Further Studies on Genetic Transformation in *Rhizobium*

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SUMMARY

A strain of *Rhizobium cowpea* transformed interspecifically and intraspecifically for the gelatinase marker showed maximum competence in the early log phase of growth which was increased further by the addition of Casamino acids and Mg^{2+} to the competence medium at pH 7.0. Ferric ions inhibited transformation and this effect was partially reversed by addition of EDTA. The DNA concentration for maximum frequency of transformation was about 20 $\mu\text{g./ml.}$ The frequency of intraspecific transformation was ten times higher than that of interspecific transformation although the G+C mole per cent of the donor and recipient was very similar, 64.0 and 64.5 respectively. A surface-bound nuclease specific for the transforming DNA could have resulted in the high donor DNA requirement.

INTRODUCTION

Genetic transformation induced by heterologous DNA was reported for the first time in *Rhizobium* by Balassa (1954). Interspecific and intraspecific transformation has since been shown to be a common phenomenon, taking place between many species of *Pneumococcus* (Bracco, Krauss, Roe & Macleod, 1957), *Streptococcus* (Pakula, Hulanicka & Walczak, 1958), *Neisseria* (Catlin, 1960) and *Bacillus* (Marmur, Seaman & Levine, 1963). The frequency of interspecific transformation is usually much lower than that of the corresponding intraspecific transformation. Although the ratio of the heterospecific transformation to that of homospecific transformation reveals kinship based on a certain degree of homology between the two types, it has been observed that the lethality of the heterospecific DNA on the recipient cell is an important factor in the low efficiency of heterospecific transformation in *Haemophilus* (Steinhart & Herriott, 1968).

Previous studies in our laboratory have been concerned with the transformation of species of *Rhizobium* using penicillinase and fructokinase production as the markers in one system (Gadre, Mazumdar, Modi & Parekh, 1967), and adenine independence in the other (Raina & Modi, 1969). While studying these biochemical characteristics, a few strains belonging to the *Rhizobium cowpea* group were found to be unable to degrade gelatin. As no other biochemical differences which could be used as genetic markers were detected, gelatin liquification was used in investigations to establish conditions for interspecific and intraspecific transformation in *Rhizobium*.

METHODS

Strains and media. The various strains and species used in the present study were kindly supplied by Dr G. Rangaswamy, Agricultural University, Bangalore, India.

The cultures were maintained on mannitol yeast extract agar slants (mannitol 1.0%, w/v, Difco yeast extract 0.1%, w/v, agar 2%, w/v) and subcultured every week. The complex media for the development of competence have been described previously (Raina & Modi, 1969). The minimal medium contained the following (w/v): K_2HPO_4 , 0.7%; KH_2PO_4 , 0.2%; NaCl, 0.05%; $MgSO_4 \cdot 7H_2O$, 0.005%; sodium citrate, 0.05%; ammonium sulphate, 0.1%; glucose, 0.2%. The recipient culture after transformation was plated on mannitol yeast extract gelatin agar medium, incubated at 30° for 24 h. and the transformants scored by overlaying the plates with 15% $HgCl_2$ in 0.6 N-HCl. The transformants formed a clear zone against an opaque background.

Characterization of cultures. Of a number of cultures tested, a green gram strain (C15) belonging to the *Rhizobium cowpea* group and unable to degrade gelatin (gelatinase⁻) was selected as the recipient. Two strains, one belonging to the groundnut species of the *R. cowpea* group (GR3) and another a french-bean species of the *R. phaseolus* group (Fb16) were able to degrade gelatin (gelatinase⁺) and were used as the donors. DNA was prepared from the two donors by the method described by Raina & Modi (1969).

Fig. 1. Competence medium was inoculated with an overnight culture of *Rhizobium cowpea* strain C15 to give 1×10^7 organisms/ml. Growth (○—○) was measured turbidimetrically at 660 nm. and competence (●—●) determined by mixing the recipient with gelatinase⁺ donor DNA (20 µg./ml.) from *R. cowpea* strain GR3 and scoring the transformants as described in Methods. Competence is expressed as % frequency of transformation.

RESULTS

Competence in relation to the growth phase of the recipient culture and the media used. Cultures of the strain C15 were made competent by the method of Raina & Modi (1969). Fig. 1 shows the growth and the competence of the culture using the gelatinase⁺ GR3 strain as the donor. Competence is attained essentially in the early log phase, and the culture has to be in an active physiological state. The addition of both Casamino acids and yeast extract to minimal medium resulted in an increased frequency of transformation (Table 1). Casamino acids (2 mg./ml.) gave the maximum effect. The

development of competence is similarly influenced by Casamino acids in *Bacillus subtilis*, and Wilson & Bott (1968) attribute this to the presence of inhibitory and stimulatory amino acids which possess chelating properties. Since the development of competence was slightly enhanced by Mg^{2+} and Ca^{2+} ions and inhibited by Fe^{3+} , Mo^{2+} and Zn^{2+} (Table 2) and since the inhibition due to ferric ions was partially reversed by EDTA, the chelating effect of Casamino acids could be the possible reason for its stimulatory effect. $MgSO_4$ at a concentration of $0.05 \mu g./ml.$ was found to give the maximum stimulatory effect.

Table 1. *Effect of medium on competence*

Medium	Frequency of transformation* (%)
Complex (control)	0.07
Minimal	0.005
Minimal + yeast extract 1 mg./ml.	0.03
Minimal + yeast extract 2 mg./ml.	0.03
Minimal + Casamino acids 1 mg./ml.	0.13
Minimal + yeast extract and Casamino acids 1 mg./ml.	0.12
Minimal + Casamino acids 2 mg./ml.	0.25
Minimal + Casamino acids 2.5 mg./ml.	0.25

* Donor: *Rhizobium phaseolus* strain FB16.

Table 2. *Effect of divalent cations on competence*

Ions added ($0.01 \mu g./ml.$)	Frequency of transformation* (%)
None	0.072
Mg^{2+}	0.090
Ca^{2+}	0.083
Zn^{2+}	0.030
Mo^{2+}	0.016
Fe^{3+}	0.002
Fe + EDTA 5 $\mu moles/ml.$	0.033

* Donor: *Rhizobium phaseolus* strain FB16.

The effect of pH on competence. pH was found to influence competence (Table 3). Maximum frequency of transformation was obtained at pH 7.0. All cultures at the beginning of incubation in competence medium contained 5×10^6 organisms/ml. The viable count at the end of this incubation was $5 \times 10^8/ml.$ at pH 7.0, but at pH 4.0, 5.0, 6.0 it was only 1 to 4×10^7 organisms/ml. At pH values greater than 7.0 viability was invariably unaffected but the frequency of transformation was reduced.

The effect of DNA concentration on transformation. Competent cultures were treated with DNA at various concentrations, incubated for 30 min. at 30° and the reaction terminated by the addition of DNAase at $50 \mu g./ml.$ and $MgCl_2$ at 5mM. Further incubation was carried out for 30 min., and after appropriate dilution samples were plated on gelatin containing agar medium. Table 4 shows the relative frequencies of transformation obtained with different concentrations of DNA; the optimum concentration appears to be $20 \mu g./ml.$ Exposure to DNA for a further 15 min. gave no further increase in frequency of transformation.

Transformation with DNA from different donors. The C15 recipient strain was transformed by the DNA from the two donors Fb16 and GR3 under optimum conditions with respect to growth phase of the recipient, pH, medium supplementation and DNA concentration. The frequency of transformation was ten times greater with a donor from the same group as the recipient than with a donor from a different group, thus showing that genetically homologous DNA has a better chance of bringing about transformation than heterologous DNA. A similar conclusion was reached by Steinhardt & Herriott (1968) and Chen & Ravin (1966).

Table 3. *The effect of pH on competence*

pH of the competence medium	Frequency of transformation* (%)
4.0	Nil
5.0	Nil
6.0	0.05
7.0 (Control)	0.08
8.0	0.055
9.0	0.018

* Donor: *Rhizobium phaseolus* strain Fb16.

Table 4. *The effect of the concentration of DNA on the frequency of transformation*

DNA (μ g./ml.)	Frequency of transformation* (%)
10.0	0.40
12.5	0.50
15.0	0.55
17.5	0.70
20.0	0.75
22.0	0.70

* Donor: *Rhizobium cowpea* strain GR3.

Using the method of Wang (1968) the G+C content of the DNA from bacteria of *Rhizobium phaseolus* group had a G+C content of 64 mole % whereas that from *R. cowpea* was 64.5 mole %. This suggests genetic homology between these species on the basis of GC content. Thus reduced frequency of interspecific transformation must implicate factors other than just DNA homology.

It is likely that the requirement for a high concentration of transforming DNA in the *Rhizobium* system, similar to that for reciprocal transformation between *Agrobacterium* and *Rhizobium* (Kern, 1969), is relevant here. Experiments with the C15 transformable recipient indicate the presence of two nucleases, one specific for transforming DNA on the cell surface which has a pH optimum of 8.5, and the other inside the cell, specific for denatured DNA and with a pH optimum of 9.0. The two enzymes also differed in their Mg^{2+} ion requirements, and effective enzyme concentrations and times of incubation, at the same substrate concentration. The two proteins have been purified three- and 12-fold respectively by ammonium sulphate fractionation, and the former purified a further tenfold by Sephadex column chromatography. The significance of such enzymes as these has been discussed by Kohoutova & Malek (1966). Membrane-bound nuclease specific for denatured DNA was reported by Birnboim (1966) in a trans-

formable species of *Bacillus subtilis*, and the author suggested that it may influence the process of transformation. The requirement for a high concentration of transforming DNA in the *Rhizobium* system may therefore be attributed to the presence of surface-bound nuclease.

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REFERENCES

- BALASSA, R. (1954). Transformation mechanisms der *Rhizobium* I-III. *Acta microbiologica Academiae scientiarum Hungaricae* **2**, 51-78.
- BIRNBOIM, H. C. (1966). Cellular sites in *B. subtilis* of nuclease which preferentially degrades single stranded nucleic acids. *Journal of Bacteriology* **91**, 1004-1011.
- BRACCO, R., KRAUSS, M. R., ROE, A. S. & MACLEOD, C. M. (1957). Transformation reaction between *Pneumococcus* and three strains of *Streptococcus*. *Journal of Experimental Medicine* **106**, 247-259.
- CATLIN, W. (1960). Interspecific transformation of *Neisseria* by cultural strains containing deoxyribonucleate. *Science, New York* **131**, 608-610.
- CHEN, K. C. & RAVIN, A. W. (1966). Heterospecific transformation of *Pneumococcus* and *Streptococcus*. I. Relative efficiency and specificity of DNA helping effect. *Journal of Molecular Biology* **22**, 109-121.
- GADRE, S. V., MAZUMDAR, L., MODI, V. V. & PAREKH, V. (1967). Interspecific transformation in *Rhizobium*. *Archiv für Mikrobiologie* **57**, 388-391.
- KERN, H. (1969). Interspecific transformation between *Agrobacterium tumefaciens* and *Rhizobium leguminosarum*. *Archiv für Mikrobiologie* **66**, 63-68.
- KOHOUTOVA, M. & MALEK, I. (1966). *The Physiology of Gene and Mutation Expression*. Edited by M. Kohoutova & J. Hubacek. Prague: Academia.
- MARMUR, J., SEAMAN, E. & LEVINE, J. (1963). Interspecific transformation in *B. subtilis*. *Journal of Bacteriology* **85**, 461-467.
- PAKULA, R., HULANICKA, E. & WALCZAK, W. (1958). Transformation reactions between streptococci, pneumococci and staphylococci. *Bulletin de l'Académie polonaise des sciences. Série des sciences biologiques* **6**, 325-328.
- RAINA, J. L. & MODI, V. V. (1969). Genetic transformation in *Rhizobium*. *Journal of General Microbiology* **57**, 125-130.
- STEINHART, W. L. & HERRIOTT, R. M. (1968). Genetic integration in the heterospecific transformation of *Haemophilus influenzae* cells by *Haemophilus parainfluenzae* deoxyribonucleic acid. *Journal of Bacteriology* **96**, 1725-1731.
- WANG, S. Y. (1968). The determination of nucleic acid base composition by chemical reactivity. In *Methods in Enzymology*, vol. 12, part B. Edited by L. Grossman and K. Moldave. New York: Academic Press.
- WILSON, G. A. & BOTT, K. F. (1968). Nutritional factors influencing the development of competence in *Bacillus subtilis*. *Journal of Bacteriology* **95**, 1439-1449.