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In vitro and *in vivo* nitrogenase activity of *Rhizobium* mutants and their symbiotic effectivity

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Abstract. A slow growing nitrogen-fixing strain of *Vigna radiata* var. *aureus* (mung bean) *Rhizobium* which expressed nitrogenase activity in a synthetic medium was isolated from its native population. Mutants with decreased and increased nitrogenase activity were derived from this strain by treatment with acridine orange and ethidium bromide. These mutants were tested for symbiotic effectivity in *vivo*. The effectivity of mutants with decreased nitrogenase activity in the culture medium was lower than the parent strain; however, the effectivity of mutants with higher nitrogenase activity did not increase above that of the parent. This suggests that the plant is perhaps a limiting factor in the full expression of rhizobial nitrogenase in the nodules.

Keywords. Nitrogenase activity; symbiotic effectivity; inoculation; mutant strains; nodulation; nitrogen fixation.

Introduction

In legume-*Rhizobium* symbioses, variabilities in symbiotic effectiveness which are either due to variations in nitrogen fixing potential of Rhizobium strains (Brockwell *et al*., 1968; Chatel and Parker, 1973; Dadarwal *et al*., 1974, 1979) or due to host genotypic compatibility (Nutman, 1969; Brockwell and Katznelson, 1976), are often observed. Since the nitrogen-fixing genes are known to be confined to the bacterial genome (Gibson, *et al.,* 1976), the host effects are mainly on symbiotic establishment and on controlling the induction and expression of nitrogenase in the nodules. The variability in the effectiveness of native *Rhizobium* isolates even on a single cultivar of a legume crop, gives the impression that the nitrogen-fixing ability of *Rhizobium* could be improved either by strain selection or by genetic manipulation. Since the nitrogen-fixing ability is expressed only in symbiotic association, it is not possible to find out whether the restriction on the bacterial gene expression is due to the bacterial genome or the plant. Recently, methods for inducing the nitrogenase under culture conditions in some slow growing *Rhizobium* strains have been reported (Child and Kurtz, 1978; Wilcockson and Werner, 1978; Kennedy and Pankhurst 1979). Having standardized the conditions for the expression of nitrogenase in a culture medium by a mung bean-*Rhizobium* strain, S24, we have derived mutants with increased and decreased nitrogenase activity as compared with the parent strain. In this paper, we report the symbiotic behaviour of these mutants in relation to the parent strain.

Materials and methods

The *Rhizobium* strain, S24, was isolated originally from the root nodules of mung bean (*Vigna radiata* var. *aureus*) cvr. Varsha. The strain, maintained on yeast extract-mannitol-agar (Vincent, 1970), was inoculated into 50 ml of filtered yeast extract-mannitol broth in 150 ml capacity flasks containing 10 µg/ml of acridine orange and ethidium bromide in separate flasks. The flasks were incubated on a shaker for 4 days at 28 ± 1°C, subcultured in the same broth with added mutagens and incubated for a further 6 days. The broth culture was then diluted and plated on yeast extract-mannitol-agar medium plates and the plates were incubated for 10 days at 28 ± 1° C. Colonies that appeared on the plates were selected and screened for nitrogenase activity on yeast-extract-mannitol-glutamate-succinate-agar slants (g/L:yeast extract, 0.2; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1; $CaCO_3$, 0.5; mannitol, 10; sodium glutamate, 0.32; sodium succinate, 1.6; agar 15; pH 7.2). The parent S24 strain showed high nitrogenase activity when grown on this medium. The procedure for determining nitrogenase activity on agar slants and its interpretation is the same as described earlier (Dadarwal *et al.*, 1980).

Selected mutant clones with higher and lower nitrogenase activity than the parent strain on yeast extract-mannitol-glutamate-succinate-agar medium, were further tested for their nitrogenase activity on media containing sugars, amino acids and citric acid cycle intermediates in different combinations.

Nodulation and nitrogen fixation

Four selected mutants were tested along with the parent strain for nodulation and nitrogen-fixing ability on mung bean var. K851. Surface sterilized seeds were inoculated with 4-day old broth culture (5 r 10^5 to 10^6 viable cells/seed) and sown in sterilized Leonard jars containing Sloger's N_2-free mineral salt solution (Sloger, 1969). Uninoculated seeds were sown as controls. Nodulation, nodule nitrogenase activity, plant weight and plant nitrogen content were determined at 15, 25 and 35 days of plant growth. Nitrogenase activity in intact nodules was determined by gas chromatography using the C_2H_2 reduction technique of Hardy *et al.* (1968). The plants were dried, weighed and the plant nitrogen determined by the conventional micro-Kjeldahl method. The ratios of plant weight and plant nitrogen were then calculated from control values.

The parent strain S24 has an electron recycling hydrogenase system (Dadarwal *et al.*, 1980). The hydrogenase in intact nodules of all the strains was also determined indirectly by determining the C_2H_2 reduction rates, both in the presence and absence of 0.1 atm. hydrogen (Emerich *et al.*, 1979).

Results

Selection of mutants with increased or decreased nitrogenase activity

A total of 2,000 clones were screened for nitrogenase activity on yeast extract-mannitol-glutamate-succinate-agar medium. All the clones obtained had nitrogenase activity on this medium. However, clones with higher and lower activity than the parent strain were observed at about 1% frequency. Two clones each with nitrogenase activity lower (S24A06, S24EB 12; 90% less) and higher

(S24A044, S24A050; 30% more) than the parent strain S24, were then selected for detailed cultural and symbiotic studies. Table 1 shows the nitrogenase activity of these strains in media containing various sugars, citric acid cycle intermediates and amino acids. The two mutants S24A06 and S24EB12 which showed low activity on yeast extract-mannitol-glutamate-succinate-agar medium, also showed comparatively low activity on all the media tested. Contrary to this, the mutant S24A044 and S24A050 showed constantly higher activity.

Table 1. Nitrogenase activity of Vigna rhizobium S24 and its mutants under culture conditions

Medium	nmol C_2H_2 reduced/h/mg cell protein				
	S24	S24EB12	S24A06	S24A044	S24A050
YEMGA[a]	3.2	4.0	3.4	4.2	4.2
a +Fumarate	82.9	6.8	8.2	116.7	124.2
a +Malate	114.2	8r2	8.4	134.2	127.4
a +Succinate	72.6	5.4	6.2	89.2	95.2
a +Pyruvate	60.3	8.4	8.6	79.2	75.2
a +Glutarate	56.5	6.2	6.4	74.5	73.2
Sugar substitutes[b]					
Galactose	120.6	5.4	6.0	142.2	128.4
Mannose	151.0	10.2	12.5	182.4	170.2
Glucose	89.4	6.5	6.4	104.4	110.3
Amino acid substitutes[c]					
Aspartate	78.4	6.2	5.4	114.2	120.2
Proline	112.2	5.2	5.2	150.2	135.4
Lysine	86.2	5.2	5.4	112.4	104.2
Serine	72.0	6.2	5.4	96.4	98.4
Glutamine	89.2	8.2	8.4	104.6	115.4
Asparagine	90.4	8.4	8.8	116.4	104.2

The citric acid cycle intermediates, sugars and amino acids were added in the YEMGA medium at 10, 55 and 2 mM concentrations, respectively. No activity was detected in media containing citrate, arabinose, xylose and ribose. C_2H_2 was injected after 10 days of growth in tubes (air: C_2H_2, 9:1). The assay tubes were further incubated for 24 h and C_2H_2 formed was analysed.

[a] Yeast extract-mannitol-glutamate-agar medium.

[b] To yeast extract-glutamate-fumarate agar medium, the sugars indicated were added.

[c] To yeast extract-glucose the amino acids indicated were added.

In media containing different levels of succinate (0 to 60 mM) the mutants S24A06 and S24EB12 showed lower activity, whereas, S24A044 and S24A050 showed high activity as compared with S24 at all the concentrations of succinate (table 2).

Table 2. Effect of succinate levels on nitrogenase activity of *Vigna* rhizobium S24 and its mutants under culture conditions.

Succinate (mM)	nmol C_2H_2 reduced/h/mg cell protein				
	S24	S24EB12	S24A06	S24A044	S24A050
0	3.5	4.0	3.9	5.2	4.4
5	45.4	6.9	7.4	52.4	39.7
10	86.2	12.1	7.1	112.4	104.2
20	95.4	14.4	13.4	123.8	115.7
40	84.2	16.6	13.2	116.4	114.8
60	74.2	14.9	12.9	98.2	79.2

Succinate was added to yeast extract-manitol-glutamate-agar medium.

Table 3 shows a comparison between the mutants and the parent, of nodule nitrogenase activity, nodule weight, plant weight and plant nitrogen ratios at different stages of growth. In all the strains, except at 15 days of plant growth, the nodule weight per plant was comparable with the parent strain indicating no change in host infectivity. The two mutants S24A06 and S24EB 12, with low nitrogenase activity under cultural conditions, showed nodule nitrogenase activity as well as plant weight and nitrogen ratios lower than the parent strain. This might mean that the nitrogenase activity in the culture medium has a close relationship with the *in vivo* nitrogen-fixing capacity of the strains. However, nodule nitrogenase activity, plant weight and plant nitrogen content of strains having increased nitrogenase activity in culture medium, were comparable with those with the parent strain S24. This shows that perhaps the plant system does not allow the full expression of the higher nitrogen-fixing capacity of these bacteria- inside nodules.

The parent strain S24 has also a hydrogen-oxidizing hydrogenase system (Dadarwal *et al.*, 1980). Emerich *et al* (1979) observed that nitrogenase activity in a bacteroid suspension of *R. japonicum* having such a hydrogenase system, is increased substantially in 10% H_2 atm. This increase in C_2H_2 reduction rates per unit weight of nodules in a hydrogen atmosphere could be taken as a measure of the hydrogenase system. When detached nodules of plants inoculated with the different strains were incubated in a 10% H_2 atm, enhanced rates of C_2H_2 reduction were observed in all the cases (table 4). This showed that the hydrogenase systems in nodules of the mutants were unaltered and that the low nitrogen fixation in the two mutants with lower activity was not due to the lack of an electron recycling system.

Table 3. Comparative effectivity of *Vigna* rhizobium S24 and its mutants on green gram var. K851.

Plant age (days)	Observation	Strains tested				
		S24	S24EB12	S24A06	S24A044	S24A050
15	Nitrogenase activity [a]	30.7	6.4	11.9	30.5	29.3
	Nodule weight [b]	6	6	10	7	12
	Shoot weight ratio [c]	1.74	1.11	1.58	1.74	2.21
	Shoot N ratio [d]	1.92	1.20	1.49	1.87	1.98
25	Nitrogenase activity	167.2	37.7	10.3	180.7	189.2
	Nodule weight	36	33	32	39	29
	Shoot weight ratio	2.39	1.63	1.47	2.50	2.45
	Shoot N ratio	2.41	1.54	1.52	2.46	2.44
35	Nitrogenase activity	211.6	35.7	28.8	179.7	182.8
	Nodule weight	63	68	57	56	60
	Shoot weight ratio	3.68	2.02	2.05	3.60	3.80
	Shoot N ratio	3.82	2.21	2.12	3.71	3.81

[a] Nitrogenase activity of intact nodules expressed as µmol C_2H_2 reduced/h/g dry weight of nodules.

[b] Nodule weight is expressed as mg/plant.

[c] $\frac{\text{Plant dry weight in inoculated}}{\text{Plant dry weight in control}}$

[d] $\frac{\text{Plannt nitrogen in inoculated}}{\text{Plant nitrogen in control}}$

The ratios are calculated from average values of 15 plants.

Table 4. Effect of added hydrogen on nitrogenase activity of detached nodules of mung bean inoculated with different strains after 25 days of plant growth.

Rhizobium strains	µmol C_2H_4 reduced/g fresh nodule weight			
	without hydrogen		with hydrogen	
	4 h	24 h	4 h	24 h
S24	4.04	5.09	6.87	7.45
S24EB12	2.74	3.74	3.66	5.07
S24A06	1.41	2.49	1.91	3.41
S24A044	4.07	5.08	6.24	7.41
S24A050	4.66	4.86	6.24	8.33

The values are the cumulative values at 4 and 24 h after injecting the C_2H_2. The nodules were incubated in air: C_2H_2 (9:1) and air: C_2H_2 : H_2 (8:1:1) atmosphere without and with H_2, respectively.

Discussion

A limited number of slow growing strains of *R. japonicum* and *Rhizobium sp*. 'cow pea group' are known to express nitrogenase activity under culture conditions (Child and Kurz, 1978; Wilcockson and Werner, 1978; Kennedy and Pankhurst, 1979). However, none of these workers have correlated the N_2-fixing ability of the strains with the nitrogenase activity under the free living conditions. One of the reasons for this is that all the *Rhizobium* strains infecting a particular legume do not express nitrogenase activity under culture conditions in the same medium.

In the present study mutants with decreased and increased nitrogenase activity were derived from a N_2 fixing strain. These mutants were not defective in nodulation. Strains S24A06 and S24EB12 with decreased nitrogenase activity under free living conditions were less effective than the parent strain in nitrogen fixation (table 3). However, contrary to expectations, the mutants S24A044 and S24A050 with increased nitrogenase activity did not show increased nitrogen fixation in the host as compared with the parent strain, although studies with different levels of succinate in the culture medium showed that both the strains had a higher specific activity of nitrogenase than the parent (table 2). The limitations, therefore, could exist in the host system where the nitrogenase potential of the rhizobial strain is not expressed beyond a certain limit. However, this does not rule out the possibility of better effectiveness of these strains in other genotypes of the same host, which have not been tested.

The nitrogenase-dependent H_2 evolution is one of the major reasons for low effectivity of rhizobia in legume symbiosis (Albrecht *et al*., 1979). The parent strain S24 as well as the four mutants possess the H_2 recycling system (table 4). Therefore, the low effectivity of the mutants S24A06 and S24EB12 was not due to a change in this enzyme system. This is the first report that correlates symbiotic effectivity with nitrogenase activity under culture conditions, showing limitations offered by the plant system to the expression of nitrogenase in nodules. However, it is possible that *in vitro* nitrogenase activity may not be an indicator of *in vivo* performance. Similar studies need to be carried out with other strains known to express nitrogenase in free living conditions.

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