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Induction of Nitrogenase and Hydrogenase Activities in the Free-Living State of *Bradyrhizobium japonicum*

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Abstract
Effect of components in the medium on the induction of the nitrogen-fixing activity in the free-living state of slow-growing soybean nodule bacteria, *B. japonicum*, was studied using five strains each of Hup+ and Hup-. Nitrogenase activity was expressed regardless of the oxygen concentration in a defined solid medium (GM) while glutamine, malic acid and molybdenum were found to be the essential components for the expression of the nitrogenase activity. Uptake hydrogenase activity was also expressed on the same nitrogenase induction medium (GM). Effect of the medium composition on the expression of the uptake hydrogenase activity was different from that for the nitrogenase activity. Glutamine, malic acid and molybdenum, were not essential for the expression of the uptake hydrogenase activity. This newly developed method on defined solid medium can be used for the evaluation of the nitrogen-fixing activity of strains in *Bradyrhizobium*.

Discipline: Soils, fertilizers and plant nutrition
Additional key words: C_2H_2 reduction, medium, nitrogen-fixing activity, root nodule, slow-growing bacteria, uptake hydrogenase

Introduction

It is generally recognized that root nodule bacteria can not fix nitrogen in the absence of host plants. However, nitrogen-fixing activity has been observed in a medium containing plant cells since 1971[6, 12, 16] and in free-living cultures in a defined medium since 1975[2, 3, 8, 11, 13, 18]. For the induction of the nitrogenase activity, nitrogen source, carbon source, trace elements and coenzymes were necessary under low oxygen conditions. However, the expression of the nitrogenase activity was observed only in a few strains of slow-growing rhizobia such as cowpea and soybean nodule bacteria. The factor for the expression of the nitrogenase activity of slow-growing nodule bacteria in the free-living state remains unclear.

Hydrogen evolution by nitrogenase is considered to be a loss of energy in symbiotic nitrogen fixation[15]. Therefore, the strains carrying an enzyme (hydrogenase) which is capable of oxidizing the evolved hydrogen by the nitrogenase are considered to be valuable rhizobia. This enzyme is designated as uptake hydrogenase and hydrogen cycling is an important parameter for the energy loss in symbiotic nitrogen fixation. Usually, the uptake hydrogenase activity is expressed under aerobic conditions[5, 9, 10, 14] in contrast to the expression of the nitrogenase activity. However, the factors required for the expression of the uptake hydrogenase activity in the free-living state have not been determined.

In these studies, solid media with different compositions were used for the induction of the nitrogenase activity in the free-living state and the effects of nitrogen, carbon and trace elements were studied. The factors for the expression of the nitrogenase and uptake hydrogenase activities in the free-living state were eventually elucidated. The methods of culture could be applied to other strains of *B. japonicum* for the evaluation of the nitrogenase and uptake hydrogenase activities.

Materials and methods

(1) **Organisms :** For the *B. japonicum* Hup^+ strains, A1017, USDA110, JIB70, A1016 and 22-7-2 and for the Hup^- strains IRJ2101, JIR33st., JIF6P, 5-28L and J1014 from the rhizobial collections of the Tokachi Federation of Agricultural Cooperatives (TFAC) were used as materials.

(2) **Media :** LNB5[7] (Table 1) for the uptake hydrogenase activity as well as CS7[11] (Table 2), CS7-Ma-50[17] (Table 2) and GM[17] (Table 3) for the nitrogenase activity were used as induction media. An aliquot of 2 m*l* of each induction medium was injected into a vial 20 m*l* in capacity and sterilized before use.

(3) **Inoculation of rhizobia :** 0.5 m*l* bacterial solution initially cultured in 50 m*l* of yeast extract sucrose (YES) liquid medium[17] for 5 days at 28°C by shaking (130 rpm) was inoculated into each solid induction medium.

(4) **Assay of nitrogenase activity :** Immediately after the inoculation of 0.5 m*l* bacterial culture into the vials, 15% (V/V) of C_2H_2 gas was injected into each vial. The vials were incubated at 28°C for 10 days. The nitrogenase activity was assayed by the acetylene-reducing method and the amount of reduced ethylene by the nitrogenase was determined by gas chromatography (Hitachi-073 type, FID).

(5) **Assay of uptake hydrogenase activity :** Immediately after the inoculation of the bacterial culture into the vials, 5% (V/V) of H_2 gas was injected into each vial, for incubation at 28°C for 10 days. Hydrogen gas concentration in the vials was determined by gas chromatography (Hitachi-063 type, TCD).

Table 1. Components of LNB5 modified medium[7]

$NaH_2PO_4 \cdot H_2O$	0.15	g
$CaCl_2 \cdot 2H_2O$	0.15	g
$MgSO_4 \cdot 7H_2O$	0.25	g
Iron EDTA	0.028	g
$MnSO_4 \cdot H_2O$	0.010	g
H_3BO_3	3.0	mg
$ZnSO_4 \cdot 7H_2O$	2.0	mg
$NaMoO_4 \cdot 2H_2O$	0.25	mg
$CuSO_4 \cdot 5H_2O$	0.04	mg
$CoCl_2 \cdot 6H_2O$	0.025	mg
KI	0.78	mg
Inositol	0.1	g
Thiamine-HCl	0.01	g
Nicotinic acid	1	mg
Prydoxal-HCl	1	mg
Sucrose	0.5	g
L-arabinose	1.0	g
Sodium gluconate	0.5	g
Sodium glutamate	0.5	g
Yeast extract	0.1	g
Agar	12.0	g
Distilled water	1.0	*l*

Adjusted to pH 6.8 with concentrated HCl.

Table 2. Components of CS7 and CS7-Ma-50 media

Component	Medium			
	CS7[11]		CS7-Ma-50[17]	
KH_2PO_4	0.3	g	0.3	g
$CaCl_2 \cdot 2H_2O$	0.1	g	0.1	g
KCl	0.067	g	0.067	g
$MgSO_4 \cdot 7H_2O$	0.035	g	0.035	g
Glutamine	0.29	g	0.29	g
Myo-inositol	1.0	g	1.0	g
Na-succinate	6.75	g		
DL-malic acid			6.75	g
L-arabinose	3.75	g		
Mannitol			9.1	g
$MnSO_4 \cdot 4H_2O$	8.7	mg	8.7	mg
H_3BO_4	5.0	mg	5.0	mg
$ZnSO_4 \cdot 7H_2O$	1.0	mg	1.0	mg
KI	1.0	mg	1.0	mg
$CuSO_4 \cdot 5H_2O$	0.2	mg	0.2	mg
$NaMoO_4 \cdot 2H_2O$	0.1	mg	0.1	mg
$CoCl_2 \cdot 6H_2O$	0.1	mg	0.1	mg
$FeSO_4 \cdot 7H_2O$	15.0	mg	15.0	mg
Na-EDTA	22.2	mg	22.2	mg
Thiamin-HCl	5.0	mg	5.0	mg
Nicotinic acid	5.0	mg	5.0	mg
Prydoxal-HCl	0.5	mg	0.5	mg
Agar	15.0	g	15.0	g
Distilled water	1.0	*l*	1.0	*l*

CS7 medium was adjusted to pH 6.8 with HCl and CS7-Ma-50 medium was adjusted to pH 6.8 with concentrated NaOH.

Table 3. Components of GM medium[17]

K_2HPO_4	0.5	g
$MgSO_4 \cdot 7H_2O$	0.2	g
NaCl	0.1	g
$Na_2MoO_4 \cdot 2H_2O$	0.1	mg
$FeSO_4 \cdot 7H_2O$	15.0	mg
Glutamine	0.3	g
DL-malic acid	6.75	g
Mannitol	10.0	g
Agar	15.0	g
Distilled water	1.0	*l*

Adjusted to pH 6.8 with NaOH.

(6) **Oxygen concentration in vials :** Oxygen concentration in each vial during the induction of the nitrogenase activity was determined also by gas chromatography (Hitachi-063 type, TCD).

(7) **Dry cell weight :** Cell weight in each vial 10 days after the incubation was determined as follows : 5 m*l* of a 0.85% NaCl solution was added into each vial, and 1.5 m*l* of suspension cells was pipetted out. The dry cell weight was determined gravimetrically with a chemical balance after removal of the cells by centrifugation at 6,000 rpm, for 10 min and drying at 28°C for 5 days.

Results

1) Nitrogenase activity

Nitrogenase activity for each of the five strains of Hup^+ and Hup^- on four different media under aerobic conditions is shown in Table 4. In the LNB5 medium, the nitrogenase activity was not observed in any of the strains and the CS7 medium induced a very low nitrogenase activity in all the strains. However, the CS7-Ma-50 and GM media induced a high nitrogenase activity in all the strains used.

Differences in the components between the CS7 and CS7-Ma-50 media were related to the carbon source ; the CS7 medium contained arabinose and succinate, while the CS7-Ma-50 medium contained mannitol and malic acid.

These observations indicated that carbon sources were the most important factor for the expression of the nitrogenase activity. Furthermore, the CS7-Ma-50 medium contained many inorganic components such as cobalt, copper and coenzymes, while the GM medium did not contain inorganic components and coenzymes except for molybdenum and iron. These results suggest that the addition of many inorganic components and coenzymes except for molybdenum and iron is not necessary for the induction of the nitrogenase activity. Expression of the nitrogenase activity was observed under aerobic conditions in the CS7-Ma-50 and GM media. Therefore, it is suggested that the oxygen concentration in the culture vial was not related to the expression of the nitrogenase activity.

Thereafter, the effects of the elimination of individual components from the GM medium were examined (Table 5). Elimination of mannitol and

Table 4. Effect of various media on the acetylene-reducing activity and growth of rhizobia

Strain	Medium			
	LNB5	CS7	CS7-Ma-50	GM
A1017	0.0[a]	1.6±0.33	187±30.2	255±27.6
	(1.8±0.17)[b]	(3.1±0.05)	(3.2±0.04)	(3.0±0.09)
USDA110	0.0	1.3±0.37	151±41.0	226±43.2
	(1.9±0.14)	(3.2±0.08)	(3.2±0.07)	(3.2±0.03)
JIB70	0.0	0.0	23±7.0	28±5.3
	(1.8±0.18)	(3.2±0.19)	(3.0±0.04)	(2.9±0.11)
A1016	0.0	4.0±2.2	393±29.9	302±46.5
	(1.4±0.12)	(3.0±0.07)	(2.8±0.03)	(2.6±0.14)
22-7-2	0.0	4.1±5.7	226±25.3	166±18.9
	(1.5±0.13)	(2.9±0.12)	(2.4±0.07)	(2.5±0.11)
IRJ2101	0.0	1.3±0.2	293±121	188±45.7
	(2.4±0.10)	(2.9±0.03)	(3.0±0.08)	(2.9±0.04)
JIR33st.	0.0	5.1±2.3	124±16.3	66±16.8
	(2.4±0.28)	(2.7±0.07)	(2.9±0.19)	(2.9±0.19)
JIF6P	0.0	0.8±0.4	260±21.0	235±27.9
	(2.6±0.09)	(3.5±0.02)	(3.1±0.01)	(3.1±0.05)
5-28L	0.0	6.0±3.2	111±29.2	85±20.7
	(1.9±0.13)	(3.4±0.05)	(3.3±0.04)	(3.1±0.03)
J1014	0.0	3.0±0.6	96±23.3	87±19.0
	(2.1±0.10)	(3.1±0.33)	(2.5±0.06)	(2.3±0.05)

Results are expressed as a value (mean ± SE) for five replicate cultures under aerobic conditions.
a) : Value is expressed as nmol of C_2H_2 per 10 days in 1 mg on a dry cell weight.
b) : Value is expressed as mg of whole dry cell weight during the 10 day incubation period per vial.

Table 5. Effect of GM medium components on the nitrogenase induction and growth of rhizobia

Removal of components in medium	Strain	
	A1017	JIF6P
Complete medium	129±11.1[a]	120±24.1
	(3.3±0.10)[b]	(2.7±0.05)
(−) Glutamine	1±0.4	1±0.1
	(2.0±0.17)	(2.0±0.26)
(−) Mannitol	126±14.9	153±9.3
	(3.2±0.06)	(2.3±0.40)
(−) Malic acid	0.0	0.0
	(4.0±0.08)	(4.1±0.25)
(−) Molybdenum	8±3.4	2±0.7
	(3.3±0.19)	(3.1±0.09)
(−) Iron	186±49.8	61±19.5
	(3.3±0.19)	(3.3±0.10)

See the footnotes of Table 4.

iron from the GM medium reduced the nitrogenase activity. However, a low nitrogenase activity was observed by the elimination of glutamine or molybdenum, and no nitrogenase activity was observed when malic acid was removed. Therefore it is concluded that glutamine, molybdenum and malic acid are essential for the induction of the nitrogenase activity. Since the growth of the cells was slow in the glutamine-free medium, glutamine appears to be an essential component for cell growth in *B. japonicum*. This observation suggests that symbiotic nitrogen-fixing bacteria such as *B. japonicum* fix a very small amount of nitrogen in the free-living state compared with the non-symbiotic nitrogen-fixing bacteria, so that *B. japonicum* can not grow sufficiently by nitrogen fixation without the supply of a nitrogen source. In other words, nodule bacteria can not fix a large amount of nitrogen in the free-living state for their cell growth. Therefore, energy (ATP) and reduction force can not be supplied for the induction of the nitrogenase activity in sufficient amounts in the free-living state.

In the medium where molybdenum was removed, the bacteria grew in almost the same way as in complete GM medium, while the nitrogenase activity was very low. Therefore, molybdenum appears to be the most important element for the induction of the nitrogenase activity in the free-living state. Malic acid was also an essential

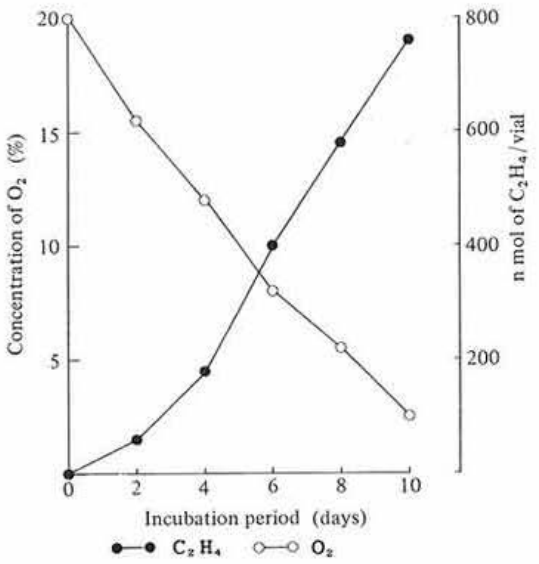

Fig. 1. Acetylene-reducing activity and oxygen consumption of A1017 strain during incubation period using vial under aerobic conditions

component for energy (ATP) and reduction force allocation to nitrogenase. In the medium where malic acid was removed, cell growth was more active than in the GM complete medium, but the nitrogenase activity was not induced.

The nitrogenase activity and oxygen concentration during the incubation of the bacteria in the GM medium under aerobic conditions containing 15% acetylene are shown in Fig. 1. The nitrogenase activity was induced at a high oxygen concentration in the gas phase, and the nitrogenase activity increased with the period of incubation. Therefore, it was possible to induce the nitrogenase activity under aerobic conditions in the GM induction medium.

2) Uptake hydrogenase activity

Uptake hydrogenase activity of each of the five Hup^+ and Hup^- strains observed on four different media under aerobic conditions is shown in Table 6. None of the Hup^- strains were able to take up hydrogen on any of the induction media, while all the Hup^+ strains took up hydrogen.

The effect of additional components on the induction of the uptake hydrogenase activity was tested using the Hup^+ strain A1017 and Hup^- strain JIF6P on the GM medium. The effects of the removal of

Table 6. Effect of various media on the uptake hydrogenase activity and growth of rhizobia

Strain	Medium			
	LNB5	CS7	CS7-Ma-50	GM
A1017	0.0[a)]	0.0	0.0	2.26±0.25
	(2.0±0.13)[b)]	(3.1±0.05)	(3.1±0.06)	(3.1±0.06)
USDA110	0.0	0.0	0.0	1.78±0.13
	(2.2±0.41)	(3.1±0.09)	(3.2±0.08)	(3.1±0.08)
JIB70	0.0	0.0	0.0	1.74±0.11
	(2.0±0.11)	(3.0±0.03)	(3.0±0.08)	(2.9±0.09)
A1016	0.0	0.0	0.0	2.74±0.18
	(1.8±0.15)	(3.0±0.20)	(2.7±0.09)	(2.6±0.08)
22-7-2	0.0	0.0	0.0	2.69±0.11
	(1.9±0.14)	(3.1±0.12)	(2.5±0.08)	(2.5±0.07)
IRJ2101	0.0	0.0	0.0	0.0
	(2.4±0.10)	(3.1±0.15)	(3.2±0.23)	(2.9±0.09)
JIR33st.	0.0	0.0	0.0	0.0
	(2.4±0.07)	(2.6±0.09)	(3.0±0.12)	(3.0±0.15)
JIF6P	0.0	0.0	0.0	0.0
	(2.6±0.10)	(3.5±0.03)	(3.1±0.10)	(3.2±0.06)
5-28L	0.0	0.0	0.0	0.0
	(2.1±0.19)	(3.4±0.08)	(3.4±0.05)	(3.3±0.13)
J1014	0.0	0.0	0.0	0.0
	(2.0±0.07)	(3.2±0.17)	(3.0±0.02)	(2.9±0.03)

a): value is expressed as μmol of H_2 per 1 day in 1 mg on a dry cell weight.
As regards the others, see the footnotes of Table 4.

Table 7. Effect of GM medium components on the uptake hydrogenase activity and growth of rhizobia

Removal of components in medium	Strain	
	A1017	JIF6P
Complete medium	1.25±0.18[a)]	0.0
	(3.6±0.01)[b)]	(3.1±0.13)
(−) Glutamine	1.51±0.25	0.0
	(2.4±0.11)	(2.2±0.02)
(−) Mannitol	1.13±0.16	0.0
	(3.4±0.01)	(3.0±0.08)
(−) Malic acid	2.13±0.26	0.0
	(3.8±0.01)	(4.0±0.02)
(−) Molybdenum	1.71±0.18	0.0
	(3.4±0.05)	(3.0±0.08)
(−) Iron	1.76±0.24	0.0
	(3.5±0.06)	(3.0±0.05)

See the footnotes of Table 6.

individual components of the medium on the uptake hydrogenase activity are shown in Table 7. Hup$^+$ strain A1017 took up hydrogen on all the media when individual components of the complete GM medium were removed, while the Hup$^-$ strain JIF6P did not exhibit the activity in any of the media. As described above, glutamine, molybdenum and malic acid were essential components for the induction of the nitrogenase activity, unlike for the induction of the uptake hydrogenase activity.

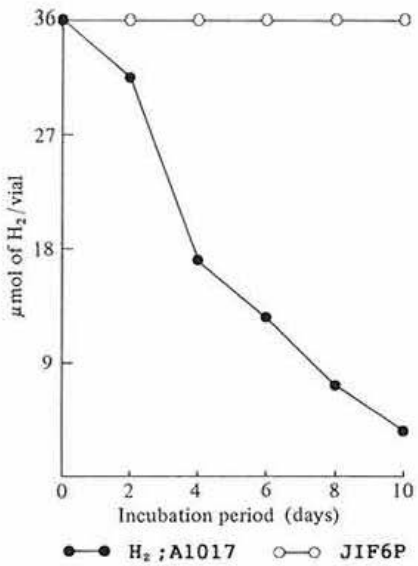

Fig. 2. Uptake hydrogenase activity of A1017 (Hup$^+$) and JIF6P (Hup$^-$) strains during incubation period using vial under aerobic conditions

Hydrogen uptake was observed at a high level on

the medium lacking glutamine regardless of the small amount of cell growth and very low induction of nitrogenase activity. This observation indicates that the induction of the uptake hydrogenase activity was not related to cell growth and that presumably energy (ATP) was produced by the hydrogen uptake and the energy was used for cell growth and for the uptake hydrogenase activity.

Hydrogen uptake was also observed on a medium lacking molybdenum which was not involved in the structure of the uptake hydrogenase protein in contrast to the nitrogenase proteins. Hydrogen uptake was also observed on the media lacking malic acid or mannitol as a sole source of energy (ATP) and reduction force of hydrogen uptake.

Time course of hydrogen uptake during the incubation observed using the Hup^+ strain A1017 and Hup^- JIF6P in vials is shown in Fig. 2. In a Hup^+ strain A1017 for which hydrogen gas had been added to the incubation vial, hydrogen was taken up into the bacterial cells and maximum hydrogen uptake was observed 4 days after incubation. On the contrary, a Hup^- strain JIF6P did not show the uptake hydrogenase activity during the incubation.

Discussion

Although the oxygen concentration is one of the major limiting factors for the expression of the nitrogenase activity in symbiotic root nodules by *B. japonicum*, the oxygen concentration was not an essential factor in the free-living state when defined solid induction media were used (Table 4). The absence of limitation of the oxygen concentration for nitrogen fixation in the new technique developed here may be ascribed to the fact that since rhizobial cells grow on the surface of a solid induction medium covered with a liquid medium, the cells can grow without being in direct contact with oxygen in the gas phase. It can be considered that the oxygen concentration of the rhizobial cells on the growing surface of the solid medium decreased by the covering with the liquid medium and the nitrogenase activity could be expressed without damage.

For the expression of the nitrogenase activity, it is necessary to supply glutamine as a nitrogen source, malic acid as a carbon source, molybdenum and iron, but not other trace elements and coenzymes. These elements can be considered to act as follows : the nitrogen source such as glutamine is used for cell growth ; the carbon source is used for a respiration substrate such as malic acid to supply ATP and the reduction force ; molybdenum and iron are used for the components of FeMo protein in the nitrogenase.

In the bacteroids growing inside of the root nodules, rhizobia can grow and fix nitrogen using organic acid instead of sucrose[1, 4]. For the induction of the nitrogenase activity in the free-living state, organic acid is also an essential element as a sole carbon source and malic acid was found to be the most effective.

For the expression of the uptake hydrogenase activity in the free-living state of *B. japonicum*, the addition of many inorganic components and coenzymes was necessary as previously reported[5-9, 10, 14]. However, the uptake hydrogenase activity was expressed in the free-living state on simple media such as GM medium. The uptake hydrogenase activity therefore, may also be expressed on the yeast extract mannitol (YEM) medium and it is not necessary to add trace elements such as molybdenum, iron, coenzymes and organic acids such as malic acid.

Recently there have been few reports on the induction of the nitrogenase and uptake hydrogenase activities in the free-living state of slow-growing *B. japonicum*. The experiments, we carried out, however indicate that the nitrogenase and uptake hydrogenase activities in the free-living state of slow-growing *B. japonicum* can be determined on an induction medium by the use of a simple GM medium. This induction technique can be applied to evaluate the ability of *B. japonicum* strains to express the nitrogenase and uptake hydrogenase activities without nodule formation if the activities in the free-living state are correlated with the symbiotic nitrogen fixation.

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