Plasmids and Host-range in *Rhizobium leguminosarum* and *Rhizobium phaseoli*

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Rhizobium phaseoli strain 1233 forms nitrogen-fixing nodules on *Phaseolus vulgaris* and produces a brown pigment (probably melanin) in plate culture. This strain contains two plasmids of molecular weight about 200×10^6 . Spontaneous deletions in the smaller plasmid abolished pigment production and the ability to nodulate *Phaseolus* beans, suggesting that genes involved in the determination of both these phenotypes are on this plasmid.

The conjugative *Rhizobium leguminosarum* plasmid pJB5JI, which carries genes that determine the ability to nodulate peas, was transferred from *R. leguminosarum* strain T3 into *R. phaseoli* strain 1233. The majority (about 97 %) of transconjugants produced pigment and nodulated both peas and *Phaseolus* beans poorly. The others (about 3 %) had received not only pJB5JI but also an extra plasmid present in strain T3 and they had lost the smaller of the two plasmids of *R. phaseoli* strain 1233, suggesting incompatibility between the plasmid transferred from T3 and that lost from the recipient. These strains did not produce pigment nor did they form nitrogen-fixing nodules on *Phaseolus* beans, but they nodulated peas as well as did *R. leguminosarum* strain T3.

Most (about 96 %) of the bacteria isolated from nodules following inoculation of peas by the pigmented transconjugants failed to produce pigment. These strains contained the larger plasmid from strain 1233, and pJB5JI, but not the smaller plasmid of strain 1233; they behaved like *R. leguminosarum* in inducing profuse nitrogen-fixing nodules on peas. The few pigment-producing bacteria isolated from pea nodules had suffered a deletion in the smaller plasmid of strain 1233; they nodulated peas poorly but could not form nitrogen-fixing nodules on *Phaseolus* beans.

INTRODUCTION

Members of the genus *Rhizobium* form nodules on and fix atmospheric nitrogen in the roots of legumes. A particular species of *Rhizobium* can usually form an effective (i.e. nitrogen-fixing) symbiosis with only a limited range of plants; e.g. *R. leguminosarum* with *Pisum*, *Vicia*, *Lathyrus* and *Lens*, and *R. phaseoli* with *Phaseolus* beans.

There have been several reports of plasmids in *Rhizobium* species (Sutton, 1974; Tshitenge *et al.*, 1975; Zurkowski & Lorkiewicz, 1976; Nuti *et al.*, 1977; Casse *et al.*, 1979). Nuti *et al.* (1979) and Ruvkun & Ausubel (1980) showed that DNA encoding part of the structural information for *Klebsiella pneumoniae* nitrogenase, the nitrogen-fixing enzyme complex, hybridized to restriction fragments of plasmid DNA from *R. leguminosarum*. There is also evidence that genes required for nodulation and nitrogen fixation may be plasmid-borne in *Rhizobium* (Higashi, 1967; Johnston *et al.*, 1978; Zurkowski & Lorkiewicz, 1978; Brewin *et al.*, 1980a; Buchanan-Wollaston *et al.*, 1980).

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Table 1. *Bacterial strains and plasmids*

<i>Rhizobium</i> strain	Origin and genetic markers	Reference
<i>R. leguminosarum</i>		
300	Wild isolate	Johnston & Beringer (1975)
1062	300 <i>ura-14 trp-16 str-86</i>	Johnston <i>et al.</i> (1978)
T3	1062 pJB5JI†	Johnston <i>et al.</i> (1978)
<i>R. phaseoli</i>		
1233	<i>rif-396</i>	Johnston & Beringer (1976)

† pJB5JI is a Tn5-marked derivative of pRL1JI, a self-transmissible, bacteriocinogenic plasmid that carries genes for pea nodulation.

In a previous study (Johnston *et al.*, 1978) the conjugative *R. leguminosarum* plasmid pJB5JI was shown to confer the ability to nodulate peas on several species of *Rhizobium*, including *R. phaseoli*; plasmid transfer occurred at high frequency (about 10^{-2}). Plasmid pJB5JI is a derivative of the conjugative, bacteriocinogenic plasmid pRL1JI (Hirsch, 1979), of molecular weight about 130×10^6 (Hirsch *et al.*, 1980), into which the transposon Tn5 (specifying kanamycin resistance) has been introduced (Beringer *et al.*, 1978). When pJB5JI was introduced into a strain (1233) of *R. phaseoli*, two classes of transconjugants were recognized (Johnston *et al.*, 1978). The majority class nodulated both peas and *Phaseolus* beans, although the onset of nodulation of both hosts was delayed and the number of nodules was reduced compared with nodulation by the parental strains of their respective hosts. The single transconjugant in the minority class nodulated peas as well as did the parental strain of *R. leguminosarum* but failed to nodulate *Phaseolus* beans.

In this paper we describe the nature of these two classes of transconjugants and a correlation of their phenotypes with their plasmid content as determined by agarose gel electrophoresis.

METHODS

Bacterial strains and plasmids. These are described in Table 1.

Bacterial manipulations. Bacteria were grown on complete (TY) or minimal (Y) medium (Beringer, 1974) supplemented as required with rifampicin ($20 \mu\text{g ml}^{-1}$), streptomycin ($200 \mu\text{g ml}^{-1}$) and kanamycin ($20 \mu\text{g ml}^{-1}$). Membrane crosses were made as described by Buchanan-Wollaston *et al.* (1980). Nodulation tests were carried out on *Pisum sativum* cv. Wisconsin Perfection and *Phaseolus vulgaris* cv. Fino. Plants were grown in axenic culture conditions (Beringer, 1974) on 1% (w/v) agar containing Fahraeus medium (Vincent, 1970) in a greenhouse at 20°C ; seeds were surface-sterilized by exposure to 12% (w/v) sodium hypochlorite for 30 min. Nitrogen fixation was measured by the reduction of acetylene to ethylene (Johnston & Beringer, 1975). The plant growth conditions were not the same as those used by Johnston *et al.* (1978). The nutrient agar medium allowed better nodulation of *Phaseolus vulgaris* by *R. phaseoli* strain 1233; however, under these new conditions *R. leguminosarum* strain T3 induced small, non-acetylene reducing (Fix^-) nodules on this variety of *Phaseolus* beans. Therefore, in this work, we only scored the ability of the transconjugants to form Fix^+ nodules on *Phaseolus* beans. *Rhizobium phaseoli* strain 1233 does not form nodules on *Pisum sativum* cv. Wisconsin Perfection even under these improved conditions.

Physical analysis of plasmids. Plasmid DNA was isolated by the technique of Hirsch *et al.* (1980). Tris/borate agarose gels (0.7%) were poured in moulds (McDonnell *et al.*, 1977) and run for approximately 16 h at 100 V and 20 mA at 4°C . Plasmid bands were visualized by staining gels with ethidium bromide ($5 \mu\text{g ml}^{-1}$) for 20 min and photographed on a shortwave u.v. transilluminator (model G61, U.V. Products Ltd) using Polaroid film.

RESULTS

In the course of this work several classes of derivatives of *R. phaseoli* strain 1233 were obtained and characterized. The derivation, phenotype and plasmid profiles of these strains are represented diagrammatically in Fig. 3.

Pigment production by R. phaseoli

Nearly all strains of *R. phaseoli* produced a dark pigment when grown on TY medium at 28 °C for 5 d and then left at room temperature for at least a further 14 d. Pigment production was most reliably seen when the strain was grown as a dense patch, although production could occur in large single colonies. No pigment was produced by any *R. leguminosarum* or *R. trifolii* strains (at least 20 of both species). Production of the pigment was not observed on minimal medium unless tyrosine (30 µg ml⁻¹) was added, indicating that it is likely to be melanin.

Since there was a correlation between the ability to produce this pigment and to nodulate *Phaseolus* beans, we examined the nine *R. phaseoli* strain 1233(pJB5JI) transconjugants described by Johnston *et al.* (1978) for pigment production. The eight transconjugants which nodulated both peas and beans were pigment-producers (Pig⁺) whereas the single transconjugant that nodulated only peas was a non-producer (Pig⁻).

We subsequently examined 341 *R. phaseoli* transconjugants inheriting pJB5JI from a cross between *R. leguminosarum* strain T3, containing pJB5JI, and *R. phaseoli* strain 1233; 11 of them (3 %) were Pig⁻. These Pig⁻ transconjugants (termed primary Pig⁻) failed to produce nitrogen-fixing nodules on *Phaseolus* beans but nodulated peas as rapidly and as effectively as did *R. leguminosarum* strain T3. The Pig⁺ transconjugants (termed primary Pig⁺), of which 17 were studied, formed acetylene-reducing nodules on peas and *Phaseolus* beans but the nodulation was poor, as had been found previously (Johnston *et al.*, 1978). There appeared, therefore, to be a correlation between loss of pigment production and loss of the ability to form nitrogen-fixing nodules on *Phaseolus* beans.

We also screened for spontaneous loss of pigment production in strain 1233; of 1421 single colonies examined, 7 (0.5 %) were non-pigmented (termed spontaneous Pig⁻). These may have been clonal since they arose from the same culture. The frequency of spontaneous Pig⁻ strains was much lower than that of primary Pig⁻ transconjugants, suggesting that the transfer of pJB5JI into strain 1233 enhanced the loss of the Pig⁺ phenotype. The spontaneous Pig⁻ strains failed to produce nodules on either *Phaseolus* beans or peas, confirming the association between the Pig⁺ and *Phaseolus* nodulation phenotypes.

Plasmids of the transconjugants

Plasmid DNA was isolated from strains T3 and 1233 and from both classes of transconjugants and was visualized following agarose gel electrophoresis (Fig. 1). Gels were run to give maximum resolution of plasmid bands and so chromosomal DNA ran off the end of the gel. Strain 1062 (track 2, Fig. 1) is a derivative of *R. leguminosarum* strain 300; there are two large plasmids (mol. wt about 200 × 10⁶) and two smaller plasmids (mol. wt about 90 × 10⁶) (Casse *et al.*, 1979; Hirsch *et al.*, 1980). Nuti *et al.* (1977) found three plasmid bands (two of mol. wt about 200 × 10⁶ and one of about 90 × 10⁶) in *R. leguminosarum* strain 897, also a derivative of strain 300, but Hirsch *et al.* (1980) showed that strain 1062 had an extra band, smaller than the smallest band of strain 300, and suggested that the smallest band in strain 300 might represent two co-migrating plasmids and that the extra strain 1062 band might have become visible as a result of a small deletion in one of the two co-migrating plasmids. The plasmids of strain 1062 were not named because of this problem. Track 6 (Fig. 1) shows strain T3, i.e. strain 1062 carrying pJB5JI. The extra band in strain T3, migrating between the two largest and two smallest strain 1062 plasmids, corresponded to that reported for pRL1JI (mol. wt about 130 × 10⁶) by Hirsch *et al.* (1980). In addition, one or two bands have been seen, in derivatives of *R. leguminosarum* strain 300, which migrate more slowly than the fourth-largest plasmid in strain 1062 (Hirsch *et al.*, 1980). The visualization of such bands on agarose gels is sporadic; one such faint band can be seen in track 6 (Fig. 1). Track 1 (Fig. 1) shows that *R. phaseoli* strain

Fig. 1. Plasmids of the *R. leguminosarum* and *R. phaseoli* parents and the *R. phaseoli* pJB5JI transconjugants, after agarose gel electrophoresis. Track 1, *R. phaseoli* strain 1233; 2, *R. leguminosarum* strain 1062; 3 and 4, *R. phaseoli* pJB5JI primary Pig^- transconjugants; 5, *R. phaseoli* pJB5JI primary Pig^+ transconjugant; 6, *R. leguminosarum* strain T3.

1233 has two large plasmids that differ slightly in relative mobility from the two largest bands of strain 1062 and, therefore, had molecular weights in the region of 200×10^6 . The smaller of the plasmids of strain 1233 was termed pRP1JI and the larger pRP2JI. These two plasmids were previously termed pRP1233-1JI and pRP1233-2JI, respectively (Beynon *et al.*, 1980).

Track 5 (Fig. 1) shows the plasmids of one primary Pig^+ transconjugant and tracks 3 and 4 (Fig. 1) those of two primary Pig^- transconjugants. All of the transconjugants had received the band corresponding to pJB5JI. The primary Pig^+ strain retained both of the *R. phaseoli* plasmids, apparently unchanged. The primary Pig^- strains, however, had lost pRP1JI and had gained one (track 4, Fig. 1) or two (track 3, Fig. 1) smaller plasmids in addition to pJB5JI. These extra bands in the primary Pig^- transconjugants appeared to co-migrate with the two smallest plasmids in strains 1062 and T3. Therefore, it would appear that the transfer of pJB5JI had been accompanied by the transfer of one or both of the smallest plasmids of strain T3 to *R. phaseoli* strain 1233.

We have examined the plasmids of 16 other primary Pig^+ and 12 other primary Pig^- transconjugants. All 14 of the primary Pig^- transconjugants contained pJB5JI plus the smallest plasmid from strain T3 and of these one had also received the second-smallest plasmid from strain T3 (track 3, Fig. 1). Among the 17 primary Pig^+ transconjugants examined, none contained the smallest plasmid from strain T3 but one had received the second-smallest plasmid. Therefore, it is the co-transfer of the smallest plasmid of strain T3 which seems to be associated with the loss of pRP1JI and of the Pig^+ and *Phaseolus* nodulation phenotypes.

Fig. 2. Plasmids of the *R. phaseoli* parent and isolates from pea nodules resulting from inoculation with an *R. phaseoli* pJB5JI primary Pig^+ transconjugant, after agarose gel electrophoresis. Track 1, *R. phaseoli* pJB5JI secondary Pig^+ transconjugant; 2, *R. phaseoli* pJB5JI secondary Pig^- transconjugant; 3, *R. phaseoli* strain 1233.

Further evidence that pRP1JI is important in determining nodulation and pigment-forming ability came from the finding that all seven of the spontaneous Pig^- derivatives of strain 1233 retained pRP2JI, apparently unchanged, but pRP1JI was replaced by a plasmid of smaller size (mol. wt about 160×10^6), indicating that it had suffered a deletion. As mentioned above, it is possible that all of these non-pigmented strains are clonally related.

Phenotype of transconjugants following passage through pea nodules

Nine of the primary Pig^+ transconjugants were used to inoculate peas and a single colony isolate was taken from each of two nodules for each transconjugant. All 18 isolates were Pig^- (termed secondary Pig^-). However, in a further test where bacteria were isolated from four nodules resulting from inoculation with a single primary Pig^+ transconjugant two of the eight isolates were found to be Pig^+ (termed secondary Pig^+). Since these secondary Pig^+ strains arose by inoculation from the same culture, they may have been clonally related.

The plasmids of one secondary Pig^- and one secondary Pig^+ transconjugants are shown in Fig. 2. Track 3 (Fig. 2) shows the two bands characteristic of strain 1233. Track 2 (Fig. 2) shows the bands from a secondary Pig^- strain. All nine secondary Pig^- strains tested had only the two plasmids seen in track 2 (Fig. 2), one plasmid migrating in the position of pRP2JI, the other in the position of pJB5JI. This indicates that pRP1JI had been lost following passage through a pea nodule. The only secondary Pig^+ strain examined (track 1, Fig. 2) had both the bands of the secondary Pig^- strains and, in addition, had a band

Fig. 3. Phenotypes and representation of plasmid profiles of strains described in the text. Phenotypes: Ph^+ , $\text{Ph}^\pm$ and Ph^- denote normal, delayed or no nodulation of *Phaseolus*; Leg^+ , $\text{Leg}^\pm$ and Leg^- denote normal, delayed or no nodulation of *Pisum*; Pig^+ and Pig^- denote pigmented and non-pigmented. Plasmids: a, b, c and d denote the plasmid bands normally observed in strain 1062 (see track 2, Fig. 1); pRP1^* and pRP1^{**} denote independent deletions of pRP1 . (Note that, for clarity, the final JI of plasmid designations has been omitted.)

migrating slightly faster than pRP1JI , indicating that this plasmid had suffered a deletion. Presumably the deletions which gave rise to the spontaneous Pig^- and the secondary Pig^+ strains were different since the latter strains retained the ability to produce the pigment.

The nine secondary Pig^- transconjugants and the single secondary Pig^+ strain were re-inoculated on to peas and *Phaseolus* beans. The secondary Pig^- transconjugants had the same symbiotic phenotype on both hosts as did the primary Pig^- transconjugants. The secondary Pig^+ transconjugants infrequently formed Fix^+ nodules on peas but did not form Fix^+ nodules on *Phaseolus* beans.

Two primary Pig^- transconjugants were used to inoculate peas and bacteria were isolated from six nodules for each transconjugant. All isolates were found to be Pig^- . One of them showed the same symbiotic phenotype on peas and beans as the primary Pig^- strains.

Phenotype of transconjugants following passage through Phaseolus nodules

Seven of the nine primary Pig^+ transconjugants inoculated on to peas were also used to inoculate *Phaseolus* beans. A single colony was purified from each of two *Phaseolus* nodules for each inoculant; all were kanamycin-resistant (indicating the presence of Tn5), suggesting that at least part of pJB5JI was still present, and were Pig^+ . Since these ex-nodule isolates appeared to be phenotypically the same as the inoculum they were not investigated further.

DISCUSSION

Johnston *et al.* (1978) suggested that the presence in the same *Rhizobium* strain of genetic information needed to nodulate host plants of different cross-inoculation groups might cause it to be impaired in its nodulation on either host. It was suggested that the single *R. phaseoli* pJB5JI transconjugant isolated in that study which only nodulated peas might have lost information required to nodulate beans. Results from the present study are consistent with those interpretations.

The resident *R. phaseoli* plasmid pRP1JI is clearly important as a determinant both of effective nodulation on *Phaseolus* and of pigment production since spontaneous derivatives of strain 1233, selected on the basis of their being non-pigmented, had lost the ability to nodulate *Phaseolus* beans effectively and had suffered a deletion in pRP1JI. Extrachromosomal inheritance of melanin production has been indicated in several species of *Streptomyces* (Gregory & Huang, 1964; Baumann & Kocher, 1976; Schrepf & Goebel, 1979).

The majority of the transconjugants resulting from the transfer of pJB5JI from strain T3 into strain 1233 (the primary Pig⁺ transconjugants) had the following characteristics (see Fig. 3): (i) their plasmid content differed from that of the strain 1233 parent only by the acquisition of an extra band, corresponding to pJB5JI (though one had also acquired the second-largest plasmid of strain 1062); (ii) they retained the ability to make the pigment produced by strain 1233; (iii) they induced acetylene-reducing nodules on peas and *Phaseolus* beans but on both hosts nodulation was delayed and reduced; (iv) when they were inoculated on to peas 96 % of the bacteria isolated from the nodules were unpigmented (secondary Pig⁻), had lost pRP1JI and nodulated peas as well and *Phaseolus* as poorly as did the *R. leguminosarum* parental strain, but the remaining 4 % of the bacteria retained the ability to produce the pigment and the one such secondary Pig⁺ strain examined appeared to have suffered a deletion in pRP1JI; it nodulated peas poorly and *Phaseolus* beans ineffectively.

These observations could be explained if the joint presence of information on pJB5JI and on pRP1JI interacted to depress the ability to nodulate either host; in order to nodulate peas the primary Pig⁺ transconjugants would need to have lost the information, apparently encoded by pRP1JI, which depresses pea nodulation. Therefore, the delay and reduction in pea nodulation induced by a primary Pig⁺ strain could be explained by the time needed for the few individuals which had spontaneously lost part or all of pRP1JI to grow to sufficient numbers in the rhizosphere to cause nodulation.

The fact that the primary Pig⁺ transconjugants were also poor at nodulating *Phaseolus* compared with the parental strain 1233 indicates that the presence of pJB5JI interferes with the nodulation of this host. One explanation for the apparent stability of the primary Pig⁺ transconjugants following passage through *Phaseolus* nodules is that the selection pressure for derivatives that have lost pJB5JI is not as great as on peas because strains of *R. leguminosarum* are themselves able to nodulate beans although they do not fix nitrogen.

Arising from the initial cross between *R. leguminosarum* strain T3 (the pJB5JI donor) and *R. phaseoli* strain 1233 were a few transconjugants that failed to produce the pigment (primary Pig⁻ strains). These strains, and also the secondary Pig⁻ strains, had lost pRP1JI, confirming that genes involved in pigment production are borne on this plasmid. The primary Pig⁻ strains all appeared to have received the smallest plasmid of strain T3 in addition to pJB5JI, suggesting that it is co-transferred with pJB5JI and is incompatible with the smaller strain 1233 plasmid. The higher frequency of the primary Pig⁻ transconjugants compared with spontaneous Pig⁻ strains would be explained by the frequency of co-transfer of the smallest plasmid of strain T3 with pJB5JI being higher than that of spontaneous deletions in pRP1JI. The second smallest plasmid of strain T3 can also be co-transferred with pJB5JI but its presence does not seem to be correlated with loss of

pRP1JI or of pigment production. The fact that the secondary Pig⁻ strains nodulated peas as well as did the *R. leguminosarum* parent suggests that it is loss of the smaller plasmid of strain 1233, and not the gain of the smallest plasmid of strain T3, which allows pJB5JI transconjugants to nodulate peas normally.

We do not know the basis of the apparent mutual inhibition of nodulation on both host plants which occurs in transconjugants of strain 1233 inheriting pJB5JI. It could be that gene expression was mutually repressed. Not all plasmid-encoded functions of the recipient were repressed by the presence of pJB5JI since pigment production still occurred in the primary Pig⁺ transconjugants. Another explanation for the delayed and reduced nodulation of the primary Pig⁺ strains may be an interference in the process of nodulation on either host by simultaneous expression of both sets of 'host-range' genes.

Rhizobium leguminosarum and *R. phaseoli* are very closely related and perhaps should be considered as a single species, together with *R. trifolii* (Graham, 1964). To our knowledge the only phenotype that distinguishes these three species, apart from their host-range, is pigment production by *R. phaseoli*. We do not know whether pigment production is required for effective nodulation of *Phaseolus* beans but clearly the genetic information for these two phenotypes is closely linked. Pigment production is not required for nodulation of *Phaseolus* since *R. leguminosarum* strains 1062 and T3 can both form Fix⁻ nodules on *Phaseolus*.

Both the primary and secondary Pig⁻ transconjugants are, by definition, strains of *R. leguminosarum*, although they carry an *R. phaseoli* chromosome and at least one *R. phaseoli* plasmid. In R68.45-mediated crosses between strains of *R. leguminosarum*, *R. trifolii* and *R. phaseoli*, haploid recombinants arose at frequencies similar to those found in crosses between derivatives of a single *R. leguminosarum* strain (Johnston & Beringer, 1977), confirming the close relationship between these three species. The fact that the chromosomes of these species are very similar and that, as described in this work, a 'species' can be constructed by the transfer of a plasmid, followed by plasmid reassortment, emphasizes that the taxonomic relationship of these species should be reconsidered.

This work and studies by other authors (Johnston *et al.*, 1978; Brewin *et al.*, 1980*a, b*) have suggested that host-range genes in certain strains of *Rhizobium* are borne on specific plasmids. The basis of host-range determination will be more amenable to study when the plasmid segments involved have been identified by fine-scale mapping of these plasmids.

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