Transfer of Symbiotic Genes with Bacteriocinogenic Plasmids in *Rhizobium leguminosarum*

By N. J. BREWIN,* J. E. BERINGER,
A. V. BUCHANAN-WOLLASTON, A. W. B. JOHNSTON AND
P. R. HIRSCH†

John Innes Institute, Colney Lane, Norwich NR4 7UH

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Transfer of derivatives of the bacteriocinogenic plasmid pRL1JI into eight symbiotically defective strains of *R. leguminosarum* resulted in suppression of the mutant phenotype in four cases. These included non-infective, ineffective and temperature-sensitive ineffective phenotypes. However, in none of these strains was the defect suppressible after high frequency transfer of derivatives of two other bacteriocinogenic plasmids, pRL3JI or pRL4JI. Nodulation ability was co-transferred at high frequency (> 95%) with bacteriocin production by the plasmid pRL1JI. The other two plasmids, pRL3JI and pRL4JI, also mediated the transfer of nodulation ability but at much lower frequency (10^{-3} to 10^{-4} per plasmid transfer). Sometimes, transconjugants that had acquired nodulation ability after the transfer of derivatives of plasmids pRL3JI or pRL4JI acted subsequently as high frequency donors for nodulation ability in a manner that was apparently similar to strains containing pRL1JI.

INTRODUCTION

Many *Rhizobium* strains harbour plasmids (Sutton, 1974; Tshitinge *et al.*, 1975; Zurkowski & Lorkiewicz, 1976; Nuti *et al.*, 1977) and genetic evidence indicates that such plasmids may carry some of the genes required for the development of the symbiosis that culminates in the fixation of atmospheric nitrogen within legume root nodules (Higashi, 1967; Nuti *et al.*, 1977; Johnston *et al.*, 1978, 1979; Casse *et al.*, 1979).

Evidence for naturally occurring transmissible plasmids in *Rhizobium leguminosarum* came from a recent survey of bacteriocin production among 97 field isolates in which bacteriocins were classified as of *small* or *medium* molecular size on the basis of their diffusion rates in agar (Hirsch, 1979). Three of the isolates (strains 248, 306 and 309) carried determinants of *medium* bacteriocins which were transmissible to non-producing strains of *R. leguminosarum* at frequencies of 10^{-1} to 10^{-2} per recipient, suggesting the presence of bacteriocinogenic plasmids. The plasmids originating from strains 248, 306 and 309 were designated pRL1JI, pRL3JI and pRL4JI, respectively. These three plasmids were similar in their transfer properties, in their ability to mobilize chromosomal markers, and in the fact that, when transferred into strains that previously produced *small* bacteriocins, each of these plasmids suppressed (or eliminated) the production of the *small* bacteriocins. Moreover, the bacteriocins specified by the three plasmids were apparently related, because the presence of any one of these plasmids in a strain conferred resistance to bacteriocin(s) produced by either of the other two.

Using a technique for transposon mutagenesis (Beringer *et al.*, 1978*b*), a derivative of pRL1JI was obtained which contains the transposon Tn5 conferring resistance to kanamycin (Berg *et al.*, 1975). When this plasmid (pJB5JI) was transferred to a non-nodulating (non-

† Present address: Max-Planck-Institut für Züchtungsforschung, 5 Köln-Vogelsang 30, W. Germany.
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Table 1. *Bacterial strains and plasmids*

<i>Rhizobium</i> strain	Bacteriocin produced	Symbiotic phenotype	Other markers	Reference
<i>R. leguminosarum</i>				
248	medium	Eff ⁺	Wild isolate	Josey <i>et al.</i> (1979)
306	medium	Eff ⁺	Wild isolate	Josey <i>et al.</i> (1979)
309	medium	Eff ⁺	Wild isolate	Josey <i>et al.</i> (1979)
300	small	Eff ⁺	Wild isolate	Johnston & Beringer (1975)
336	small	Eff ⁺	Wild isolate	Hirsch (1979)
1027	small	Inf ⁻	300 <i>phe-1 ade-27</i> <i>rif-45</i>	Pain (1979), Hirsch (1979)
1062	small	Eff ⁺	300 <i>ura-14 trp-16</i> <i>str-86</i>	Johnston <i>et al.</i> (1978)
897	small	Eff ⁺	300 <i>phe-1 trp-12</i> <i>str-37</i>	Johnston & Beringer (1976)
6015	small	Inf ⁻	300 <i>phe-1 trp-12</i> <i>rif-392 str-37</i> <i>inf-6007</i>	Johnston <i>et al.</i> (1978)
16015	small	Inf ⁻	300 <i>str-37 spc-54</i> <i>inf-6007</i>	This laboratory
3634	small	Inf ⁻	300 <i>phe-1 str-276</i> <i>spc-57 inf-3628</i>	This laboratory*
7577	small	Eff ⁻	300 <i>phe-1 eff-7577</i>	This laboratory*
7062	small	Eff ⁻ at 25 °C	300 <i>phe-1 eff-7062</i>	This laboratory*
7021	small	Eff ⁻	300 <i>phe-1 eff-7021</i>	This laboratory*
7186	small	Eff ⁻	300 <i>phe-1 eff-7186</i>	This laboratory*
7155	small	Eff ⁻ at 25 °C	300 <i>phe-1 eff-7155</i>	Beringer <i>et al.</i> (1977)
<i>R. trifolii</i>				
6662	small	Eff ⁺	<i>str-170</i>	Rothamsted†

Plasmid	Derivation	Properties determined			Reference
		Medium bacteriocin production	Suppression/ elimination of small bacteriocin production	Kanamycin resistance	
pRL1JI	Strain 248	+	+	—	Johnston <i>et al.</i> (1978)
pRL3JI	Strain 306	+	+	—	Hirsch (1979)
pRL4JI	Strain 309	+	+	—	Hirsch (1979)
pJB5JI	pRL1JI	—	+	+	Johnston <i>et al.</i> (1978)
pVW1JI	pRL1JI	—	+	+	This paper
pVW3JI	pRL3JI	—	+	+	This paper
pVW5JI	pRL4JI	—	+	+	This paper
R68.45		—	—	+	Haas & Holloway (1976)

* These strains were all isolated from the same mutagenized stock described by Beringer *et al.* (1977).

† Strain 6662 is a *str* mutant of strain 12 from the Rothamsted culture collection, Harpenden, Herts.

infective) strain of *R. leguminosarum*, normal nodulating ability was restored. Furthermore, strains of *R. trifolii* and *R. phaseoli* that received pJB5JI acquired the ability to nodulate peas (Johnston *et al.*, 1978).

In the present study, the transfer of symbiotic genes by pRL1JI is examined further and the properties of this plasmid are compared with those of two other bacteriocinogenic plasmids, pRL3JI and pRL4JI.

METHODS

Bacterial strains, plasmids and bacteriophage. Strains and plasmids are listed in Table 1. The bacteriophage used was RL38 (Buchanan-Wollaston, 1979).

Bacterial manipulations. Culture conditions and plant nodulation tests were described by Beringer (1974).

Membrane crosses were described by Beringer *et al.* (1978a). Transduction by the bacteriophage RL38 was described by Buchanan-Wollaston (1979). The production of *small* and *medium* bacteriocins was tested as described by Hirsch (1979), using *R. leguminosarum* strain 248 as the indicator for *small* bacteriocins and strain 336 as the indicator for *medium* bacteriocins.

Direct selection for the transfer of nodulating ability. The donors in these crosses were derivatives of strain 1027 which is non-infective on peas because of its requirement for adenine (Pain, 1979); the recipient was the non-infective strain 16015. Membrane crosses were incubated overnight on complete medium (TY) plates and the bacteria were resuspended in 3 ml water to give a viable count of about 2×10^8 ml⁻¹ for each parent. Samples of a 10^{-1} dilution from the crosses were spread on TY plates supplemented with streptomycin (200 µg ml⁻¹) to counterselect the donors. [In some cases, kanamycin (20 µg ml⁻¹) was also included to eliminate recipients that had not received *kan*.] After growth at 28 °C for 3 d, the confluent lawns were resuspended in 3 ml distilled water to give a final concentration of about 7×10^9 viable bacteria ml⁻¹. The suspensions were serially diluted and peas were inoculated in duplicate with 0.1 ml samples from each dilution.

RESULTS

Transfer of symbiotic gene functions with pRL1JI and pJB5JI

Eight symbiotically defective mutants of *R. leguminosarum* were used as recipients for pJB5JI in order to determine how many symbiotic gene functions might be transferred with this plasmid. These mutants included examples of non-infective (Inf⁻), ineffective (Eff⁻) and temperature-sensitive ineffective phenotypes. For four of the eight mutants, the introduction of pJB5JI resulted in suppression of the mutant phenotype in the transconjugant clones (Table 2), indicating that a range of symbiotic gene functions, and not just those for infectiveness, may be associated with this plasmid.

No attempts have yet been made to map any of the symbiotic mutations that were suppressible by pJB5JI. However, it may be significant that for one of the non-infective mutants, 6015, the largest of the three plasmid bands detected by gel electrophoresis of lysates of this mutant was considerably smaller in size than for its parent strain, 897 (P. R. Hirsch, unpublished observations), implying that the symbiotic defect may have been due to a large deletion from this plasmid. A prototrophic derivative of the same mutant strain that gave rise to strain 6015 (strain 16015) was used to inoculate more than 100 pea plants but never induced nodulation. Since there is very strong selection for nodulating revertants (see Table 5), this result implies that the reversion rate to *inf*⁺ was very low or zero, a result compatible with the presence of a deletion mutation.

In addition to these nodulation tests on peas, strain 16015 was tested on the sweet pea (*Lathyrus odoratus*), another member of the cross-inoculation group normally nodulated by *R. leguminosarum*. Strain 16015 was unable to nodulate sweet peas whereas strain 300 formed pink effective nodules, showing that the same gene (or genetic region) was required for nodulation of both *Pisum* and *Lathyrus*. A derivative of strain 16015 containing pJB5JI was

Table 2. *Introduction of pJB5JI into symbiotically defective strains of R. leguminosarum*

Recipient strain	Phenotype* of recipient strain on peas	Phenotype of pJB5JI transconjugants
6015	Inf ⁻	Eff ⁺
3634	Inf ⁻	Eff ⁺
1027	Inf ⁻	Inf ⁻
7577	Eff ⁻	Eff ⁺
7062	Eff ⁻ (25 °C)	Eff ⁺ (25 °C)
7021	Eff ⁻	Eff ⁻
7186	Eff ⁻	Eff ⁻
7155	Eff ⁻ (25 °C)	Eff ⁻ (25 °C)

* Five clones of recipients and transconjugants were re-purified twice and tested on peas in duplicate.

Table 3. *Transfer of plasmids to strain 6015*

Donor strain	Selected plasmid marker	Frequency of marker transfer (%)	No. of recipients tested on peas*	No. of infective recipients†
248	<i>Medium</i> bacteriocin	10	10	10
1062(pRL1JI)	<i>Medium</i> bacteriocin	7	10	10
1027(pVW1JI)	Kanamycin resistance	1	5	5
306	<i>Medium</i> bacteriocin	0.5	1	0
1062(pRL3JI)	<i>Medium</i> bacteriocin	15	5	0
1027(pVW3JI)	Kanamycin resistance	1	5	0
309	<i>Medium</i> bacteriocin	5	10	0
1062(pRL4JI)	<i>Medium</i> bacteriocin	10	5	0
1027(pVW5JI)	Kanamycin resistance	1	5	0

* Recipients were streaked twice to allow selection of stable transconjugants that had received *medium* and lost *small* bacteriocin production. Only stable recipients of *medium* bacteriocin production or kanamycin resistance were tested.

† Each recipient was tested on two peas.

able to nodulate both *Pisum* and *Lathyrus*, implying that the genetic information transferred with this plasmid was sufficient to permit nodulation of both hosts.

When the native bacteriocinogenic plasmid pRL1JI was transferred into the non-infective strain 6015, the 20 *medium* bacteriocin-producing recipients tested all formed pink effective nodules on peas (Table 3), whereas a similar number of clones of the parent strain 6015 showed no nodulating ability. The same result was obtained regardless of whether 6015 received pRL1JI directly from its original host strain (248) or from a derivative of strain 300 (strain 1062) containing this plasmid. These results show that the high frequency transfer of nodulating ability is a property associated with the natural bacteriocinogenic plasmid pRL1JI as well as with pJB5JI, its derivative containing the transposon Tn5 (Johnston *et al.*, 1978).

Transfer of pRL3JI and pRL4JI to 6015

The bacteriocinogenic plasmids pRL3JI and pRL4JI transferred between strains of *R. leguminosarum* at similar frequencies to those observed with pRL1JI, and all three plasmids eliminated (or suppressed) *small* bacteriocin production and resistance from strains into which they were transferred. However, in contrast to pRL1JI, when pRL3JI or pRL4JI was transferred into strain 6015, none of the *medium* bacteriocin-producing recipients tested was able to nodulate peas (Table 3). The donors used in these crosses were the original host strains 306 or 309, or derivatives of strain 1062 containing the bacteriocinogenic plasmids which had previously been transferred to them by conjugation; all the donors were able to nodulate peas normally.

Transduction of markers between bacteriocinogenic plasmids

In order to facilitate studies with pRL3JI and pRL4JI, it was useful to obtain kanamycin-resistant derivatives of these plasmids which would be analogous to pJB5JI, the derivative of pRL1JI obtained by transposon mutagenesis (Beringer *et al.*, 1978a). In pJB5JI, Tn5 probably inserted into genes determining *medium* bacteriocin production because *kan* was cotransduced with inactivation of *medium* bacteriocin from pJB5JI to pRL1JI (Johnston *et al.*, 1978). These two phenotypes could also be cotransduced at high frequency from pJB5JI when pRL3JI or pRL4JI was present in the recipient (Table 4). The transductants obtained transferred *kan* by conjugation to further recipients at frequencies expected for pRL3JI and pRL4JI transfer, indicating that Tn5 had been introduced into the plasmid of the recipient. When strain 1062 was used as a recipient, in transduction from pJB5JI, *kan* transductants were not observed (frequency < 10⁻⁸). These results provide evidence for

Table 4. Transduction of *kan* to bacteriocinogenic plasmids using bacteriophage RL38 grown on strain 6015(pJB5JI)

Recipient strain	Frequency of transduction of <i>kan</i>	No. of transductants producing <i>medium</i> bacteriocin	Frequency of transfer of <i>kan</i> to further recipients from transductants†
1062(pRL1JI)	5×10^{-7}	0/100	10^{-2}
1062(pRL3JI)	1×10^{-7}	1*/39	10^{-2}
1062(pRL4JI)	1×10^{-7}	2*/62	10^{-2}
1062	1×10^{-9}	0/1*	$< 10^{-8}$

* In these clones *kan* was not transmissible at a frequency greater than 10^{-8} per recipient: they might have arisen either as spontaneous *kan* mutants of the recipient or perhaps by transposition of Tn5 to a different site.

† At least two transductants were used from each transduction, except for strain 1062, where only one kanamycin-resistant derivative was obtained.

DNA sequence homology between the three plasmids in the region determining production of *medium* bacteriocin; however, the slightly lower frequencies of transduction to pRL3JI and pRL4JI compared to pRL1JI suggest imperfect homology.

The kanamycin-resistant derivatives of pRL1JI, pRL3JI and pRL4JI obtained by transduction from pJB5JI were designated pVW1JI, pVW3JI and pVW5JI, respectively. It is not known how large a region of DNA was transduced from pJB5JI into the recipient plasmids. However, in all the properties so far studied, the derived plasmids closely resembled the original bacteriocinogenic plasmids (except for bacteriocin production itself). Transfer of pVW1JI, pVW3JI and pVW5JI to strain 6015 always resulted in elimination of *small* bacteriocin production; as expected, recipients of pVW1JI were all infective whereas recipients of pVW3JI and pVW5JI were non-infective. Similarly, when transferred to *R. trifolii* strain 6662, pVW1JI transferred the ability to nodulate peas but pVW3JI and pVW5JI did not (Johnston *et al.*, 1978). In addition, pVW3JI and pVW5JI were unable to suppress the three other symbiotically defective mutations that were shown in Table 2 to be suppressible by pJB5JI and pVW1JI.

Direct selection for the transfer of nodulating ability

Very few strains of *R. leguminosarum* have been found with bacteriocinogenic plasmids that transfer at high frequency ($> 10^{-2}$) and only one of the three known bacteriocinogenic plasmids (pRL1JI) has been shown to transfer symbiotic genes at high frequency. There is therefore a need to select directly for the transfer of nodulating ability in crosses by a method that is independent of the selection for transfer of bacteriocinogenic plasmids. One such method exploits the ability of the plant to select for nodulating clones among a large population of a non-infective strain (16015). Reconstruction experiments (Table 5) using various mixtures of 16015 and a derivative of 16015 containing pJB5JI showed that an inoculum containing fewer than 10 infective bacteria could induce nodule formation even in the presence of 10^7 non-infective cells; as expected the bacteria recovered from these nodules were all *kan* derivatives of strain 16015. Thus, using strain 16015 as a recipient in crosses, it was possible to select for nodulating transconjugants by taking the entire mixture from a cross (following counterselection against the donor) and inoculating peas so that infective rhizobia could be recovered from any nodules that were formed.

It was also possible to estimate the frequency of infective transconjugants present in the inoculum by serial dilution of the cross mixtures prior to inoculation on peas (Vincent, 1970): at the end-point there would be approximately 1 to 10 infective cells per inoculum (Table 5). The total number of viable rhizobia added per inoculum was determined by plating dilutions on TY medium, and hence the frequency of infective transconjugants could be estimated.

Table 5. *Nodulation of Wisconsin Perfection peas using inocula containing mixtures of strain 16015(pJB5JI) with an excess of the non-nodulating strain 16015*

Single plants were used for each inoculation and examined 3 weeks later for the presence (+) or absence (–) of nodules.

Inoculum mixture (no. of viable bacteria in undiluted inoculum)		Dilution of inoculum						
16015	16015 (pJB5JI)	10 ⁰	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶
3 × 10 ⁷	0	–	–	–	–	–	–	–
0	1 × 10 ⁶	+	+	+	+	+	+	+
3 × 10 ⁷	1 × 10 ⁶	+	+	+	+	+	+	–
3 × 10 ⁷	1 × 10 ⁵	+	+	+	+	+	+	–
3 × 10 ⁷	1 × 10 ⁴	+	+	+	+	–	–	–
3 × 10 ⁷	1 × 10 ³	+	+	+	–	–	–	–
3 × 10 ⁷	1 × 10 ²	+	+	+	–	–	–	–
3 × 10 ⁷	1 × 10 ¹	+	–	–	–	–	–	–

Table 6. *Transfer of nodulating ability to strain 16015 from derivatives of strain 1027 containing various plasmids*

Donor strain	Frequency of Kan ^r or medium bacteriocin producing transconjugants per recipient	Approximate frequency of Inf ⁺ transconjugants per recipient	Approximate frequency of Inf ⁺ among Kan ^r transconjugants	Proportion of Kan ^r or medium bacteriocin producing clones among Inf ⁺ transconjugants
1027	—	< 10 ⁻⁶	—	—
1027(pRL1JI)	1 × 10 ⁻²	10 ⁻²	—	17/20
1027(pRL3JI)	1 × 10 ⁻²	10 ⁻⁶	—	4/5
1027(pRL4JI)	1 × 10 ⁻²	10 ⁻⁶	—	5/5
1027(pVW1JI)	2 × 10 ⁻³	10 ⁻³	10 ⁰	49/50
1027(pVW3JI)	5 × 10 ⁻³	10 ⁻⁶	10 ⁻³	43/50
1027(pVW5JI)	5 × 10 ⁻³	10 ⁻⁶	10 ⁻⁴	10/20
1027(R68.45)	3 × 10 ⁻³	< 10 ⁻⁶	< 10 ⁻⁶	—

The validity of this end-point dilution method was tested in reconstruction experiments for mixtures containing 16015(pJB5JI) and 16015 in ratios of 1:10³ and 1:10⁵.

When strain 1027 containing no bacteriocinogenic plasmids was the donor in such crosses there was no detectable nodulation of peas by derivatives of the recipient implying that, if transfer of nodulation ability occurred, the frequency must have been less than 10⁻⁶ per recipient (Table 6). However, when the donor contained pRL1JI, nodulation was obtained even when the diluted inoculum contained fewer than 10³ viable rhizobia. Hence the frequency of transfer of nodulation ability was approximately the same as that of pRL1JI itself.

Similar crosses were performed using strain 1027 containing pVW1JI, the kanamycin-resistant derivative of pRL1JI. Before inoculation of peas, the cross mixtures were grown on TY plates containing either streptomycin to counterselect the donor or streptomycin plus kanamycin which enriched specifically for derivatives of 16015 that had received pVW1JI. In the former case the transfer frequency for nodulating ability was about 10⁻³ per recipient, but when selection was made for kanamycin-resistant transconjugants, all were infective.

When derivatives of strain 1027 carrying pRL3JI or pRL4JI were used as donors in crosses to 16015, the frequency of infective transconjugants was estimated to be 10⁻⁶ per recipient by the end-point dilution technique, but nodulation was not obtained consistently from these crosses, indicating that the limits of detection were being approached. A similar

result was obtained using the kanamycin-resistant derivatives pVW3JI and pVW5JI, and in these cases prior enrichment of the progeny for kanamycin-resistant transconjugants resulted in a 10^3 -fold enhancement in the frequency of infective cells (Table 6). The contents of surface-sterilized nodules obtained from these crosses were examined. All clones isolated were sensitive to rifampicin (to which the donor was resistant) and contained the genetic markers expected for the recipient, 16015. Most of these isolates (50 to 90%) were infective when re-tested on peas and a large proportion contained markers of the bacteriocinogenic plasmids or their derivatives. However, some clones were obtained which had acquired nodulation ability without the accompanying plasmid markers *kan* or *medium* bacteriocin production (Table 6), and in other nodule re-isolates these plasmid markers had been acquired without the stable inheritance of nodulation ability (1/80 for pVW1JI, 8/60 for pVW3JI and 3/60 for pVW5JI).

Using recovery from nodules as the selective system, derivatives of 16015 were obtained which had acquired nodulation ability along with pVW3JI or pVW5JI. Some of these clones were used as donors to 6015 to determine whether nodulation ability was now transferred at high frequency with pVW3JI and pVW5JI. Selection was made for the transfer of *kan* and five transconjugant clones that had received pVW3JI or pVW5JI were re-purified twice from single colonies and tested for nodulation ability on peas. Of the donors examined, 2/6 of the 16015(pVW3JI) derivatives and 3/6 of the 16015(pVW5JI) derivatives transferred nodulation ability at high frequency such that for each cross all five of the 6015 *kan* transconjugant clones nodulated peas (and the bacteria re-isolated from these nodules had the expected genetic markers).

The apparent transfer of symbiotic gene functions by pRL3JI and pRL4JI at frequencies of about 10^{-6} might perhaps be due to the rare activation (as a consequence of plasmid transfer) of latent symbiotic genes normally present on pRL3JI and pRL4JI in silent form. In order to test this possibility non-nodulating clones of 6015(pRL3JI) and 6015(pRL4JI) were used as donors in crosses similar to those described above. Thus donor and recipient (16015) carried the same *inf* mutation in these crosses; no nodulating transconjugants were detected by the plant selection method. It is therefore likely that the symbiotic genes transferred by pRL3JI and pRL4JI are derived from the genome of the nodulating host strain.

The bacteriocinogenic plasmids pRL3JI and pRL4JI are known to mobilize chromosomal markers at frequencies of 10^{-7} to 10^{-8} per recipient (Hirsch, 1979). For comparison, the P group R plasmid, R68.45, which mobilizes chromosomal genes at frequencies of about 10^{-6} to 10^{-7} (Beringer & Hopwood, 1976), was introduced into strain 1027 and used in crosses with 16015. Although the frequency of R68.45 transfer (3×10^{-3} per recipient) was comparable to that for the bacteriocinogenic plasmids, and the transfer of a chromosomal marker (*rif*) occurred at about the same frequency (10^{-7} per recipient), no transfer of nodulating ability was detected using R68.45 even after enrichment for kanamycin-resistant transconjugants (Table 6). This result implies that the frequency of transfer of nodulation ability of R68.45 could not have been greater than 10^{-7} per recipient.

Incompatibility between bacteriocinogenic plasmids

The kanamycin-resistant plasmids pJB5JI, pVW1JI, pVW3JI and pVW5JI all suppressed or eliminated production of *small* bacteriocins when transferred into derivatives of strain 300. In addition, the introduction of pJB5JI eliminated *medium* bacteriocin production from strains containing pRL1JI, pRL3JI or pRL4JI (Hirsch, 1979) and similarly the introduction of pVW1JI, pVW3JI or pVW5JI eliminated bacteriocin production from strains carrying pRL1JI (Table 7). The transfer frequencies of all the kanamycin-resistant plasmids were reduced 10 to 100-fold by the presence of a *medium*-bacteriocin producing plasmid in the recipient strain (Tables 3 and 7), indicating superinfection inhibition between these three plasmids and their derivatives.

Table 7. *Studies on incompatibility between bacteriocinogenic plasmids*

Expt A. Transfer of nodulation ability with pJB5JI (a derivative of pRL1JI) into derivatives of the non-infective strain 6015 containing the bacteriocinogenic plasmids pRL1JI, pRL3JI or pRL4JI.

Expt B. Failure to eliminate nodulation ability with *medium* bacteriocin production from strain 6015 containing pRL1JI following the introduction of pVW1JI, pVW3JI or pVW5JI (which are Kan^r non-bacteriocinogenic derivatives of pRL1JI, pRL3JI and pRL4JI, respectively).

Donor strain	Recipient strain	Frequency of transfer of <i>kan</i>	No. of Kan ^r transconjugants that were Inf ⁺	No. of Inf ⁺ Kan ^r transconjugants producing <i>medium</i> bacteriocin
Expt A				
1062(pJB5JI)	6015(pRL1JI)	1×10^{-4}	5/5	0/5
1062(pJB5JI)	6015(pRL3JI)	1×10^{-4}	5/5	0/5
1062(pJB5JI)	6015(pRL4JI)	1×10^{-4}	5/5	0/5
Expt B				
1027(pVW1JI)	6015(pRL1JI)	5×10^{-4}	5/5	0/5
1027(pVW3JI)	6015(pRL1JI)	8×10^{-4}	5/5	0/5
1027(pVW5JI)	6015(pRL1JI)	2×10^{-4}	5/5	0/5

When pJB5JI was introduced into derivatives of 6015 containing pRL3JI or pRL4JI, all the kanamycin-resistant transconjugants tested (five for each cross) had lost the ability to produce *medium* bacteriocin but had acquired the ability to nodulate peas (Table 7). Because pRL1JI transfers both bacteriocin production and infectivity to strain 6015, it might have been expected that both properties would be eliminated together from strain 6015 containing pRL1JI following the introduction of pVW3JI or pVW5JI. Surprisingly (Table 7), the five re-purified kanamycin-resistant transconjugant clones tested from each cross were all able to nodulate peas (and rhizobia extracted from the resulting nodules had the expected genetic markers). Moreover, when these transconjugants were used as donors in further crosses with 16015 and selection was made for the transfer of kanamycin resistance, it was found that infectivity was transferred with *kan* in all the clones examined (10/10). Thus the determinants of infectivity which had been acquired with pRL1JI were not eliminated with bacteriocin production when pVW3JI or pVW5JI was introduced and subsequently transferred at high frequency with *kan*, a marker for these latter plasmids.

DISCUSSION

The bacteriocinogenic plasmid pRL1JI and its derivatives pJB5JI and pVW1JI transferred at high frequency a number of genes concerned with nodule initiation (*inf*) and nodule function (*eff*). The symbiotic gene(s) corresponding to those missing in strain 6015 were transferred along with plasmid markers when the selected marker was bacteriocin production or kanamycin resistance; when selection was made directly for the transfer of infectivity, the transfer frequency for bacteriocin production or kanamycin resistance among the infective transconjugants was greater than 95%. Such very high co-transfer frequencies suggest a physical linkage between the symbiotic genes and the determinants of bacteriocin production, although the fact that the two phenotypes can occasionally become separated raises an element of doubt about this simple model. An alternative explanation involves the high frequency transfer of two distinct plasmids, and it will require a physical analysis of the plasmid profiles from various strains in order to distinguish between these possibilities.

Unlike pRL1JI, the other two bacteriocinogenic plasmids, pRL3JI and pRL4JI, did not transfer symbiotic genes at high frequency. Instead, these two plasmids promoted the transfer of nodulating ability from strain 1027 at a frequency of about 10^{-6} per recipient,

whereas a chromosomal marker (*rif*) was mobilized at 10^{-7} to 10^{-8} per recipient in the same crosses. Following enrichment for the transfer of the bacteriocinogenic plasmid, the frequency of nodulating clones among the recipients was raised to 10^{-3} to 10^{-4} per recipient, indicating that the transfer of nodulating ability was usually associated with the transfer of the bacteriocinogenic plasmid. When the donor strain 1027 contained no bacteriocinogenic plasmid no transfer of nodulating ability was detectable (frequency $< 10^{-6}$ per recipient). Even the presence of R68.45 in the donor did not promote detectable transfer of nodulating ability, despite the fact that this plasmid mobilized the chromosomal marker (*rif*) at frequencies that were slightly higher than for pRL3JI or pRL4JI. These results indicate that the bacteriocinogenic plasmids pRL3JI and pRL4JI may have a preferential interaction with these symbiotic genes.

The exact role of the bacteriocinogenic plasmid in promoting *inf* transfer remains obscure. Some transconjugants were obtained (Table 6) that had acquired nodulation ability without the stable inheritance of markers from the bacteriocinogenic plasmid (*medium* bacteriocin or *kan*). This result might be taken to imply that the transfer of the bacteriocinogenic plasmid was not itself necessary for the transfer of nodulation ability. However, the point at which transconjugant clones could be analysed for genetic markers was only after recovery from mature root nodules after many cell generations had elapsed: thus it is possible that the bacteriocinogenic plasmid, although originally present, was not stably inherited in these nodulating clones.

Where nodulation ability had been transferred at low frequency with pVW3JI or pVW5JI, the ability to transfer nodulation ability with *kan* in subsequent crosses was sometimes raised to the very high levels characteristic of pJB5JI. It is therefore possible that the normal method for mobilization of nodulating ability by pRL3JI or pRL4JI is by means of a co-integrate plasmid containing the bacteriocinogenic plasmid together with the nodulation genes, but that this co-integrate can break up in the recipient. On this model pRL1JI might be a more stable co-integrate of a bacteriocinogenic plasmid containing the nodulation genes.

None of the symbiotic mutations that can be suppressed by pJB5JI has yet been mapped genetically and so we do not know whether the genes are chromosomal or plasmid-borne in strain 300. However, there is some circumstantial evidence from physical studies that the mutation of strain 6015 may result from a deletion in the largest of the three plasmids seen in strain 300 (P. R. Hirsch, unpublished observations).

The special properties of pRL1JI raise the possibility that this plasmid might be a stable co-integrate of the bacteriocinogenic plasmid with the symbiotic genes. In any case, it is likely that the symbiotic genes transferred with pRL1JI are of distant origin (i.e. from strain 248 or earlier), whereas in the case of the low frequency transfer by pRL3JI and pRL4JI the relevant genes are probably mobilized from the genome of the immediate donor strain. These two plasmids, pRL3JI and pRL4JI, may therefore be useful as a means of mobilizing symbiotic genes from strains which have no endogenous transfer ability.

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