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Plasmid-Mediated Control of Nodulation in *Rhizobium trifolii*

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Abstract. The nodulation ability was effectively eliminated from different *Rhizobium trifolii* strains incubated at elevated temperature (Żurkowski and Lorkiewicz, 1978). Non-nodulating (Nod$^-$) mutants were stable and no reversion of Nod$^-$ to Nod$^+$ phenotype was observed. Strains *R. trifolii* 24 and T12 which showed a high percentage of elimination of nodulation ability were examined in detail. Two plasmids were detected in strain 24 using neutral and alkaline sucrose gradient centrifugation of plasmid preparations. Molecular weights of the plasmids pWZ1 and pWZ2 were 460 Mdal and 190 Mdal, respectively. *Rhizobium* lysates labeled with ^{3}H-thymidine and ultracentrifuged in caesium chloride – ethidium bromide gradients demonstrated a 40% reduction of the plasmid DNA content in *R. trifolii* 24 Nod$^-$ mutants in comparison with the nodulating wild type strain 24. It was found further that non-nodulation of mutants 24 Nod$^-$ was due to the absence of plasmid pWZ2. Sucrose gradient data also demonstrated that strain T12 contained two plasmids with molecular weights corresponding to those of pWZ1 and pWZ2, respectively. In Nod$^-$ mutant clones derived from strain T12, pWZ2 plasmid was missing.

Key words: *Rhizobium trifolii* – Nodulation – Non-nodulating mutants – Plasmids – Molecular weight of plasmid – Elimination of plasmid.

The mechanism of genetic regulation of symbiotic nitrogen fixation was difficult to study previously because of the complex interactions of the symbiont with its host plant. The results of recent studies indicate that some stages of symbiosis can be mediated by extrachromosomal DNA. The occurrence of endogenous plasmids in different rhizobia was demonstrated using physico-chemical methods (Sutton, 1974; Klein et al., 1975; Tshitenge et al., 1975; Dunican et al., 1976; Żurkowski and Lorkiewicz, 1976; Kowalczuk and Lorkiewicz, 1977; Nuti et al., 1977; Bechet and Guillaume, 1978; Palomares et al., 1978). Several authors determined the plasmid size by sedimentation analysis (Tshitenge et al., 1975; Dunican et al., 1976; Żurkowski and Lorkiewicz, 1976; Nuti et al., 1977; Palomares et al., 1978) or by electron microscopy (Żurkowski and Lorkiewicz, 1976). Unfortunately, functions of the detected plasmids were in most cases not determined. Cole and Elkan (1973) and Dunican et al. (1976) found in rhizobia plasmids being R factors. The results of experiments carried out by Dunican et al. (1976) suggested plasmid-borne control of nitrogen fixation by *Rhizobium*. However, it was demonstrated recently (Skotnicki and Rolfe, 1978) that the strain used by Dunican was more related taxonomically to *Agrobacterium* than to *R. trifolii*.

The genetical studies by Higashi (1967) and Johnston et al. (1978) indicate indirectly that the nodulation specificity of *Rhizobium* is mediated by a plasmid. We have assumed (Żurkowski et al., 1973) on basis of the studies on elimination of the nodulation character that the ability to nodulate was controlled in *R. trifolii* by plasmids. Parijskaya (1973) obtained similar results in elimination of the nodulation character from *Rhizobium meliloti*.

In extensive studies on *R. trifolii* of different symbiotic properties we have detected plasmid DNAs in all the eighteen examined strains (in preparation). In many strains more than one single-size plasmid species were demonstrated. Among *R. trifolii* strains treated with acridine dyes non-nodulating clones were not found. On the other hand *R. trifolii* strains incubated at elevated temperature were cured from the nodulation trait (Żurkowski and Lorkiewicz, 1978). In this paper

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Non Standard Abbreviations. CCC = covalently closed circular; OC = open circular; Sarkosyl = sodium N-lauroylsarcosinate

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the hypothesis of the plasmid control of nodulation in *R. trifolii* was proved by genetical and physico-chemical methods.

Materials and Methods

Bacterial Strains. The nodulating wild-type *Rhizobium trifolii* strain 24 was obtained from the Institute of Cultivation and Fertilization of Soil at Pulawy, strain T12 was isolated from clover nodules. Both cultures were derived from single clones and maintained on YM agar slants. Non-nodulating (Nod^-) mutant strains were isolated from cultures incubated at elevated temperature (Żurkowski and Lorkiewicz, 1978).

Labelling Procedure of Bacterial DNA. Overnight cultures of *R. trifolii* strains were diluted thrice with fresh yeast-mannitol (YM) medium (Żurkowski and Lorkiewicz, 1978) supplemented with [6-^{3}H] thymidine up to 10 μCi/ml. Deoxyadnosine was not added to the medium since wild type strains of *R. trifolii*, contrary to some bacteria of other genera, readily utilized exogenous thymidine similarly to the thymine-less mutants of *R. trifolii* (Żurkowski, 1977). Following the addition of thymidine, bacteria were incubated with aeration for 4–6 h.

Preparation of Bacterial Lysates. Rhizobium cultures (8 ml) labeled with ^{3}H-thymidine were centrifuged at 20,000 × g for 10 min using Unipan 310 centrifuge. Cells were washed with TE buffer (0.05 M Tris and 0.01 M EDTA, pH = 8.0), sedimented and resuspended in 1 ml of 25% sucrose (nucleases free, E. Merck) solution in TE buffer. 0.5 ml lysozyme (BDH) in concentration 3 mg per ml of 0.25 M buffer Tris-HCl pH = 8.0 was added to the mixture. Lysozyme solution was preincubated at 37° C for 1.5 h before use. After 15 min of incubation bacteria were lysed by addition of 1.5 ml 2% Sarkosyl (0.01 M Tris, 0.05 M Na_2EDTA, 0.05 M NaCl, 2% sodium N-lauroylsarcosinate, pH = 8.0). The mixture was then incubated at 20° C for 15 min. Afterwards lysates were very gently pipetted 5–10 times at flow rate less than 0.2 ml/s using a 5 ml pipette with an orifice of 0.7–0.8 mm.

Ultracentrifugation in Caesium Chloride – Ethidium Bromide Gradients. 2.5 ml of labeled cells lysate was added to 6 g of CsCl dissolved in 3.4 ml TES (0.05 M Tris, 0.05 M Na_2EDTA, 0.05 M NaCl, pH = 8.0) in a 10 ml ultracentrifuge tube. Subsequently 0.4 ml of ethidium bromide solution (2 mg per ml of TES buffer) was added. The preparation was then mixed by gentle rotation of the tube and overlayered with parafin oil. Preparations were centrifuged for 44 h using a Ti 10 × 10 ml rotor in a MSE 65 ultracentrifuge at 15° C and 44,000 rpm. Seven-drop fractions were collected from the bottom of the tube. Samples (10 μl) for radioactivity counting were prepared as described earlier (Żurkowski and Lorkiewicz, 1977).

Ultracentrifugation of Plasmid DNA in Neutral Sucrose Gradients. Fractions containing plasmid DNA were dialysed against TES buffer (0.05 M Tris, 0.005 M EDTA, 0.05 M NaCl, pH = 8.0) for 8–12 h at 0–4° C. Samples of the dialysed plasmid DNA were mixed with ^{32}P labelled DNA of phage P22, and 0.2 ml of the preparation was layered on linear 5–20% (w/v) neutral sucrose gradient in TES buffer (0.05 M Tris, 0.005 M EDTA, 0.5 M NaCl, pH = 8.0). Gradients were centrifuged for 60 min using Ti 3 × 6.5 ml swing-out rotor in MSE 65 ultracentrifuge at 20° C and 32,000 rpm. Gradients were fractionated by collecting six-drop fractions directly onto Whatman GF/C microfibre discs, and radioactivity was measured after precipitation with trichloroacetic acid. For preparative purposes, fractions of gradients were collected in tubes, and then 25 μl of the material were removed to determine the radioactivity. In preparative gradients the undiluted material collected from caesium chloride – ethium bromide gradients was layered onto sucrose gradients.

Alkaline Sucrose Gradients. Linear 5–20% (w/v) alkaline sucrose gradients, containing additionaly 0.2 M NaOH and 0.7 M NaCl, were centrifuged for 20 min in an MSE 65 Ti swing-out rotor at 20° C and 32,000 rpm.

Determination of the Molecular Weight of Plasmids. Sedimentation coefficients of plasmid DNA preparations were estimated on basis of the linear dependence between distances of the DNA peaks from the meniscus, and sedimentation coefficient (Clowes, 1972). ^{32}P labelled DNA of phage P22 was employed as the marker. The molecular weight of P22 DNA molecule is 28×10^6 daltons (Tye et al., 1974). The sedimentation coefficient for linear duplex of this size, using the formula $S_{20,w} = 2.8 + 0.00834\ M^{0.479}$ (Clowes, 1972), corresponds to 33.6S. Molecular weights of tested plasmids were estimated on basis of the sedimentation coefficient of the covalently closed circular (CCC) form of plasmid DNA employing the formula of Bazaral and Helinski (1968).

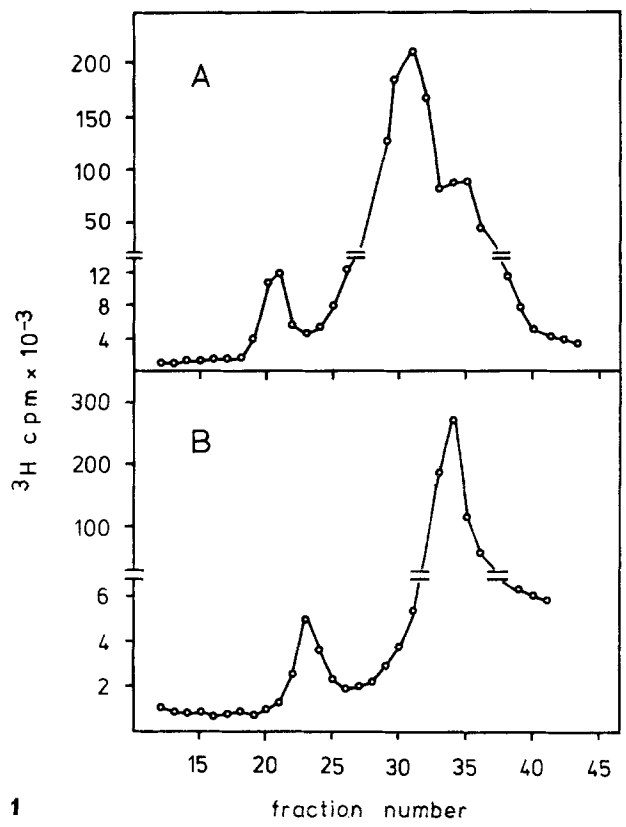
Results

Elimination of Plasmids from R. trifolii Incubated at Elevated Temperature

R. trifolii strain 24 and T12 characterized by a high rate of elimination of nodulation at elevated temperature were employed in molecular studies on curing of bacteria from the nodulation ability. The culture 24 and seven tested Nod^- mutants derived from it showed the presence of plasmid DNA in caesium chloride – ethidium bromide density gradient centrifugation of Sarkosyl lysates. A typical sample of gradient fractionation is presented in Fig. 1. It was estimated that the amount of the plasmid DNA isolated under standard conditions from Nod^- mutants was approximately 40% lower than that in the wild type strain 24. In the culture 24 the average yield (mean of 7 experiments) of the isolated plasmid DNA relative to chromosomal DNA was 3.8% (± 1.1), whereas in Nod^- mutants 2.1% (± 0.4).

Plasmid DNAs isolated by caesium chloride – ethidium bromide gradient centrifugation were re-centrifuged through linear 5–20% neutral sucrose gradients. Plasmid DNA of *R. trifolii* strain 24 was separated into 5 fractions of different sedimentation coefficients (Fig. 2A). Values of sedimentation coefficients of the fractions determined in eight independent experiments were as follows: I – 173S (± 5.6); II – 119S (± 1.9); III – 95S (± 1.1); IV – 70S (± 1.7); V – 46S (± 2.3). Plasmid DNAs of the seven tested Nod^- mutants derived from the strain 24 were separated into three fractions of different sedimentation coefficients. In all the seven tested Nod^- mutants, fractions II and IV were missing. A typical sedimentation profile of plasmid DNA isolated from the Nod^- mutants is presented in Fig. 2B.

Plasmid DNA of *R. trifolii* strain T12 demonstrated also five fractions like the wild type strain 24 (Fig. 3A). Sedimentation coefficients of the fractions from I to V were as follows (mean of 3 independent experiments):



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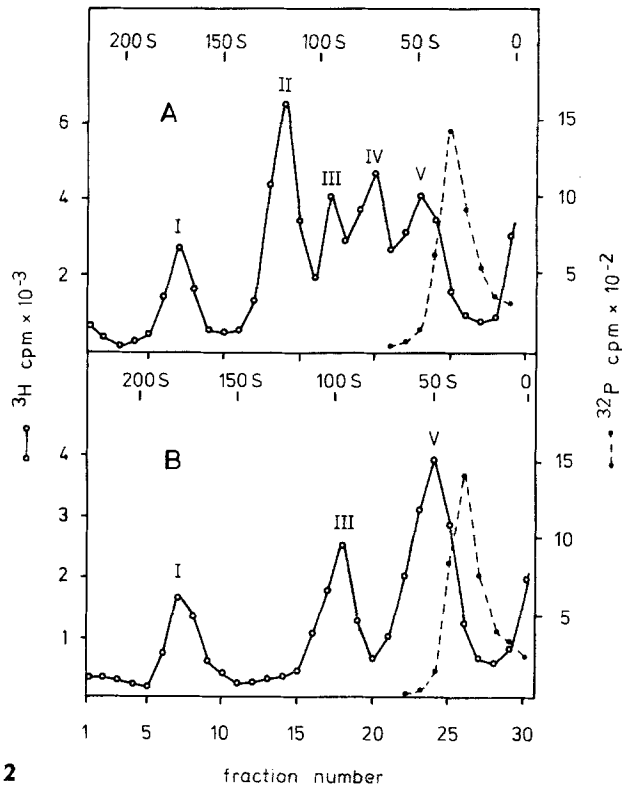

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Fig. 1. Caesium chloride – ethidium bromide gradient ultracentrifugation of Sarkosyl lysates of ^{3}H-thymidine labeled nodulating *Rhizobium trifolii* strain 24 (**A**) and non-nodulating mutant 24 Nod$^-$ (**B**)

Fig. 2. Neutral 5–20% sucrose gradient centrifugation of plasmid DNA of nodulating *Rhizobium trifolii* strain 24 (**A**) and non-nodulating mutant 24 Nod$^-$ (**B**)

181S, 121S, 92S, 73S and 42S. Sedimentation coefficients of the corresponding plasmid fractions isolated from the strain 24 Nod$^+$ were alike to those of T12 Nod$^+$. In the examined T12 Nod$^-$ mutant the fractions II and IV were missing (Fig. 3B).

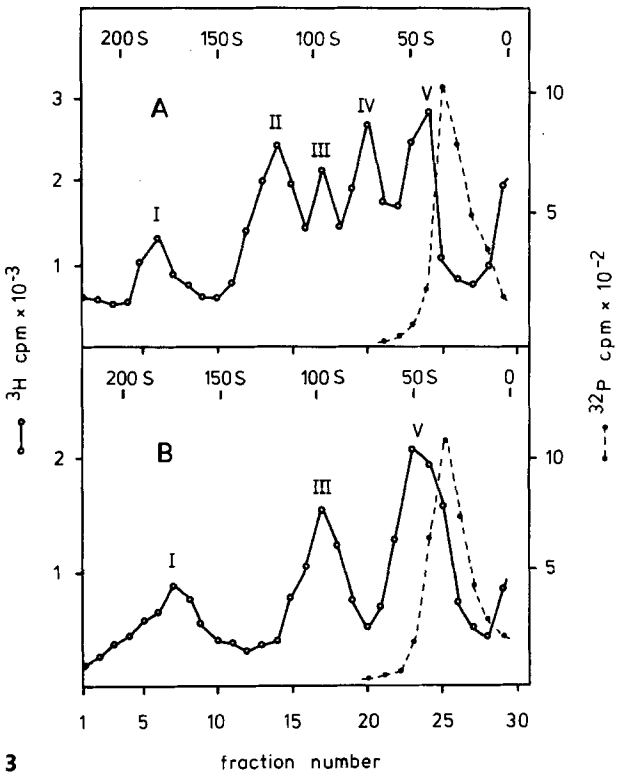
Molecular Characteristic of R. trifolii Plasmids

Plasmid DNA isolated from *R. trifolii* strain 24 was subjected to further analysis. Additional experiments were carried out to determine properties and the character of particular DNA fractions separated by sucrose gradient ultracentrifugation. For that purpose sedimentation profiles of plasmid DNA stored at 4°C were determined. Additionally, DNA of particular fractions isolated from preparative sucrose gradients were resedimented in neutral and alkaline sucrose gradients. In neutral sucrose gradient centrifugation two plasmid forms are usually detected, fast sedimenting CCC form and slowly sedimenting OC form (Clowes, 1972). Storage or ionizing radiation brings about the transformation of the CCC to OC form and subsequently to linear form of DNA.

In order to obtain the preparation as open circular forms, plasmid DNA isolated from the strain 24 was stored at 4°C for 4 days. This DNA subjected to sedimentation analysis in neutral sucrose gradient showed a reduction of the content of fractions I and II (Fig. 4). Sedimentation analysis of the freshly isolated DNA is presented in Fig. 2A. The results indicated that fractions I and II were CCC forms of two different plasmids.

The plasmid DNA isolated from marked peaks of preparative neutral sucrose gradient (Fig. 5) were resedimented in neutral (Fig. 6) and alkaline sucrose gradients (Fig. 7). DNA collected from the peak I was separated by neutral sucrose gradient centrifugation in two fractions which corresponded to the fractions I and III (Fig. 6A and 5). DNA of the peak II was also separated in two fractions. Sedimentation coefficients of these fractions corresponded with the coefficients of the fractions II and IV (Fig. 6B and 5). In profiles of the resedimented material from the fractions III and V no additional peaks were observed (Fig. 6C, D and 5).

In alkaline sucrose gradients the CCC form of DNA sediments three to four times more rapidly than linear



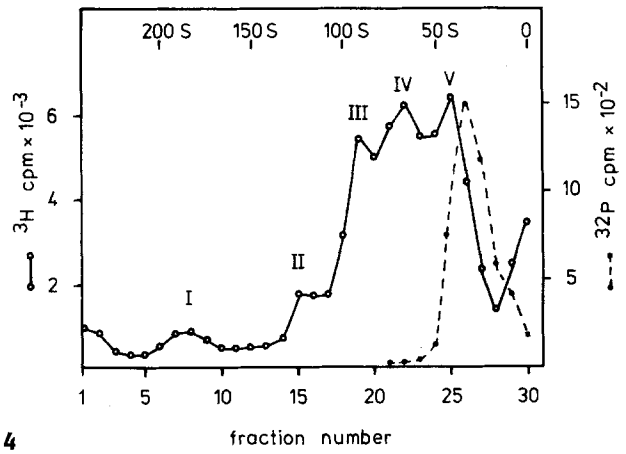


Fig. 3. Neutral 5–20% sucrose gradient centrifugation of plasmid DNA of nodulating *Rhizobium trifolii* strain T12 (**A**) and non-nodulating mutant T12 Nod$^-$ (**B**)

Fig. 4. Neutral 5–20% sucrose gradient centrifugation of plasmid DNA of *Rhizobium trifolii* strain 24, stored for 4 days at 4°C

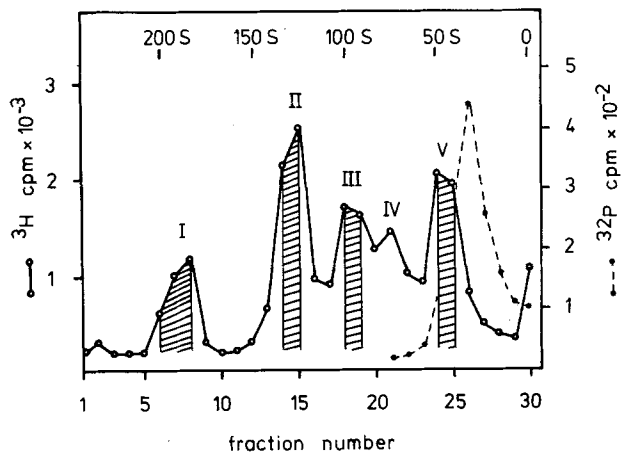


Fig. 5. Preparative neutral 5–20% sucrose gradient centrifugation of plasmid DNA of *Rhizobium trifolii* strain 24. Marked fractions were collected for resedimentation

or open circular DNA (Clowes, 1972). DNAs isolated from the peaks I and II sedimented rapidly in alkaline sucrose gradient (Fig. 6A and B).

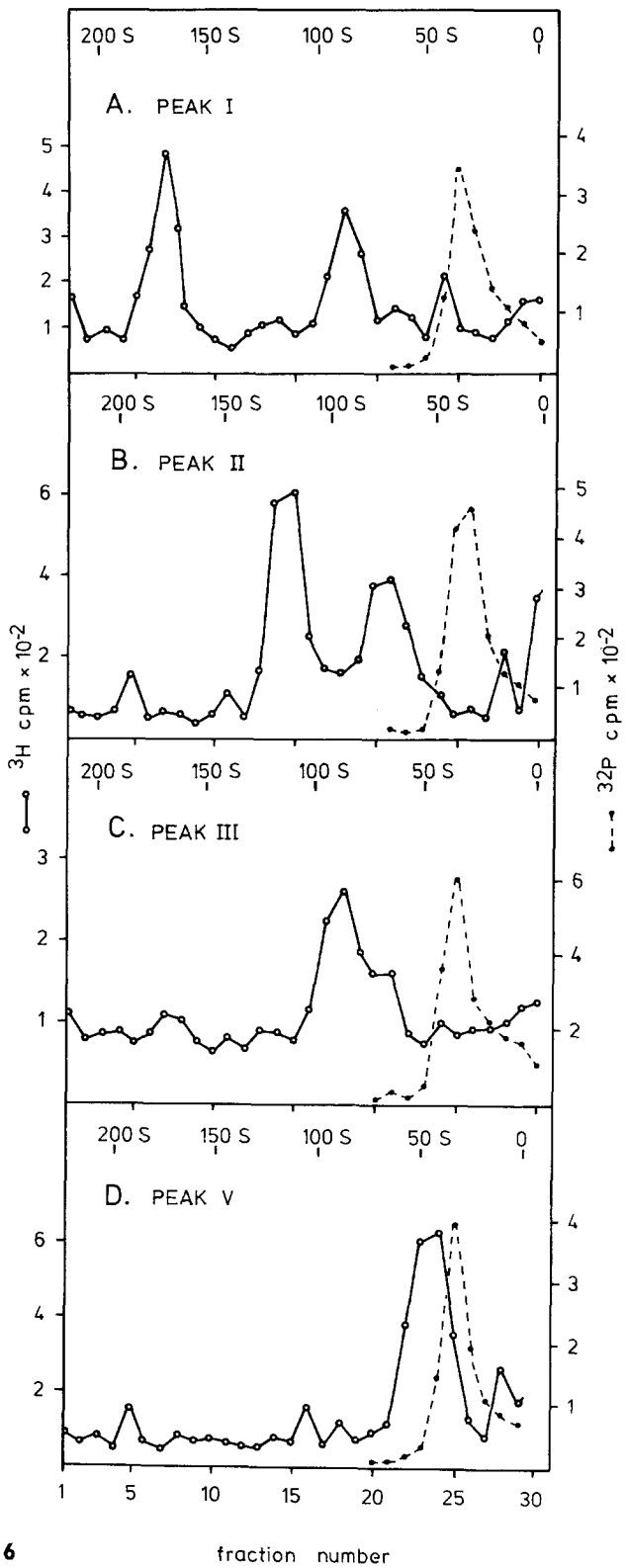
It was shown above by three different tests that the fractions I and III were CCC and OC forms, respectively of one plasmid, designated according to Nowick et al. (1977) as pWZ1, whereas fractions II and IV were configurational forms of the other plasmid designated pWZ2.

It seems probable that deficiency of the fractions II and IV in Nod$^-$ mutants resulted from the loss of one plasmid, pWZ2. The fraction V centrifuged in alkaline sucrose gradient sedimented slowly. It is more likely that this fraction contained the degraded DNA of large plasmids and/or fragments of the chromosomal DNA but not CCC DNA of a smaller plasmid.

By application of the Bazaral and Helinski (1968) formula, molecular weights of the plasmids carried by *R. trifolii* strain 24 were estimated. DNA of the CCC and OC forms of the respective sedimentation coefficients 173S (± 5.6) and 95S (± 1.1) corresponded to the large plasmid pWZ1 of the molecular weight 457 (± 35) Mdal. The molecular weight of the plasmid pWZ2 having sedimentation coefficients of 119S (± 1.9) for CCC and 70S (± 1.7) for OC form was 191 (± 7) Mdal.

Stability of the Nod$^-$ Mutants

Many tests have shown that for seedlings of leguminous plants grown in or on agar, 1–2 rhizobia per tube



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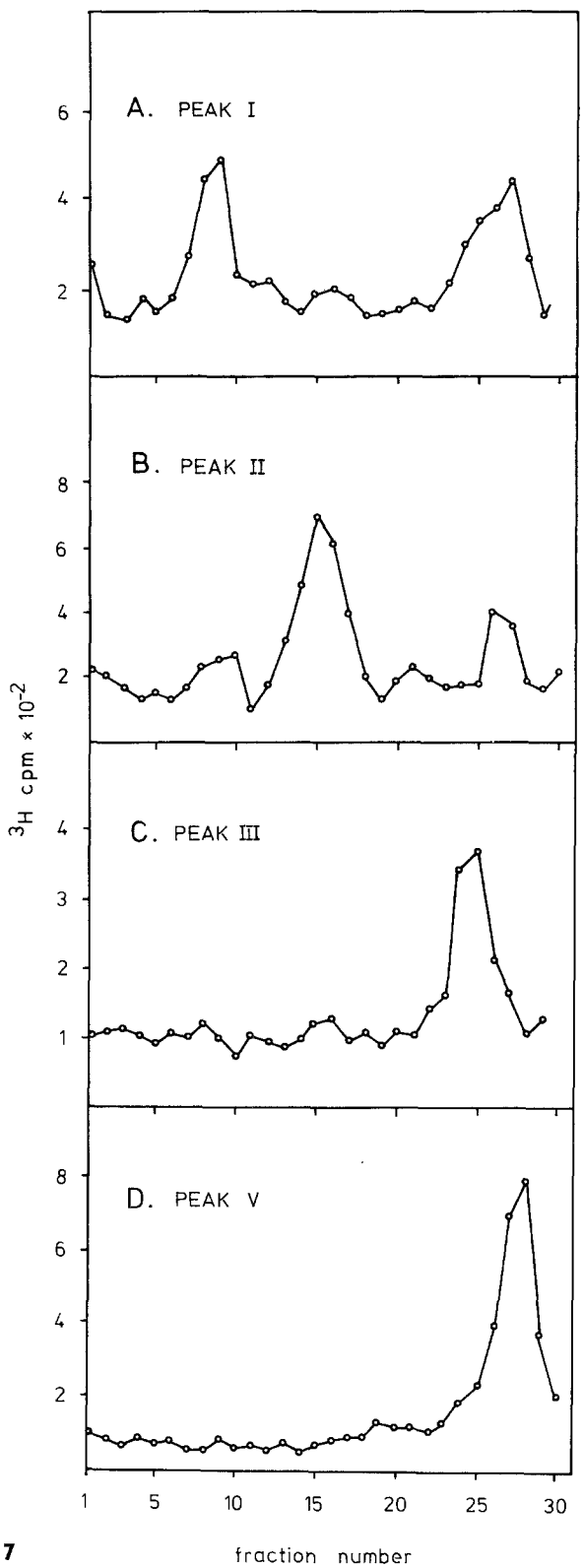

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Fig. 6. Resedimentation in neutral sucrose gradient of plasmid DNA collected from neutral sucrose gradient from peaks marked on Fig. 5

Fig. 7. Resedimentation in alkaline sucrose gradient of plasmid DNA collected from neutral sucrose gradient from peaks marked on Fig. 5

being usually sufficient for a positive result of nodulation (Vincent, 1970). Approximately 550 clover seedlings were inoculated with cultures of *R. trifolii* 24 Nod$^-$ and *R. trifolii* T12 *Nod*$^-$ mutants. Among them 100 plants were inoculated with a large inoculum (8×10^9 cells/ml) of one *R. trifolii* 24 Nod$^-$ mutant. In any of these experiments as well as in tests with Nod$^-$ mutants isolated from other strains of *R. trifolii* by incubation at elevated temperature, no reversions to Nod$^+$ were observed.

Discussion

It was found that many strains of *Rhizobium trifolii* incubated at elevated temperature lost their nodulating ability (Żurkowski and Lorkiewicz, 1978). Growth at elevated temperature was first employed by May et al. (1964) and Terawaki et al. (1967) to cure Staphylococci and *Proteus* from plasmids.

Two strains, *R. trifolii* 24 and T12, which showed a high rate of elimination of the nodulating ability by incubation at elevated temperature, were examined in detail in the present study. Plasmid DNAs of both strains sedimented in neutral sucrose gradient were separated into five fractions of different sedimentation coefficients. It was shown by three different tests (changes in sedimentation profiles of stored plasmid DNA, resedimentation of individual fractions of plasmid DNA in neutral, or in alkaline sucrose gradients) that fractions I and III were CCC and OC forms, respectively of one plasmid designated pWZ1, whereas fractions II and IV were configurational forms of the other plasmid designated pWZ 2. In seven tested Nod$^-$ mutants, isolated from the strain 24 after incubation at elevated temperature, the fractions II and IV were missing. Deficiency of both DNA fractions was also observed in the Nod$^-$ mutant isolated from strain 24 by treatment with UV irradiation. On the other hand in one tested nodulating clone isolated from *R. trifolii* strain 24 after 7 days of incubation at 35° C, both plasmids, pWZ1 and pWZ 2, were detected. The above results indicate to a correlation between the loss of the nodulation character and the elimination of the plasmid pWZ 2. The disappearance of the nodulation ability of *R. trifolii* strain T12 was also correlated with the loss of the plasmid.

The mechanism of the infection of leguminous plants with *Rhizobium* and the nodule formation are not well known. The detected by us model of the plasmid control is suitable to study these processes.

It is interesting to note that also in *Agrobacterium* which is taxonomically related to *Rhizobium*, a large plasmid responsible for infectiveness and tumor formation in plants was detected (Van Larebeke et al., 1974).

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