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NOTES

Heat Curing of a Sym Plasmid in a Fast-Growing *Rhizobium* sp. That Is Able To Nodulate Legumes and the Nonlegume *Parasponia* sp.

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Genes involved in nodulation of both legumes and the nonlegume *Parasponia* sp., as well as nitrogenase genes, reside on a large plasmid in a fast-growing *Rhizobium* sp. from *Lablab purpureus*. This plasmid can be cured by incubation at elevated temperatures and can be mobilized by the P1 group plasmid RP1::Tn*501*.

Rhizobia involved in the nitrogen-fixing symbiosis with leguminous plants have been divided into two main groups: the so-called fast growers and the slow growers. However, it has been shown recently that a number of slow-growing rhizobia isolated from cowpea-type plants are able to nodulate the nonlegume *Parasponia*, a member of the *Ulmaceae*. Similarly, a number of isolates from wild *Parasponia* nodules proved to be typical slow-growing organisms which had a variable host range among the cowpea plants (15).

Of over 200 isolations made in New Guinea from nodules of cowpea group plants (which are normally nodulated by slow growers), Trinick (14) found only one fast-growing bacterium. This strain, NGR234, resembles fast-growing rhizobia from the tropical tree *Leucaena leucocephala* and is able to nodulate a broad range of cowpea group plants, as well as lucerne (*Medicago sativa*), *Leucaena*, and *Acacia farnesiana* (14). Remarkably, this fast-growing *Rhizobium* sp. is able to nodulate the nonlegume *Parasponia* (15). In contrast to the slow growers from *Parasponia*, the symbiosis between *Parasponia* and strain NGR234 is not effective in nitrogen fixation. Clearly, the genotype of this organism allows it to nodulate a wide range of plants, crossing the boundaries that separate not only the fast- and slow-growing rhizobia but also the legume and *Parasponia*.

It has been shown that the symbiotic properties of many fast-growing rhizobia from temperate regions can be lost through a process of prolonged exposure to elevated temperatures. The eradication of the symbiotic capability is attributed to the loss of large endogenous plasmids, or to internal deletions in these plasmids (18). We report here the isolation of nonnodulating derivatives of strain NGR234 by heat curing a single large resident plasmid. This plasmid is also shown to encode the endogenous nitrogenase of this *Rhizobium* sp.

Approximately 10^{10} cells from a log phase culture of strain ANU240 (a spontaneous streptomycin-resistant mutant of NGR234) grown on tryptone-yeast extract medium (2) were evenly spread on the surface of solid YM medium (18). The cultures were incubated at 37°C for 7 days, during which no growth occurred. The YM plates were placed at room temperature, and after 5 days, about 100 single colonies arose per plate. No inhibition of growth was noted in similarly treated cultures incubated at 30 and 35°C. The single colonies from the cultures treated at 37°C were purified and checked for the antibiotic resistance pattern and growth characteristics of the strain to ensure that they were not contaminants. The purified clones were tested for the ability to nodulate the tropical legume siratro (*Macroptilium atropurpureum*) by the plate method (3, 10). Approximately 25% of the tested clones failed to nodulate.

Seven nodulated and seven nonnodulated plant tests were chosen at random, and bacteria were reisolated from the agar surface. No evidence of contamination was found, and invariably the reisolated bacteria had the characteristics of strain ANU240. The reisolated strains were retested for nodulation of siratro, using six plants per test. The nodulating (Nod^+) and nonnodulating (Nod^-) phenotypes were found to be

stable and nonreverting through subculture. Plasmid profiles of these seven Nod^+ and seven Nod^- strains were made in triplicate by the Ekhardt technique (4); one such isolate is shown in Fig. 1A. In all cases, the Nod^+ strains possessed a plasmid of approximately 200 megadaltons (designated pNM3AN). Invariably, the 200-megadalton plasmid was not detected in the Nod^- strains, nor was a deleted form of this large plasmid observed. Two small plasmids of approximately 20 (pNM1AN) and 25 (pNM2AN) megadaltons could be resolved in all NGR234 derivatives, whether Nod^+ or Nod^-, if the gel technique was modified from Ekhardt's original procedure (Fig. 1B). These two small plasmids were not identified by using the usual Ekhardt technique, since they comigrated with linear chromosomal fragments. The 200-megadalton plasmid was never detected, by either technique, in the heat-cured Nod^- strains.

One Nod^- strain, ANU264, and one Nod^+ strain chosen at random were tested for the ability to nodulate a number of plants usually nodulated by NGR234. Strain ANU264 was found to be unable to nodulate the following plants: *Vigna unguiculata*, *Lablab purpureus*, *Macroptilium lathyroides*, *Leucaena leucocephala*, and *Parasponia andersonii*; the Nod^+ strain and ANU240 were able to nodulate all of these tested plants.

The slide method of Fahraeus (5) was used to microscopically examine the roots of siratro plants inoculated with either strain ANU240 or ANU264. This was done to determine the stage at which the process of nodulation was blocked in the heat-cured strain ANU264. Whereas strain ANU240 was able to induce curling, extreme distortions, and infection threads on root hairs, the mutant strain had no observable effect on root hair morphology (Fig. 2).

When the self-transmissible plasmid pBR1AN (9), which codes for the nodulation of clovers and carries the kanamycin resistance transposon Tn*5*, was transferred to ANU264, the resultant strain was able to form ineffective nodules on white and subterranean clovers (*Trifolium repens* and *T. subterraneum*, respectively), but not on siratro. However, ANU240 carrying pBR1AN was able to nodulate siratro effectively and formed small ineffective nodules on both clover types. Ekhardt plasmid gels of these strains consistently showed a single plasmid of 130 megadaltons in strain ANU264(pBR1AN), whereas strain ANU240(pBR1AN) showed both the 130-megadalton pBR1AN and the endogenous 200-megadalton plasmid. An analogous result was found by using the plasmid pJB5JI, which codes for the nodulation of peas (7). These results show that when exogenous Sym plasmids are introduced into the heat-cured strain ANU264, the nodulation functions encoded on such plasmids can still be expressed.

The electrophoretic banding patterns of restriction endonuclease-treated total genomic DNA can be used to "fingerprint" strains of bacteria, since for a given endonuclease, and any given genome, the pattern is unique (11). Therefore, to show that strain ANU264 and strain ANU240 were of the same genetic background, total DNA of both strains was treated with the restriction endonucleases *Eco*RI, *Hin*dIII, *Sal*I,

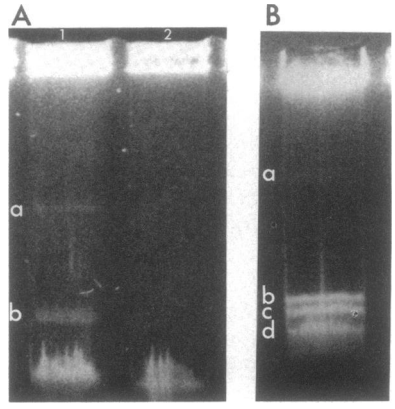

FIG. 1. (A) Visualizations of plasmids. Lane 1, strain ANU240 carrying (a) the endogenous plasmid pNM3AN and (b) the 38-megadalton plasmid RP4 (1), as a size standard. Lane 2, the heat-cured strain ANU264. The procedure used was the crude lysate gel electrophoresis technique of Ekhardt (4). (B) Plasmid profile, using a modified Ekhardt technique, which has resolved two small plasmids of approximately 20 and 25 megadaltons. Bands on the gel are (a) the 200-megadalton Sym plasmid pNM3AN; (b) the 25-megadalton plasmid pNM2AN; (c) the 20-megadalton plasmid pNM1AN; (d) linear chromosomal DNA. Approximately 10^8 early log phase cells grown on tryptone-yeast extract medium at 30°C were washed once in TAE buffer (40 mM Tris–5 mM sodium acetate–1 mM EDTA, pH 7.8) and protoplasted in 50 μl of lysozyme (1 mg/ml)–ribonuclease A (100 μg/ml)–Ficoll-70 (160 mg/ml) in TAE buffer for 10 min at 37°C. Protoplasts were lysed with the addition of 50 μl of sodium dodecyl sulfate (0.02 mg/ml)–Ficoll-70 (160 mg/ml) in TAE buffer. After 5 min at 37°C, 10 μl of proteinase K (5 mg/ml) was added, and digestion was allowed for 10 min at 37°C. Lysates were then carefully loaded onto horizontal 0.6% agarose gels in TAE buffer and electrophoresed for 5 min at 70 mA. The gel was then flooded with buffer to a depth of 2 mm, and electrophoresis continued for 8 h at 45 mA. Gels were stained in 4 μg of ethidium bromide per ml for 10 min and photographed under short-wavelength UV illumination, using Polaroid type 107 film and a Kodak Wrattengelatin filter (no. 23A). All chemicals and reagents were from Sigma Chemical Co., except for proteinase K, which was from Boehringer Mannheim Corp.

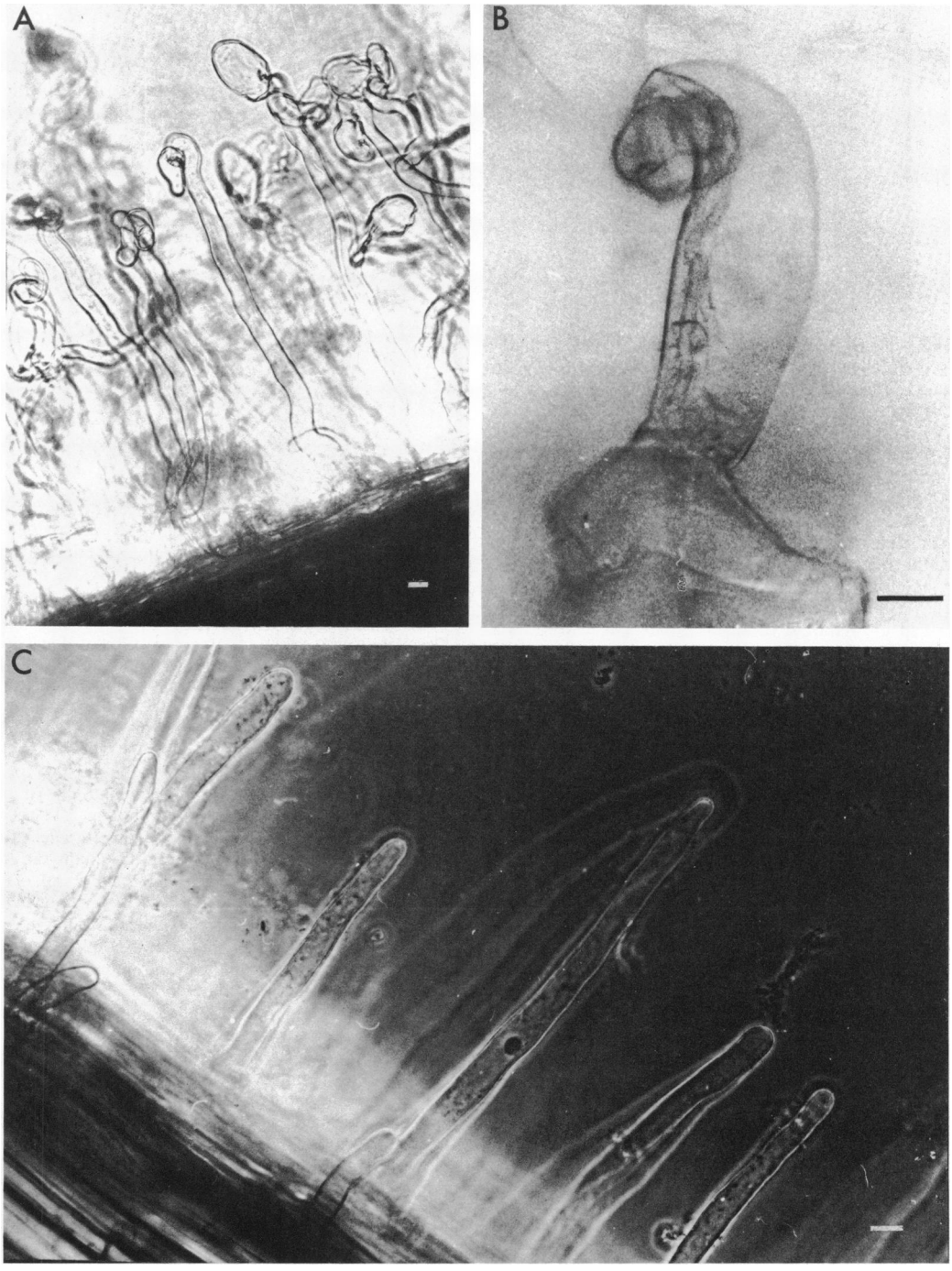

FIG. 2. (A) Root hair curling induced by strain ANU240 on siratro. (B) Infection threads in a root hair of siratro plant inoculated with ANU240. A root nodule subsequently developed at this site. (C) Siratro roots inoculated with strain ANU264. No root hair curling, gross morphological aberrations, or infection threads are present. Plants were grown at 30°C under lighting of 300 μE m^{-2} s^{-1}. Roots were examined after 5 days under phase and Nomaski differential interference contrast, using a Zeiss photomicroscope III. Bar scale is 20 μm in (A) and 10 μm in (B).

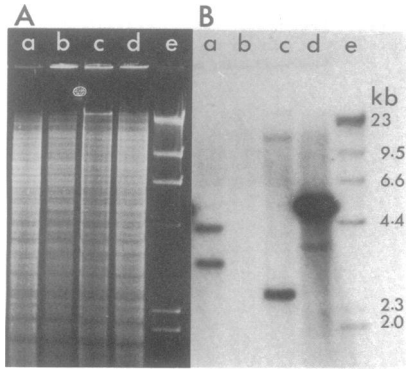

FIG. 3. (A) *Eco*RI restriction endonuclease analysis of total genomic DNA. Lane a, strain ANU240. Lane b, the heat-cured strain ANU264. Lane c, strain ANU264 carrying the plasmid pJB5JI. Lane d, strain ANU264 carrying the plasmid pBR1AN. Lane e, 1 μg of phage λ DNA cut with *Hin*dIII and mixed with radioactively end-labeled *Hin*dIII-cut phage λ DNA. The restriction fragments were resolved in 1.0% agarose gels run at 20 mA for 20 h in TAE buffer. DNA was visualized as described in the legend to Fig. 1. (B) Southern hybridization analysis of DNA in Fig. 1A, probed for specific hybridization to the *R. trifolii nif*H gene. Southern hybridization conditions were as described by Whitfeld et al. (17). The DNA used as a probe was a recombinant pBR322 derivative containing a 3.5-kilobase (kb) segment of the *nif*H region of *R. trifolii* (K. Scott, unpublished data). Radioactivity was incorporated into the probe by the random primer method of Taylor et al. (13). Approximately 10^6 cpm of incorporated label was allowed to hybridize for 12 h at 65°C. Autoradiography was done at −70°C for 24 h, using Kodak type XS-5 X-ray film and intensifying screens.

*Bam*HI, and *Xho*I. Both strains gave identical electrophoretic banding patterns for each endonuclease (Fig. 3A). No change in the banding pattern could be attributed to the lack of the 200-megadalton plasmid in strain ANU264, indicating that this method may not be able to detect such changes in total DNA content. Moreover, strains ANU240 and ANU264 both were found to be immunologically identical to the parent strain, NGR234, using the tube agglutination test as detailed by Vincent (16) (data not shown).

Southern hybridization techniques (12) demonstrated that radioactively labeled DNA from a cloned fragment of the *R. trifolii nif*H gene hybridized to the DNA of strain ANU240 but not to that of strain ANU264. The *nif*H gene region of strain ANU240 appeared to be contained on two *Eco*RI-generated fragments of approximately 3 and 4 kilobases (Fig. 3B). DNA from strains ANU264(pBR1AN) and ANU264(pJB5JI) were included in the analysis as controls, since these plasmids have previously been shown to encode nitrogenase genes (7, 9). A 7-kilobase cloned DNA fragment of a region coding for root hair curling in *R. trifolii* was also used as a probe for Southern analysis. This probe only hybridized to DNA from strain ANU264(pBR1AN), indicating that no homology exists between the hair-curling gene region of *R. trifolii* and DNA from strains ANU240, ANU264, and ANU264(pJB5JI) (data not shown).

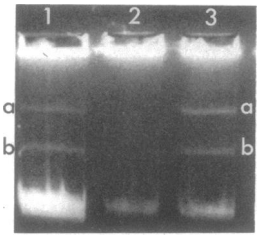

FIG. 4. Mobilization of the Sym plasmid pNM3AN. Lane 1, strain ANU239 (RP1::Tn*501*), a rifampin-resistant, streptomycin- and spectinomycin-sensitive derivative of NGR234. Plasmids are (a) the Sym plasmid pNM3AN and (b) the P1 group plasmid RP1::Tn*501*. Lane 2, strain ANU265, a spectinomycin-resistant derivative of ANU264. Lane 3, plasmids in a streptomycin- and spectinomycin-resistant, rifampin-sensitive, nodulating transconjugant derived from a mating between ANU239 (RP1::Tn*501*) and ANU265. This strain has the Sym plasmid pNM3AN (a) and plasmid RP1::Tn*501* (b). Strain ANU239 (RP1::Tn*501*) was mated on filters with ANU265 for 4 h at 30°C. Total cells were diluted and plated on tryptone-yeast extract medium containing 250 μg of streptomycin, 250 μg of spectinomycin (to select for the recipient ANU265), and 200 μg of kanamycin (to select for the transfer of RP1::Tn*501*) per ml. The transfer frequency of RP1::Tn*501* was 10^{-2}. Total cells on the cross plates were purified twice, en masse, by replica plating onto selective medium. Total cells were washed off the selective plates, and appropriate dilutions were used to inoculate siratro plants. These dilutions were also plated onto rifampin-containing medium to check for the presence of the donor strain. A few rifampin-resistant colonies arose, but when purified they all failed to nodulate siratro and were probably spontaneous mutants of ANU265, as they were also resistant to streptomycin and spectinomycin. Nodules arose only on plants inoculated with high cell numbers of total ANU265 selected cells from the mating, whereas the control ANU239 (RP1::Tn*501*) nodulated well at low cell numbers. The appearance of nodules was delayed in comparison with control experiments done with ANU239(RP1::Tn*501*). The former took 3 weeks for the first appearance of nodules, whereas the latter gave good nodulation within 10 days. Bacteria were reisolated from 38 nodules and tested for their antibiotic resistances. All were streptomycin and spectinomycin resistant, like ANU265, and none was rifampin resistant. Sixteen strains were tested, and all had the same plasmid profile as described above.

restored to the heat-cured strain by reintroducing pNM3AN. This was achieved by mobilizing the Sym plasmid with the P1 group plasmid RP1::TN*501* (8) from ANU239 (a rifampin-resistant NGR234 derivative) to ANU265 (a spectinomycin-resistant ANU264 derivative). Ekhardt plasmid gels showed that the nodulating transconjugants contained both the Sym plasmid pNM3AN and RP1::Tn*501* (Fig. 4). No evidence of stable cointegration of the two plasmids was found. All attempts to show self-transmissibility of pNM3AN were unsuccessful, and no mobilization of pNM3AN by the P1 group plasmid R68:45 (6) was found. The method used is described in the legend to Fig. 4.

These results indicate that the 200-megadalton plasmid of ANU240 is required for the formation of infection threads and root hair curling on siratro and for the nodulation of a range of different legumes and the nonlegume *Parasponia*. The pattern of hybridization of cloned *nif*H DNA to total genomic DNA blots indicates that the genes for the nitrogenase enzyme complex also reside on the 200-megadalton plasmid pNM3AN.

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