Genetic Transformation of Rhizobium leguminosarum by Plasmid DNA

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We demonstrated the genetic transformation of Rhizobium leguminosarum by R68.45 plasmid DNA by freezing and thawing cell suspensions in the presence of R68.45 plasmid DNA and 20 mM MgCl2. Clones resistant to kanamycin and tetracycline were recovered at a frequency of 10^-8 per recipient cell. No colonies that were doubly drug resistant were recovered in parallel control experiments.

Clone banks of Rhizobium DNA in Escherichia coli have been constructed (5, 7), but it has not been easy to identify the function of any particular rhizobial DNA fragment. This is because the genes in Rhizobium spp. are not likely to be expressed in E. coli; Johnston et al. (12) have shown that Rhizobium trp genes are not ordinarily expressed in E. coli, and Neve et al. (13) have shown that Rhizobium promoters do not function efficiently for an E. coli plasmid gene.

Genetic transformation of Rhizobium spp. is a more direct route for the transfer of DNA than mating individual E. coli clones back into Rhizobium spp. Although several reports of transformation have appeared (2, 6, 14), no reproducible results have been obtained to date. In this communication, we demonstrate genetic transformation of Rhizobium leguminosarum by the use of a modification of the freeze-thaw technique developed by Holsters et al. (9) for the transformation of Agrobacterium tumefaciens; independent control experiments ruled out possible artifacts.

The transforming DNA used in this study was plasmid R68.45, isolated from R. leguminosarum 2345 by a modification of the method described by Adachi and Iyer (1). Cells (1 liter) were grown to an optical density of 1 (at 550 nm) in TY medium (4) with 10 mM CaCl2 plus 20 µg of kanamycin per ml and 5 µg of tetracycline per ml (to maintain the R68.45 plasmid bearing the genes for ampicillin, kanamycin, and tetracycline resistance). The cells were harvested by centrifugation and washed once at 4°C with 25% (wt/vol) sucrose containing 0.05 M Tris at pH 8.0. The pellets were resuspended in 150 ml of the same buffer. After this, 3 ml of 5 M NaCl and 6 ml of 0.5 M EDTA (pH 8.0) were added, followed by 15 ml of sucrose-Tris buffer containing 10 mg of lysozyme per ml. The suspension was held at 4°C for 15 min. Lysis was completed by adding 2 ml of 20% sodium dodecyl sulfate and holding the suspension for 30 min at room temperature. The lysate was chilled on ice for 1 to 3 h to precipitate DNA-membrane complexes and then centrifuged for 10 min at 11,000 × g at 4°C. The DNA-membrane pellet was suspended in 80 ml of 10 mM Tris buffer (pH 8.0) containing 1 mM EDTA and was then added to 10 ml of 5 M NaCl. The suspension was extracted with a 50% volume of chloroform-isoamyl alcohol (24:1); the aqueous phase was then mixed with a 50% volume of distilled phenol saturated with 10 mM Tris buffer (pH 8.0). After centrifugation, the aqueous phase was mixed with an equal volume of cold isopropanol, and the viscous precipitate was spooled onto a glass rod. The nucleic acid was dissolved in 80 ml of Tris-EDTA buffer, and heat treated pancreatic RNase (free of DNase activity) was added to a final concentration of 10 µg/ml. The suspension was incubated for 1 h at 37°C to digest RNA. Solid CsCl was added to give a final density of 1.61 g/cm³ and a 1/25 volume of ethidium bromide (dissolved at 6 mg/ml in dimethyl sulfoxide) was added. The solution was loaded into Beckman Quickseal tubes (39 ml) and centrifuged at 170,000 × g for 24 h in a Beckman VTi50 vertical rotor. The DNA bands in the tubes were located by fluorescence after exposure to UV light, and the lower band (plasmid DNA) was removed with a syringe. Ethidium bromide was removed by four extractions with CsCl-saturated isopropanol. The dye-free DNA solution was dialyzed against at least three changes of Tris-EDTA buffer (pH 8.0). This technique reproducibly yielded 30 to 50 µg of
plasmid DNA per liter of cells. The integrity of the plasmid DNA was confirmed by electron microscopy and agarose gel electrophoresis.

This plasmid fraction was used to transform strain 300 (wild type) or 897 (phe trp Str). The strains were grown in TY medium with 10 mM CaCl$_2$ to a density of 0.4 units of absorbancy at 550 nm and harvested by centrifugation at 4°C. The cells were washed in 10 mM Tris buffer, pH 7.5, at 4°C and recentrifuged. The pellet was resuspended in TY broth containing 10 mM CaCl$_2$ and 20 mM MgCl$_2$ to give a cell density of 10$^{10}$ colony-forming units/ml. A 0.2-ml portion of this suspension was added to 0.1 ml of plasmid DNA (10 μg/ml) dissolved in 10 mM Tris buffer, pH 8.0, containing 1 mM EDTA. This mixture was frozen immediately in an ethanol-dry ice bath at −70°C. After 5 min, it was transferred for 25 min to a 30°C water bath. The contents of the tube were diluted fivefold in TY medium without MgCl$_2$ and incubated at 25°C for 5 to 90 min to allow for phenotypic expression of the drug markers. Although we were able to recover transformants by plating the mixture as early as 5 min after it had thawed, we routinely waited 90 min for maximum expression before plating the mixture (we observed a twofold increase in transformants between the 5- and 90-min time points). No increase in viable counts was seen until 120 min or more had passed. Portions containing 2 × 10$^7$ to 2 × 10$^9$ colony-forming units were plated with kanamycin soft agar on plates containing 30 μg of kanamycin per ml. After incubation at 28°C for 96 h, all drug-resistant colonies were picked and restreaked on TY agar containing 30 μg of kanamycin per ml and 7.5 μg of tetracycline per ml. Colonies resistant to both antibiotics were scored as genetic transformants. Ampicillin resistance was not used as a selective marker for *R. leguminosarum* because Ap$^+$ is expressed poorly in the strains used for this study.

For transfection with RL4-1 DNA, the same steps were followed until thawing. The entire thawed mixture of cells and DNA was plated immediately on TY plates. Plaques were scored after 72 h at 28°C.

Genetic transformation to kanamycin and tetracycline resistance depended on intact plasmid DNA and frozen-thawed recipient cells (Table 1). Neither cells alone nor DNA yielded any colonies resistant to both drugs. The addition of DNase just before the freezing step completely destroyed the transforming activity of the plasmid; however, transformation was resistant to both RNase and proteinase K. Plasmid DNA isolated from *E. coli* hosts never yielded any transformants out of 2 × 10$^{10}$ cells plated; this suggests that the *Rhizobium* host inactivated the foreign plasmid DNA (15). In summary, the data of Table 1 show that R68.45 plasmid DNA was the transforming agent.

To confirm the presence of the intact plasmid in transformant clones, we tested 11 kanamycin- and tetracycline-resistant transformants for the presence of the R68.45 donor plasmid. In filter mating experiments (11), all 11 clones transferred the three drug resistance markers to *E. coli* KF$^{+}$ 933 r$^{-}$ m$^{-}$ and *B. leu*, with an efficiency of 10$^{-3}$ to 10$^{-2}$ per recipient.

### Table 1. Transformation of *R. leguminosarum* 897 phe trp Str$^+$ by R68.45 plasmid DNA$^a$

<table>
<thead>
<tr>
<th>Condition</th>
<th>Transforms/recipient cell</th>
<th>Km$^+$ Tc$^+$ colonies/plate (2 × 10$^5$ cells plated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>10$^{-8}$</td>
<td>21</td>
</tr>
<tr>
<td>DNA alone</td>
<td>&lt;10$^{-11}$</td>
<td>0</td>
</tr>
<tr>
<td>Cells alone</td>
<td>&lt;10$^{-11}$</td>
<td>0</td>
</tr>
<tr>
<td>plasmid alone</td>
<td>&lt;10$^{-11}$</td>
<td>0</td>
</tr>
<tr>
<td>No freeze-thawing</td>
<td>&lt;10$^{-11}$</td>
<td>0</td>
</tr>
<tr>
<td>DNase added</td>
<td>&lt;10$^{-11}$</td>
<td>0</td>
</tr>
<tr>
<td>RNase added</td>
<td>10$^{-8}$</td>
<td>15</td>
</tr>
<tr>
<td>Proteinase K added</td>
<td>10$^{-8}$</td>
<td>17</td>
</tr>
<tr>
<td>Plasmid DNA isolated from <em>E. coli</em> B</td>
<td>&lt;5 × 10$^{-11}$</td>
<td>0</td>
</tr>
</tbody>
</table>

*a* R68.45 DNA from *R. leguminosarum* 2345 was isolated as described in the text. DNA (1 μg; 7.5 molecules per cell) was added to 2 × 10$^9$ cells of *R. leguminosarum* 897 phe trp Str$^+$ and frozen in dry ice-ethanol. After thawing at 30°C and incubation for 5 to 90 min at 25°C, the mixture was plated on kanamycin agar plates. Controls showed no increase in the number of colony-forming units on nonselective medium until 120 min after thawing. For all experiments, a two-step selection procedure (J. Beringer, personal communication) was employed; primary selection on tetracycline (6.5 μg/ml) yielded drug-resistant clones 3 h after thawing. All Tc$^+$ clones were also resistant to kanamycin. During the initial selection, about half of the kanamycin-resistant colonies appeared to be spontaneous drug resistance mutations which were not tetracycline resistant. After 96 h, kanamycin-resistant colonies were transferred to tetracycline-kanamycin plates. Doubly resistant colonies were scored as transformants. Five plates were assayed per experiment for each of the conditions listed. Of the transformed colonies, 11 were picked, and all of these mated Ap$^+$ Km$^+$ Tc$^+$ back into *E. coli*. Plasmid DNA from transformants could be used to transform 897 to Km$^+$ Tc$^+$.

Pilot experiments with RL4-1 phage DNA, a linear molecule of 64 × 10$^6$ daltons (H. J. Burkhardt, personal communication), showed that transfection occurred at a frequency of 1 × 10$^{-8}$ to 2 × 10$^{-8}$ PFU per recipient cell when saturating amounts of DNA were used (75 molecules per cell). In the medium used for freezing the DNA-cell mixture, 20 mM Mg$^{2+}$ ions was required. No loss in viable counts of recipient cells was seen after the freeze-thaw step. In the control experiments, we added enzymes at 10 μg/ml before the freezing step. DNase added less than 10 s after the freezing step had no effect on genetic transformation or transfection.
We also mated the original plasmid donor strain (2345) and several transformed clones back into E. coli B recipients and reisolated the plasmid DNA from exconjugants carrying resistance to ampicillin, kanamycin, and tetracycline. Figure 1A shows an agarose gel of native plasmid DNA; the plasmid DNA derived from the cross with the transformants had the same electrophoretic mobility as the plasmid DNA from the cross with the original donor strain. Figure 1B shows that the Smal restriction digests of the plasmid DNAs were also indistinguishable; the Smal patterns of these DNAs are characteristic of R68.45. As evidenced by the observation that the restriction enzyme digests were identical, the entire plasmid was transferred during transformation without major rearrangements.

In addition, we tested the ability of transformed R. leguminosarum 897 colonies to infect host plants. Using the methods described by Vincent (18), we showed that 12 of 12 such clones nodulated the Early Perfection variety of peas; no nodules were observed when the two uninfected controls were examined. Bacteria isolated from the nodules still carried the 897 phe trp Str" and plasmid Tc' Km' markers.

We added pancreatic DNase at various times after the plasmid DNA and the recipient cells were mixed to determine when irreversible uptake of DNA had occurred. In all experiments, the cells became completely resistant to DNase immediately (≤10 s) after the mixture had been frozen. Thus, DNase sensitivity was present only if the enzyme was added before the cell-DNA mixture was frozen. The same result was obtained when RL4-1 phage DNA was used to transfect R. leguminosarum.

We next mixed various concentrations of R68.45 DNA with a constant number of recipient cells. The number of transformants reached a maximum frequency of 5 × 10⁻⁸ per recipient at 33 μg of DNA per ml, which corresponds to 75 DNA molecules per cell. The data also suggest a linear dependence of transformation on lower DNA concentrations. A similar result was observed when transfection with RL4-1 DNA was performed.

Since the frequency of genetic transformation was low (though reproducible), this technique should not be used with most recipients carrying revertible point mutations. However, when selection for two markers is available, transformants are easily identified. Both pRK290 (5) and RP4 (K. F. Scott, P. M. Hughes, B. G. Greshoff, B. G. Rolfe, and J. Shine, International Congress on Nitrogen Fixation, Canberra, 1980, abstract no. 124) have easily identifiable drug resistance markers which are not inactivated after digestion with either EcoRI or BglII and insertion of cloned fragments (5). Given a Rhizobi-