JOURNAL OF BACTERIOLOGY, Dec. 1988, p. 5925–5927
0021-9193/88/125925-03$02.00/0

Vol. 170, No. 12

High-Frequency Transformation of *Rhizobium meliloti*

JOSIANE COURTOIS,* BERNARD COURTOIS, AND JEAN GUILLAUME

Laboratoire de Microbiologie, U.F.R. de Biologie, SN2, Université des Sciences et Techniques de Lille Flandres-Artois, 59655 Villeneuve d'Ascq Cédex, France

Received 16 May 1988/Accepted 23 September 1988

Transformation of R factor RP4 and its derivative pRK290 from *Escherichia coli* to *Rhizobium meliloti* is reported. The efficiency of transformation was in the range of 10^{-5} per viable cell. In addition, chromosomal DNA prepared from one *R. meliloti* strain resistant to streptomycin was transferred to the isoleucine-valine-requiring mutant susceptible to streptomycin.

Genetic transformation is a more direct route for the transfer of DNA than mating, but this technique has not yet been described for many strains.

In the genus *Rhizobium*, although several reports of transformation techniques for chromosomal characters have been reported earlier (1, 7, 14), no reproducible results have been obtained to date.

Transformation of plasmid DNA isolated from *Rhizobium meliloti* to overcome the restriction of foreign DNA to *R. meliloti* 41 (10) and *R. meliloti* JJ1 (15) has been reported, with a low frequency of 10 to 54 cells per μg of a 10.3-kilobase plasmid DNA and 2×10^{-8} cells per μg of a 56-kilobase plasmid, respectively. If transformation was performed with plasmid DNAs isolated from *Escherichia coli*, no colony formation appeared on selective plates (10).

High-frequency transformation of *R. meliloti* by plasmid DNA isolated from another genus donor strain has not been described in the literature. In this work, we report a transformation system for *R. meliloti*, using foreign plasmid DNA RP4 and pRK290 extracted from *Escherichia coli*; the technique could be used for transferring chromosomal DNA prepared from one *R. meliloti* strain resistant to streptomycin (Sm^r) to the isoleucine-valine-requiring (Ilv^-) mutant susceptible to streptomycin (Sm^s).

Bacterial strains used as recipients are listed in Table 1.

Plasmids used in this study, prepared according the method of Jouanin et al. (9), were isolated from *E. coli* J.53(RP4) (5) and *E. coli* HB101(pRK290) (6) grown to logarithmic phase in Luria broth (11) and from *R. meliloti* M5N1(pRK290) [obtained from *R. meliloti* M5N1, *E. coli* HB101(pRK290), and *E. coli* HB101(pRK2013) according to the procedure of Ditta et al. (6)] grown to logarithmic phase in TY medium (2). The integrity of plasmid DNA was confirmed by agarose gel electrophoresis. Total DNA was isolated from *R. meliloti* M5N1 Sm^r grown to logarithmic phase in TY medium (2) according to the method described by Miura (12). Plasmid (60 μg/ml) and total (100 μg/ml) DNA were stored at 4°C in 0.15 M NaCl (pH 7) over chloroform.

Transformation of *R. meliloti* M5N1 by R factor was performed as follows. The strain was grown up in *R. meliloti* competence (RMC) medium containing (per liter) K_2HPO_4, 3.6 g; KH_2PO_4, 0.4 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; $(NH_4)_2SO_4$, 1 g; glucose, 10 g; yeast extract, 1 g; and Casamino Acids 1 g (pH 7.2), in a rotary bath shaker (60 revolutions per min) at 30°C to a density of 10^8 CFU/ml, and it maintained static at 30°C. After 3 h of incubation, the cells were harvested by centrifugation at 3,000 × *g* for 10 min and suspended in *R. meliloti* transformation (RMT) medium containing (per liter) KNO_3, 0.6 g; Na_2SO_4, 0.6 g; $(NH_4)_2SO_4$, 0.1 g; $CaCl_2$, 5.5 g; and $MgCl_2$, 1.9 g (pH 7.2), to give a cell density of 10^9 CFU/ml.

TABLE 1. *R. meliloti* strain used as recipients for genetic transformation

Strain	Characteristic	Source
M5N1	Wild type	Isolated from *M. sativa* (our collection)
	Ilv^-	Obtained by nitrosoguanidine treatment
	Sm^r	Spontaneous Sm^r mutant of M5N1
21	Wild type	F. Pichinoty (Center International Agricultural tropical Colombia)
2011	Wild type	J. Denarie (Toulouse, France)

A 0.2-ml portion of this suspension was added to 0.1 ml of DNA dissolved in 0.15 M NaCl (pH 7). The mixture was chilled rapidly at 0°C. After 15 min at 0°C, it was transferred for 5 min at 37°C then at 0°C. After 40 min, the mixture was incubated for 30 min in a 30°C water bath. Then, to allow phenotypic expression of the drug markers, the cells were diluted in RC medium (4) and spread on sterilized membrane filters (type HA, 0.45-μm-pore size; Millipore Corp., Bedford, Mass.) placed on RC agar (15‰) plates. After 120 min of incubation at 30°C, the filters were transferred onto RC agar plates containing 10 μg of tetracycline per ml. Transformant colonies were scored after 4 days of incubation at 30°C.

TABLE 2. Importance of cell density before transfer of cells in RMT medium[a]

Cell density[b] on RMC medium before transfer to RMT medium	Transformation frequency/viable cell
10^7	2×10^{-6}
5×10^7	8×10^{-6}
10^8	2.2×10^{-5}
5×10^8	4×10^{-6}
10^9	10^{-7}

[a] Cells were grown in RMC medium at 30°C over a rotary shaker (60 revolutions per min). During growth, samples were used for transformation, according to the procedure described in the text. In each case, cell density in RMT medium was constant at 10^9 cells per ml.

[b] Expressed as bacteria per ml in RMC medium.

* Corresponding author.

TABLE 3. Transformation of *R. meliloti* M5N1 by RP4 plasmid DNA isolated from *E. coli*

Condition[a]	Transfer frequency[b]
Complete system[c]	2.2×10^{-5}
Cells alone[d]	—[e]
No static culture before transformation[f]	9.6×10^{-6}
DNA added after freezing-thawing[g]	10^{-6}
Cells sprayed on the filter placed on RC agar plate[h]	
Just after the freeze-thaw step	0.9×10^{-6}
30 min after (complete system)	2.2×10^{-5}
1 h after	7.5×10^{-6}
2 h after	10^{-6}
Transformation in RMC medium[i]	7×10^{-9}
DNA treated with DNase before addition to cells[j]	—
Cells grown in TY medium before transformation[k]	$<10^{-9}$

[a] M5N1 strains were grown in RMC medium on a rotary shaker at 30°C; when cell density was 10^8, the cells were used under the indicated conditions.

[b] Transfer frequency expressed per viable cell.

[c] Cells were placed under static conditions at 30°C for 3 h, centrifuged for 10 min at 3,000 × *g*, and transferred to RMT medium. The cell density in the transformation medium was 10^9. Then, 0.2 ml of cells was mixed with 0.1 ml of DNA, so that the final DNA concentration was 20 μg/ml. After being frozen and thawed, the transformation mixture was diluted in RC medium before being sprayed onto filters placed on RC agar medium. After 120 min of incubation at 30°C, the filters were placed on RC medium containing tetracycline (10 μg/ml). The incubation was continued for 4 days at 30°C.

[d] Cells were treated as in the complete system, without the addition of DNA.

[e] —, No colonies were observed when 10^9 bacteria were assayed in the absence of DNA or with DNA treated with pancreatic DNase.

[f] Cells grown in RMC medium over a rotary shaker at 30°C were harvested by centrifugation and suspended in RMT medium before DNA was added.

[g] DNA was added after the freezing and thawing steps; cell treatment was as in the complete system.

[h] The transformation procedure was as in the complete system except for the indicated difference in time of spraying the transforming mixture onto filters placed on RC agar plates.

[i] After centrifugation, the cells were suspended in RMC medium (cell density was 10^9) and used as described in footnote *c*.

[j] DNA was treated with pancreatic DNase (10 μg/ml) at 37°C for 5 min in the presence of 10 mM $MGCl_2$ before use in transformation assay.

[k] M5N1 strains were grown in TY medium on a rotary shaker at 30°C. When cell density was 10^8, cells were placed under static conditions for 3 h, centrifuged, and used in the complete system.

The transformed *R. meliloti* M5N1(RP4) expressed the drug resistance genes coded by the R factor as shown by the concomitant acquisition of resistance to kanamycin (50 μg/ml) and tetracycline (10 μg/ml). The foreign RP4 plasmid could be detected in transformants by using the method of Eckhardt (8) (data not shown).

No transformants were observed on the control plates.

Plant nodulation tests on *Medicago sativa* were performed to confirm the identity of transformed *Rhizobium* strains.

Genetic transformation of *R. meliloti* by R factor RP4 depended on the cell density before transfer of cells in the RMT medium (Table 2), the growth conditions before addition of DNA, the time of DNA addition (before or after freezing and thawing the cell suspension), and the cell incubation period at 30°C before transfer onto filters staying on the RC agar plates; prior treatment of the DNA preparation with pancreatic DNase completely eliminated the transforming ability (Table 3).

The high frequency of transfer reported here was only due to transformation and not to transformation followed by conjugation as shown by the similar frequency of transfer for the tetracycline resistance marker of 3.7×10^{-5} obtained with the non-self-transmissible derivative of RP4:pRK290 when isolated from *E. coli* HB101(pRK290). When the same plasmid DNA was isolated from *R. meliloti* M5N1(pRK290), the frequency of transformation was 1.5×10^{-3}. These results revealed that the high frequency of transfer obtained with plasmid DNA isolated from *E. coli* was only due to an increase in the competence of the rhizobial cells during the procedure used and not to an inactivation of restriction mechanisms in *R. meliloti*.

TABLE 4. Transformation of *R. meliloti* M5N1 by chromosomal DNA

Condition	Transfer frequency[a]	
	Ilv	Sm^r
Complete system[b]	7×10^{-4}	10^{-5}
DNA treated with DNase before addition to cells[c]	—[d]	8×10^{-9}
Cells alone[e]	—	5×10^{-9}

[a] Transfer frequency expressed per viable cell.

[b] Complete system was as in Table 3, footnote *c*, except the selective medium on which filters were placed was R agar plates (3), and RC agar plates containing streptomycin (200 μg/ml) were used for selecting Ilv^+- and Sm^r-transformed cells.

[c] DNA was treated with pancreatic DNase (10 μg/ml) at 37°C for 5 min in the presence of 10 mM $MgCl_2$ before use in transformation assay.

[d] No colonies were observed.

[e] Cells were treated as in the complete system without the addition of DNA.

In addition to strain M5N1, the procedure could be used successfully on two other *R. meliloti* strains, *R. meliloti* 21 and *R. meliloti* 2011. The frequencies of transfer for the tetracycline resistance marker were 1.3×10^{-5} and 0.95×10^{-5}, respectively.

This technique was applicable to *R. meliloti* M5N1 Sm^r chromosomal DNA transfer to *R. meliloti* M5N1 Ilv^-. Ilv^+ and Sm^r transformants were scored on filters placed on R agar plates (3) and RC medium containing streptomycin (200 μg/ml), respectively, after 4 days of incubation at 30°C. The transfer frequency of the Ilv character was 7×10^{-4}, and it was 7×10^{-5} for antibiotic resistance (Table 4).

In this note, we report a system of transformation of *R. meliloti* by plasmid DNA prepared from another genus. The frequency was in the region of 10^{-5}, which is important with regard to the DNA size and its origin compared with the frequency obtained with other techniques described earlier for *R. meliloti* (10, 15) and with the frequency obtained for R factor RP4 transfer from *E. coli* to *Rhizobium trifolii* (13). In addition, we show that this technique is applicable for transformation of *R. meliloti* by chromosomal DNA.

LITERATURE CITED

1. **Balassa, G.** 1963. Genetic transformation of *Rhizobium*: a review of the work of R. Balassa. Bacteriol. Rev. **27**:228–243.
2. **Beringer, J. E.** 1974. R factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. **84**:188–198.
3. **Courtois, B., J. Courtois, A. Heyraud, and M. Rinaudo.** 1986. Effect of biosynthesis conditions on the chemical composition of the water soluble polysaccharides of fast growing Rhizobia. J. Gen. Appl. Microbiol. **32**:527–534.
4. **Courtois, B., J. P. Hornez, J. Courtois, and J. C. Derieux.** 1983. Mise en évidence d'une propriété métabolique de *Rhizobium meliloti* utilisable pour sa classification. Ann. Microbiol. (Paris) **134**:141–147.
5. **Datta, N., R. W. Hedges, E. J. Shaw, R. B. Sykes, and M. H. Richmond.** 1971. Properties of an R factor from *Pseudomonas aeruginosa*. J. Bacteriol. **108**:1244–1249.
6. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77**:7347–7351.
7. **Drozanska, D., and L. Lorkiewicz.** 1978. Genetic transformation

in *Rhizobium trifolii*. Acta Microbiol. Pol. **27**:81–88.
8. **Eckhardt, T.** 1978. A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. Plasmid **1**:584–588.
9. **Jouanin, L., P. Delajudie, S. Bazetoux, and T. Huguet.** 1981. DNA sequence homology in *Rhizobium meliloti* plasmids. Mol. Gen. Genet. **182**:189–195.
10. **Kiss, G. B., and Z. Kalman.** 1982. Transformation of *Rhizobium meliloti* 41 with plasmid DNA. J. Bacteriol. **150**:465–470.
11. **Luria, S. E., and J. W. Burrous.** 1957. Hybridization between *Escherichia coli* and Shigella. J. Bacteriol. **74**:461–476.
12. **Miura, K. I.** 1967. Preparation of bacterial DNA by the phenol pH 9 RNases method. Methods Enzymol. **12**:543–545.
13. **O'Gara, F., and L. K. Dunican.** 1973. Transformation and physical properties of R-factor RP4 transferred from *Escherichia coli* to *Rhizobium trifolii*. J. Bacteriol. **116**:1177–1180.
14. **Raina, J. L., and V. V. Modi.** 1971. Further studies on genetic transformation in Rhizobium. J. Gen. Microbiol. **65**:161–165.
15. **Selveraj, G., and V. N. Iyer.** 1981. Genetic transformation of *Rhizobium meliloti* by plasmid DNA. Gene **15**:279–283.