. W., HOMBRECHER, G. & JOHNSTON, A. W. B. (1982). Plasmid determined nodulation and nitrogen fixation abilities in *Rhizobium phaseoli*. *Molecular and General Genetics* **186**, 449-452.
- LONG, S. R. (1989). *Rhizobium*-legume nodulation: life together in the underground. *Cell* **56**, 203-214.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning, a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MERRICK, M., FILSER, M., KENNEDY, C. & DIXON, R. (1978). Polarity of mutations induced by insertion of transposons Tn5, Tn7 and Tn10 into the *nif* gene cluster of *Klebsiella pneumoniae*. *Molecular and General Genetics* **165**, 103-111.
- MILLER, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- NEWTON, J. W., WILSEN, P. W. & BURRIS, R. H. (1953). Direct demonstration of ammonia as an intermediate in nitrogen fixation by *Azotobacter*. *Journal of Biological Chemistry* **204**, 445-451.
- NOVEL, G. & NOVEL, M. (1973). Mutants d'*Escherichia coli* K12 affectés pour leur croissance sur méthyl β -D-glucuronide: localisation du gène de structure de la β -D-glucuronidase (*uidA*). *Molecular and General Genetics* **120**, 319-335.
- RONSON, C. W., NIXON, B. T., ALBRIGHT, L. M. & AUSUBEL, F. M. (1987). *Rhizobium meliloti* *ntrA* mutants fail to utilize succinate as a carbon source. *Journal of Bacteriology* **169**, 2424-2431.
- ROSSEN, L., SHEARMAN, C. A., JOHNSTON, A. W. B. & DOWNIE, J. A. (1985). The *nodD* gene of *Rhizobium leguminosarum* is autoregulatory and in the presence of plant exudate induces the *nodA*, *B* and *C* genes. *EMBO Journal* **4**, 3369-3373.
- RUVKUN, G. B. & AUSUBEL, F. M. (1981). A general method for site-directed mutagenesis in prokaryotes. *Nature, London* **289**, 85-88.
- SANTERO, E., TOUKDARIAN, A., HUMPHREY, R. & KENNEDY, C. (1988). Identification and characterization of two nitrogen fixation regulatory regions, *nifA* and *nfrX*, in *Azotobacter vinelandii* and *Azotobacter chroococcum*. *Molecular Microbiology* **2**, 303-314.

- STACHEL, S. E., GYNHEUNG, A., FLORES, C. & NESTER, E. W. (1985). A Tn3 *lacZ* transposon for the random generation of β -galactosidase gene fusions: application to the analysis of gene expression in *Agrobacterium*. *EMBO Journal* **4**, 891–898.
- TOUKDARIAN, A. & KENNEDY, C. (1986). Regulation of nitrogen metabolism in *Azotobacter vinelandii*: isolation of *ntr* and *glnA* genes and construction of *ntr* mutants. *EMBO Journal* **6**, 399–407.
- WOOD, W. B. (1966). Host specificity of DNA produced by *E. coli* bacterial mutation affecting the restriction and modification of DNA. *Journal of Molecular Biology* **16**, 118–133.