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Determination of the Hydrogenase Status of Individual Legume Nodules by a Methylene Blue Reduction Assay†

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We adapted a method for the rapid screening of colonies of free-living *Rhizobium japonicum* for hydrogenase activity to determine the hydrogenase status of individual soybean nodules. Crude bacteroid suspensions from nodules containing strains known to be hydrogen uptake positive (Hup^+) caused a localized decolorization of filter paper disks, whereas suspensions from nodules arising from inoculation with hydrogen uptake-negative (Hup^-) mutants or strains did not decolorize the disks. The reliability of the method was demonstrated by its successful application to 29 slow-growing rhizobia. The Hup phenotype on methylene blue filters agreed with that determined amperometrically with either methylene blue or oxygen as the electron acceptor.

The hydrogen uptake (Hup) system possessed by some *Rhizobium* species can potentially increase the efficiency of energy utilization during N_2 fixation and act as a source of O_2 protection of nitrogenase. At least in the case of *R. japonicum* USDA 122DES, the hydrogen uptake system can result in a significant increase in plant yields and nitrogen contents if the plants are grown to maturity and the inoculant strains are otherwise isogenic (Evans et al., Proceedings of the World Soybean Conference, Ames, Iowa, 1985, in press).

The application of recombinant DNA technology to the identification (2), characterization, and transfer (1) of hydrogen uptake genes in *Rhizobium* species requires rapid, reliable methods for determining the hydrogen uptake status of both the free-living bacteria and of nodule bacteroids. Haugland and co-workers (3) have discussed existing techniques for measuring H_2 uptake and have developed a method which is particularly amenable to the rapid screening of large numbers of individual colonies of the free-living bacteria. These authors showed that methylene blue reduction can be used as an indicator of hydrogenase activity, provided that malonic and iodoacetic acids are added to prevent methylene blue reduction by the respiratory electron transport processes.

The goal of the present work was to adapt this methylene blue reduction colony assay to provide a method for determining the hydrogen uptake status of individual soybean nodules. Plant growth and inoculation conditions were analogous to those reported previously (2), and amperometric H_2 determination was as described by Lepo et al. (4). A simple method which can be used to screen hundreds of nodules simultaneously in a relatively short period of time is reported.

Several methods for determining the hydrogen uptake status of nodules by methylene blue reduction in solution or on filters (Whatman, Inc., Clifton, N.J.) were investigated. The time course of the simplest and most successful method is presented in Fig. 1. Nodules were placed on gridded Whatman filters previously impregnated with a solution containing methylene blue and respiratory inhibitors and equilibrated as described by Haugland and co-workers (3) (Fig. 1A). The nodules were then crushed with a flat, hard surface such as the end of a glass rod (Fig. 1A). The crushed cortical tissues of the nodule were removed from the filter with forceps. The pigmented suspension from the nodules caused a slight discoloration of the blue filter which varied in extent with the size of the nodule. In our photograph, this is apparent as a localized darkening of the filter paper (Fig. 1B). A problem encountered in preliminary experiments was that while most of the liquid emanating from the nodule was absorbed by the filter paper beneath the nodule, some liquid emitted under pressure spurted to other portions of the filter paper, which can confuse results in the subsequent assay. This was prevented simply by placing a portion of a plastic cuvette (1 by 1 by 1 cm), open at top and bottom, around each nodule before crushing the nodule (Fig. 1A). In experiments in which it was desired to test hundreds of nodules simultaneously, precautions were necessary to prevent dehydration and buckling of the filters impregnated with the solution containing methylene blue during the time taken to crush all the nodules. By simply replacing the petri dish lids over the open plates containing the filters, dehydration was satisfactorily minimized.

An important difference between this method for nodules and the assay for free-living colonies (3) is that methylene blue must be applied to the filter before the addition of the nodule suspension. Colonies of *Rhizobium* species had sufficient cohesion to resist dispersion when the methylene blue solution was applied to the filter, but in preliminary experiments we found that nodule suspensions were dispersed on the filters by washing with methylene blue solution, invalidating the subsequent assay. For the same reason an attempt to restore the dark blue coloration of the filter after crushing the nodules (Fig. 1B) by adding a drop of methylene blue proved unsuccessful.

After 15 min of equilibration, the filter paper (Fig. 1B) was placed in the screening tray as described by Haugland and co-workers (3). Hup^+ suspensions were clearly visualised by the localized decoloration of the blue filter (Fig. 1C) which was evident by 30 min to 2 h after gassing with H_2 commenced. The most striking difference between Hup^+ and Hup^- soybean nodules was obtained after a 2 to 6 h incubation (e.g., see Fig. 1C). In this experiment, distinctly different Hup phenotypes were tested: SR, PJ17-1, and PJ18-1 (all Hup^+), PJ17 and PJ18 (both Hup^-) (4). Moreover, the hydrogen uptake status of the nodules determined by this

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methylene blue reduction method agreed with that determined amperometrically. Crude bacteroid suspensions from nodules of strains SR, PJ17-1, and PJ18-1 showed hydrogen uptake rates of 442, 424, and 420 nmol/min per mg of protein, whereas PJ17 and PJ18 showed no detectable activity (less than 5 nmol/min per mg of protein). The results were determined amperometrically with oxygen as the acceptor on suspensions prepared from soybean nodules as described by Lepo et al. (4) and represent the means of five determinations.

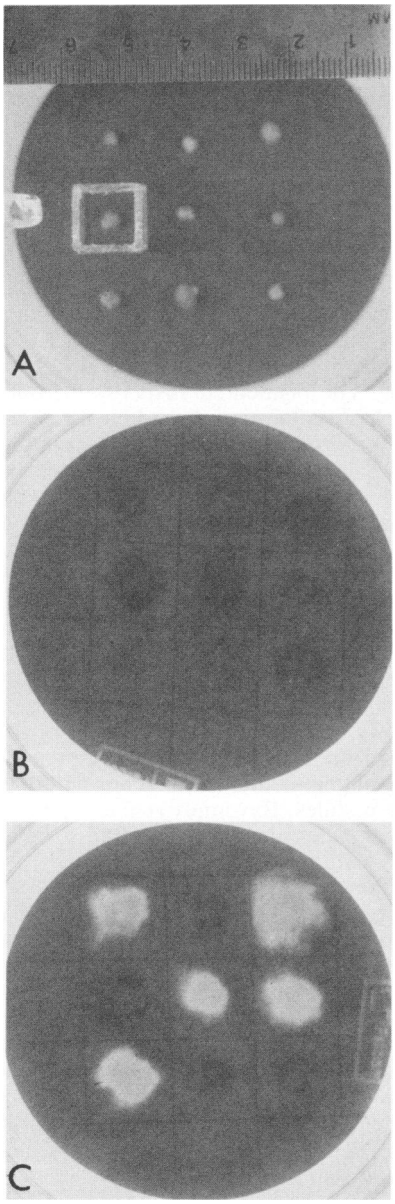

FIG. 1. Time course of differentiation of Hup$^+$ and Hup$^-$ nodule suspensions by the methylene blue assay. (A) Intact nodules were placed on Whatman filters impregnated with methylene blue inhibitor solution. The inoculant *R. japonicum* strains were SR, PJ18, and PJ18-1 (top row, left to right); PJ17, PJ17-1, and SR (middle row); and PJ17-1, PJ18, and PJ18 (bottom row). The plastic wall and glass rod used in crushing the nodule (middle left) are shown. Illumination for photography was from above the filter. (B) Appearance of the filter after the nodules were crushed and before gassing commenced. (C) Appearance of the filter after 4 h of flushing with hydrogen gas. Illumination for photography in panels B and C was from beneath the filter.

TABLE 1. Hydrogenase phenotype of bacteroid suspensions by methylene blue assay and amperometric measurement

Rhizobial strain	Hup phenotype in free-living state[a]	Hup phenotype with methylene blue nodule assay[b]	O_2-dependent hydrogenase activity (nmol · min^{-1} · mg of protein^{-1})[c]	Methylene blue-dependent hydrogenase activity (nmol · min^{-1} · mg of protein^{-1})[d]
USDA 122DES	+	+	618	108
SR1	−	−	<1	ND[e]
SR2	−	−	<1	ND
SR3	−	−	<1	ND
WA5099-1-1	+	+	433	ND
61A24	−	−	<1	ND
61A76	−	−	<1	ND
3I1b6	+	+	599	ND
3I1b142	+	+	674	ND
3I1b143	+	+	707	192
3I1b144	−	−	<1	<1
USDA 16	−	−	<1	ND
USDA 31	−	−	<1	ND
USDA 38	−	−	<1	ND
USDA 62	−	−	<1	ND
USDA 110	+	+	918	385
USDA 117	−	−	<1	<1
USDA 120	±	−	<1	ND
USDA 136	+	+	697	ND
USDA 138	−	−	<1	<1
176A28	+[f]	+	116	942
41Z2	−[f]	−	<1	<1
32H1	+[f]	+	75.2	180.5
61B11	+[f]	+	289.9	204.6

[a] Determined previously with a methylene blue colony assay on the free-living bacteria derepressed for hydrogenase activity (3). +, Hup$^+$ phenotype; ±, weak Hup activity; −, Hup$^-$ phenotype.
[b] Results were consistent for two nodules of each strain tested.
[c] Determined amperometrically.
[d] Determined amperometrically with 200 μM methylene blue.
[e] ND, Not determined.
[f] Inferred from the results of Schubert et al. (5). See text for explanation.

We applied this method to determine the nodule hydrogenase phenotype of 24 other slow-growing rhizobia. The host for strains 176A28, 41Z2, 32H1, and 61B11 was the cowpea California blackeye, and *Glycine max* (cv. Williams 82) was used for all other strains. Plants were harvested after 4 weeks of growth, and fresh nodules were used for the filter assays. The hydrogenase phenotype determined for the nodules in this experiment agreed with the earlier determination of Hup phenotype using the methylene blue assay on colonies of the free-living bacteria (3) (Table 1, columns 2 and 3). The only difference was in the case of *R. japonicum* USDA 120, which showed a weak Hup activity in the free-living state (3) but no detected activity in the present nodule assay (Table 1, column 3). In the case of the four cowpea rhizobia used, the previously determined Hup phenotype (Table 1, column 2) was inferred from the relative efficiency of the cowpea plants (*Vigna unguiculata* [cv. Whippoorwill]) inoculated with these strains (5); it agreed with the nodule phenotype determined here (Table 1, column 3).

The results obtained from the nodule assay agreed with the amperometric determinations on the bacteroid suspensions with oxygen as the acceptor (Table 1, columns 3 and 4) or methylene blue (columns 3 and 5), when tested. Hup$^+$

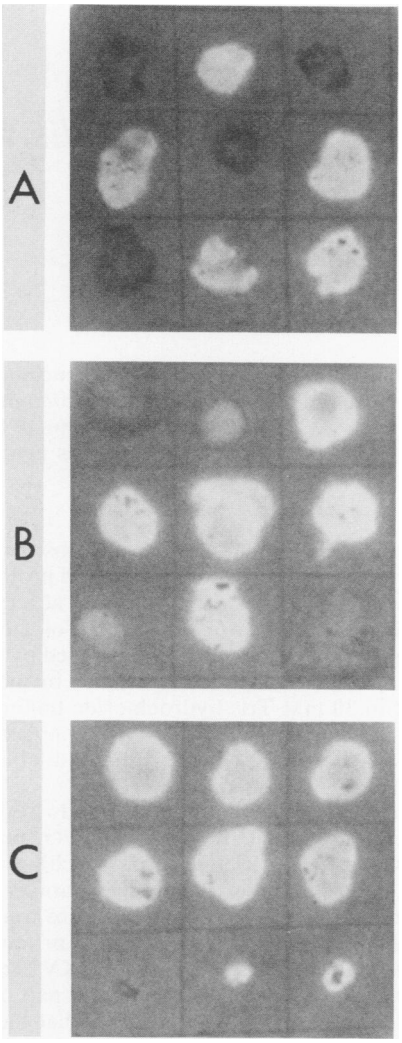

FIG. 2. Determination of the Hup phenotype of nodule suspensions of diverse slow-growing rhizobia. Photographs were taken after 6 h of flushing with hydrogen gas. Illumination was from beneath the filter. (A) Nodule suspensions from soybean (*G. max*). The inoculant *R. japonicum* strains were 61A76, 3I1b6, and USDA 117 (top row, left to right); WA5099-1-1, USDA 16, and USDA 122DES (middle row); and USDA 138, USDA 136, and USDA 110 (bottom row). (B) Nodule suspensions from the cowpea *V. unguiculata* (California blackeye). The inoculant rhizobial strains were 41Z2, 32H1, and 176A28 (top row, left to right); 61B11, 176A28, and 61B11 (middle row); and 32H1, 61B11, and 41Z2 (bottom row). (C) Nodule suspensions from soybeans inoculated with *R. japonicum* USDA 122DES. Larger nodules (top and middle rows) and small nodules (bottom row) were used. Details are described in the text. The nodules used in panels A and B were 2 to 4 mm in diameter before they were crushed.

nodule suspensions were clearly distinguished from Hup^- suspensions for both *G. max* nodules (Fig. 2A) and cowpea rhizobia (Fig. 2B), except in the case of strain 32H1, which showed a weak Hup^+ response in the nodule assay (Fig. 2B) and a low activity (75.2 nmol/min per mg of protein) of O_2-dependent H_2 uptake on the H_2 electrode (Table 1, column 4). This result gives an approximation of the lower limit of Hup activity which can be detected by the nodule assay, but this will, of course, vary with the size of the nodule and the length of the assay.

The nodule Hup assay can also be used to examine a large number of nodules formed by the same strain. The application of the assay to six large nodules (about 3 mm in diameter) of *R. japonicum* USDA 122DES taken from the crown of the root system and three small nodules (about 1 mm in diameter) taken from smaller lateral roots is shown in Fig. 1C. Hup activity is evident for all six large nodules and for two of the three small nodules. An identical assay for *R. japonicum* USDA 110, WA5099-1-1, and 3I1b143 showed the presence of Hup activity in six large nodules (about 3 mm in diameter) from each strain but no detectable activity in each of the three small nodules (about 1 mm in diameter). All nine nodules tested for *R. japonicum* USDA 138 were Hup^-.

This method should prove equally applicable to other legume nodules. Although most fast-growing rhizobia do not possess an active H_2 uptake system, this method can potentially be used to screen the nodule hydrogenase phenotype of genetically altered fast-growing strains and to identify Hup^+ transconjugants against a predominantly Hup^- background. Hundreds of individual nodules can be screened simultaneously in a relatively short period of time without the need for expensive equipment.

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