Identification and Characterization of Plasmids in Hydrogen Uptake Positive and Hydrogen Uptake Negative Strains of *Rhizobium japonicum*

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**Abstract.** Modifications were made of published procedures to allow routine isolation of plasmids from *Rhizobium japonicum*. The plasmid profiles of a series of H₂ uptake positive and H₂ uptake negative strains were compared. None of the strains of *R. japonicum* with high H₂ uptake activities exhibited discernible plasmids, while most of the strains, with little or no H₂ uptake activity, showed plasmids with molecular weights ranging from approximately 49 - 290 x 10⁶. An examination of H₂ uptake negative mutants derived from an H₂ uptake positive parent revealed two discernible plasmid bands in nonrevertible mutants but no detectable plasmids in revertible mutants or in the parent strain from which mutants were derived.

**Key words:** *Rhizobium japonicum* - Hydrogenase - Endogenous plasmids - Hydrogen uptake - Knallgas reaction

Bacteroids formed by some strains of *Rhizobium* are capable of synthesizing a hydrogenase complex that catalyzes the oxidation of H₂ to H₂O (Dixon 1967, 1968; Schubert and Evans 1976). Strains of *Rhizobium* which possess this characteristic have the ability to recover a portion of the energy expended by nitrogenase-catalyzed H₂ evolution (Dixon 1972; Emerich et al. 1979). In greenhouse and field experiments soybean plants inoculated with groups of hydrogenase-negative (Hup-) strains of *R. japonicum* have been reported to contain a higher percentage of N in their tissues and seeds than plants inoculated with groups of hydrogenase-positive (Hup+) strains (Albrecht et al. 1979; Hanus et al. 1981). As a consequence, we have been interested in determining the factors that control the hydrogen uptake phenotype in *R. japonicum*.

Some evidence supports the view that uptake hydrogenases in certain species of H₂-oxidizing bacteria may be encoded on plasmids. For example, Reh and Schlegel (1975), Schlegel (1976) and Pootjes (1977) have shown that loss of the ability to grow chemolithotrophically and loss of hydrogenase activity were associated with the exposure of *Nocardioida* species and *Pseudomonas facilis* to plasmid curing agents. Furthermore, Reh and Schlegel (1975) demonstrated conjugal transfer of autotrophic capability and NAD-dependent hydrogenase activity between different species of *Nocardioida*. The simultaneous loss of hydrogenase activity and a high molecular weight plasmid after exposure of *Alcaligenes eutrophus* to mitomycin C has been reported by Lim et al. (1980). The transfer of this large plasmid to the mitomycin treated strains resulted in the restoration of hydrogenase activity (Lim et al. 1980). More importantly, the determinants for the uptake hydrogenase in *Rhizobium leguminosarum* strain 128C53 have been shown by Brewin et al. (1980) to be genetically linked to the determinants for nodulation, which appear to be plasmid encoded (Johnston et al. 1978). The uptake hydrogenase of this member of the fast-growing *Rhizobium* species therefore appears to be encoded on a plasmid with a molecular weight of approximately 180 x 10⁶.

There have been extensive studies on the plasmid content and genes encoded on plasmids in the fast-growing *Rhizobium* species (Tshitenga et al. 1975; Prakash et al. 1980; Johnston et al. 1978; Nuti et al. 1977; Beringer 1980; Brewin et al. 1980). Plasmids also have been detected in slow-growing species such as *R. japonicum* (Klein et al. 1975; Prakash et al. 1980) and one extensive study has been made of plasmids in non-mucoid, extra-slow-growing strains of *R. japonicum* (Gross et al. 1979).

The present work was initiated to determine whether a correlation exists between plasmid content and the Hup+ phenotype in *R. japonicum*. The procedure of Currier and Nester (1976) for isolation of plasmids was modified to allow the routine isolation of plasmids from both the non-mucoid, extra-slow-growing and mucoid strains of *R. japonicum*.

**Materials and Methods**

**Bacterial Strains.** *Rhizobium japonicum* strains and their sources are listed in Table 1. The following plasmids of known molecular weights were kindly supplied by Eugene Nester and Frank White of the University of Washington: RP4, molecular weight of 36 x 10⁶ in *Escherichia coli* strain J53; a cryptic plasmid of 60 x 10⁶ in *Salmonella typhimurium* strain LT2; pAr15834a of 107 x 10⁶ and pAt-C58 of about 300 x 10⁶ in *Agrobacterium tumefaciens* strain FHT6; and pAr15834b::Tn5 of 154 x 10⁶ and pAt-C58 in *A. tumefaciens* strain FGT14.

**Growth and Preparation of Bacteria.** *R. japonicum* was grown in a yeast extract, mannitol medium (Hanus et al. 1979) to early log phase (A₅₄₀ of 0.1 - 0.2) unless otherwise indicated. Cell suspensions were incubated in 1 M NaCl by the procedure of Casse et al. (1979) and after collection by centrifugation, washed twice with a volume of TE buffer (50 mM Tris-HCl, 20 mM EDTA, pH 8.0) equal to one-half the original culture volume. Suspensions of cells in TE buffer, in most cases, were frozen in liquid nitrogen and stored at -70°C until used.

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Chemolithotrophically cultured cells were grown in the medium of Repaske and Repaske (1976) which was continuously sparged with H₂, CO₂, and O₂. The procedure for preparation of these cells for plasmid isolation was the same as that described for heterotrophically cultured cells.

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### Preparation of Bacteroids

The procedure for preparation of bacteroids from soybean nodules is that of Evans et al. (1972) with modifications as described by Ruiz-Argueso et al. (1979).

### Purification and Analysis of Plasmid DNA

Unless otherwise indicated, all steps of plasmid isolation were carried out as described by Currier and Nester (1976). Some of the modifications which were incorporated into the method are based on the procedure of Casse et al. (1979). The final procedure which resulted in the most reproducible recovery of plasmids is described below.

Lysis was performed on approximately 2 x 10^10 cells in 16 ml of TE buffer containing 1% (w/v) sodium dodecyl sulfate (Bio-Rad, electrophoresis purity) and 0.5 mg/ml pre-digested pronase (Calbiochem, B grade). The shearing procedure of Currier and Nester (1976) was omitted. Lysates were adjusted to pH 12.3 to 12.4 and incubated for 12 to 18 min with gentle stirring (Currier and Nester 1976) then adjusted to pH 8.5 to 8.6 with 2 M Tris-HCl, pH 7.0 and stirred for an additional 5 to 20 min. After adjustment to 3% (w/v) NaCl, samples were extracted with a one-half volume of phenol (Mallinckrodt, AR, loose crystals) freshly equilibrated with a 3% NaCl solution. Phenol extraction was performed by stirring samples sufficiently to obtain complete mixing for 10 s followed by 2 min stirring at 100 to 150 rev/min then centrifugation at 4,300 x g for 15 min to separate the phases. Extraction of excess phenol from the aqueous phase was performed by adding chloroform equal to one-half the lysis volume and gently inverting each lystate 20 times per min for 2 min. The phases were separated by centrifugation at 4,300 x g for 5 min and DNA in the aqueous phase was precipitated by addition of one volume of cold 95% ethanol followed by incubation overnight at -20°C. The precipitate was collected by centrifugation at 4,300 x g for 15 min at -10°C. Ethanol was removed from each tube and the precipitate redissolved in 0.2 ml of a solution containing 3 parts TES buffer (50 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl, pH 8.0) and one part tracking dye solution composed of 20% Ficoll (Sigma, type 400), 0.2% SDS, and 0.05% bromophenol blue. Each tube was evacuated for 2 min to remove residual ethanol.

The DNA samples were analyzed by agarose gel electrophoresis according to the procedure of Meyers et al. (1976). Gels were stained overnight with 0.5 μg/ml ethidium bromide and visualized on an Ultra-violet Products C61 Transilluminator.

### Hydrogenase Assays

Agar plates of the H₂-uptake medium of Maier et al. (1978a) were inoculated with 0.1 to 0.2 ml of mid-log phase cultures grown in the H₂-uptake medium. Plates were then incubated 5 days in an atmosphere of 1% O₂, 4% H₂, 5% CO₂, and 90% N₂. Cells were washed off the plates with a sterile solution of the H₂-uptake medium without carbon components and further derepressed for 2 days with shaking in the atmosphere listed above. Suspensions were then assayed amperometrically as described by Maier et al. (1978a).

### Results

Several different procedures and combinations of procedures were initially compared for the isolation of plasmid DNA from *Rhizobium japonicum* (Ledeboer et al. 1976; Currier and Nester 1976; Nusba et al. 1977; Hansen and Olsen 1978; Casse et al. 1979). The most reproducible procedure for isolation of plasmid DNA from *R. japonicum* was obtained when early log phase cells were washed by the procedure of Casse et al. (1979) then treated by a modification (see Materials and Methods) of the procedure of Currier and Nester (1976). The series of Hup⁺ and Hup⁻ strains of *R. japonicum* was obtained from four wild-type strains, USDA 117, USDA 120, USDA 3, and USDA 311b, with modifications as described by Casse et al. (1979). The final procedure described by Currier and Nester (1976). Some of the modifications which were incorporated into the method are based on the procedure of Casse et al. (1979). The final procedure which resulted in the most reproducible recovery of plasmids is described below.

Lysis was performed on approximately 2 x 10^10 cells in 16 ml of TE buffer containing 1% (w/v) sodium dodecyl sulfate (Bio-Rad, electrophoresis purity) and 0.5 mg/ml pre-digested pronase (Calbiochem, B grade). The shearing procedure of Currier and Nester (1976) was omitted. Lysates were adjusted to pH 12.3 to 12.4 and incubated for 12 to 18 min with gentle stirring (Currier and Nester 1976) then adjusted to pH 8.5 to 8.6 with 2 M Tris-HCl, pH 7.0 and stirred for an additional 5 to 20 min. After adjustment to 3% (w/v) NaCl, samples were extracted with a one-half volume of phenol (Mallinckrodt, AR, loose crystals) freshly equilibrated with a 3% NaCl solution. Phenol extraction was performed by stirring samples sufficiently to obtain complete mixing for 10 s followed by 2 min stirring at 100 to 150 rev/min then centrifugation at 4,300 x g for 15 min to separate the phases. Extraction of excess phenol from the aqueous phase was performed by adding chloroform equal to one-half the lysis volume and gently inverting each lystate 20 times per min for 2 min. The phases were separated by centrifugation at 4,300 x g for 5 min and DNA in the aqueous phase was precipitated by addition of one volume of cold 95% ethanol followed by incubation overnight at -20°C. The precipitate was collected by centrifugation at 4,300 x g for 15 min at -10°C. Ethanol was removed from each tube and the precipitate redissolved in 0.2 ml of a solution containing 3 parts TES buffer (50 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl, pH 8.0) and one part tracking dye solution composed of 20% Ficoll (Sigma, type 400), 0.2% SDS, and 0.05% bromophenol blue. Each tube was evacuated for 2 min to remove residual ethanol.

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