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SHORT PAPER

Effective method for the isolation of non-nodulating mutants of *Rhizobium trifolii*

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SUMMARY

Non-nodulating mutants of *Rhizobium trifolii* were found in cultures incubated at 35 °C for 7 days. From among ten strains incubated at the elevated temperature six became non-nodulating. The frequency of Nod$^-$ mutants in *R. trifolii* cultures varied from 1 to 75% depending on the strain tested. It is suggested that gene(s) controlling nodulation are located on a large plasmid.

1. INTRODUCTION

Nodulation is a necessary step towards nitrogen fixation in the symbiotic *Rhizobium*/leguminous-plants system. The molecular basis for the development of nodules on plant roots is unknown, and *Rhizobium* mutants with altered nodulation activity would be helpful in studies of this process. Effective methods for isolation of non-nodulating mutants were previously restricted to single strains of *Rhizobium* (Higashi, 1967; Parijskaya, 1973; Zurkowski, Hoffman & Lorkiewicz, 1973). In this paper we present a new method for isolation of these mutants that was found to be effective in numerous strains of *R. trifolii*.

2. MATERIALS AND METHODS

The strains of *R. trifolii* used in this study are listed in Table 1. The optimal temperature for the growth of these *Rhizobium* strains in our standard laboratory experiments was 28 °C. We found that incubation of some of these bacteria at higher temperature lead to selection of non-nodulating (Nod$^-$) mutants. The standard conditions for selection of such mutants were as follows: overnight cultures growing in liquid YM medium (K_2HPO_4 1·0 g; KH_2PO_4 0·5 g; $MgSO_4.7H_2O$ 0·2 g; NaCl 0·1 g; $CaCl_2$ 40 mg; yeast extract (Difco) 1·0 g; mannitol 10·0 g; H_2O 1 l; pH = 6·8) were diluted to about 2×10^8 cells/ml, and incubated without shaking at 35 °C for 7 days. The cultures were aerated twice each day by a brief intense shaking. Bacteria were then plated on agar medium and single colonies were transferred to agar slants for further testing. Nodulation ability was determined in tube tests by inoculation of sterile red clover seedlings (Vincent, 1970). The sensitivity of microorganisms to phages was tested by spotting phages (5×10^8 plaque forming units/ml) on to bacterial lawns on YM agar plates.

3. RESULTS AND DISCUSSION

The optimum temperature for growth of the strains tested was approximately 28 °C. During 24 h of incubation at 35 °C the number of bacteria fluctuated slightly, whereas after prolonged incubation a decline of bacterial population was found. A typical survival curve for one strain grown at elevated temperature is shown in Fig. 1, and initial and final numbers of cells for all strains tested are given in Table 1. When small inocula were used, no survivors were found after prolonged incubation at 35 °C; large inocula were therefore always used. Though there was essentially no multiplication of micro-organisms, the size of the bacteria and the optical density increased during the first day of incubation at 35 °C, suggesting that macromolecular syntheses were still going on.

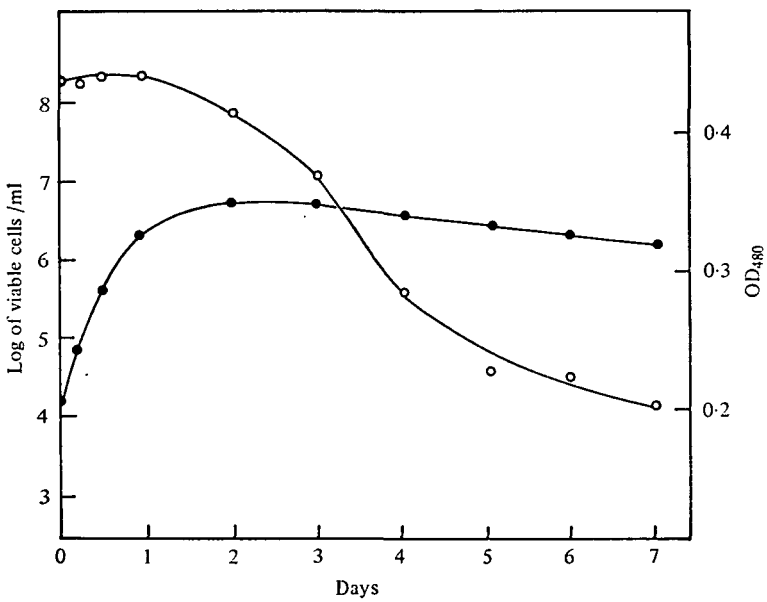

Fig. 1. Effect of incubation of *R. trifolii* T12 at 35 °C. ○—○, Viability of cells; ●—●, optical density at 480 nm.

Results for the loss of nodulation ability of single clones isolated from *R. trifolii* strains are presented in Table 1. From ten strains incubated at the elevated temperature in six cultures each, Nod⁻ mutants were detected. The frequency of Nod⁻ mutants varied from 1 to 75%, depending upon the strain tested. Nod⁻ mutants were not detected at 28 °C under standard experimental conditions. Three strains, C101, T12 and 24, which showed a high proportion of Nod⁻ mutants at 35 °C were further tested in control experiments at 28 °C, but Nod⁻ mutants were not detected after incubation for 7 days (Table 1). The Nod⁻ mutants isolated remained continuously non-nodulating; reversion to the Nod⁺ phenotype was not observed. Nod⁻ mutants were as sensitive to elevated temperature as wild strains. The provenance of the Nod⁻ mutants has been confirmed by several tests to exclude the possibility that there are contaminants. For example, wild-type strains and their Nod⁻ mutants were typed using three different rhizobiophages (Staniewski, 1970; table 2). All the Nod⁻ mutants demonstrated the same pattern of sensitivity to phages as the corresponding wild-type strains.

Further detailed studies on two strains, T12 and 24, showed that elimination of the nodulation character was accompanied by the loss of large plasmids from bacterial cells

(manuscript in preparation). Large plasmids were detected by Nuti *et al.* (1977) and by us (manuscript in preparation) in numerous *Rhizobium* strains. The elimination of plasmids by elevated temperatures in other bacteria has been demonstrated by May,

Table 1. *Loss of the nodulation character from* R. trifolii

Strain	Source	Initial and final no. of viable cells/ml	No. of tested clones	Nod⁻ clones (%)
	Incubation at 35 °C for 7 days			
C5	R. Staniewski	3×10^8 to 7×10^2	180	5
C32	R. Staniewski	2×10^8 to 8×10^2	163	12
C46	R. Staniewski	2×10^8 to 3×10^3	148	0
C95	R. Staniewski	1×10^8 to 4×10^5	100	1
C101	R. Staniewski	1×10^8 to 5×10^4	120	16
T11	Isolated from clover nodules by us	1×10^8 to 3×10^3	115	0
T12	Isolated from clover nodules by us	2×10^8 to 2×10^4	384	63
CC2480	E. A. Schwinghamer	2×10^8 to 4×10^4	133	0
T1	E. A. Schwinghamer	1×10^8 to 1×10^3	161	0
24	IUNG, Puławy	2×10^8 to 4×10^4	632	75
	Incubation at 28 °C for 7 days			
C101		1×10^8 to 2×10^9	236	0
T12		1×10^8 to 8×10^8	291	0
24		1×10^8 to 7×10^8	367	0

Table 2. *Susceptibility of wild-type strains and Nod⁻ mutants of* Rhizobium trifolii *to bacteriophages*

Strains	Pattern of phage lysis 1H	3H	1P	No. of Nod⁻ mutants tested	No. of mutants with wild phage type
C5	±	±	−	9	9
C32	+	+	−	20	20
C95	+	±	−	1	1
C101	+	+	−	19	19
T12	+	+	−	68	68
24	+	+	−	68	68

+, Complete lysis; ±, semiconfluent lysis; −, no lysis.

Houghton & Perret (1964) in *Staphylococcus*, and by Tarawaki, Takayasu & Akiba (1967) in *Proteus*. The loss of nodulation properties from *R. trifolii* incubated at an elevated temperature may similarly be due to temperature-sensitive replication of a plasmid which controls nodulation.

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