Screening for Mutants of *Rhizobium japonicum* with Defects in Nitrogen Fixing Ability

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Mutants of *Rhizobium japonicum* with reduced *ex planta* nitrogenase activity could be isolated with high frequency by direct screening of ultra-violet mutagenized bacteria growing as spots on the surface of an appropriate agar medium permitting derepression of nitrogenase synthesis. Small glass chambers fitted with a serum cap were pushed into the agar around each spot of growth, forming a small enclosed gas space which was made to 10% acetylene, permitting assessment of nitrogenase activity by the acetylene reduction test. Four mutants were isolated from a total of 305 screened spots. Two mutants had almost no *ex planta* activity, one of which had no symbiotic activity despite normal nodulation (ineffective), the other had only somewhat reduced activity symbiotically. Two other mutants with less than half wild-type activity *ex planta* were normal in symbiosis.

Introduction

It has been suggested that direct screening of small cultures of rhizobia growing on air exposed agar surfaces could be used to identify mutants with defects in nitrogen fixation [1]. The isolation of mutants with defects leading to a failure in the normal differentiation of the symbiosis between *Rhizobium* and legume is a promising approach to a better understanding of this economically very important symbiosis. The failure of wild-type rhizobia to support their own growth by the nitrogen they fix, precludes the possibility of replica-plating from agar plates containing a nitrogen source to those lacking one, as can be done for identifying mutants in *Klebsiella* and *Azotobacter*.

Rhizobium japonicum 61-A-101 growing as a 10–15 mm spot on an appropriate agar surface, will reduce acetylene at 5–10% of the rate of the same strain in liquid medium under optimal conditions. The latter generally means having a gas atmosphere with low oxygen content passing over the medium. The ability of rhizobia to fix nitrogen under air, when growing as a small mass, indicates a measure of self-protection, since liquid cultures do not fix under air. The fact that the rapidly growing *Klebsiella pneumoniae* will fix at up to 30% of the maximum anaerobic rate when exposed to air as a spot of growth on agar [2], is probably a reflection of the rapid respiration and consequent oxygen consumption by this organism, relative to the much slower growing *R. japonicum*. Acetylene reducing ability of up to seven spots of growth on the surface of agar in an ordinary (∅ 90 mm) petri dish, can be very quickly measured by pushing a glass ring fitted with a serum cap into the agar around the spot and making the atmosphere to 10% acetylene. After a short incubation time, ethylene production by nitrogenase activity can be assessed by gas chromatography. Nitrogen fixing ability is a multigene function [3] and thus a relatively large target for a mutagenizing treatment. Since several hundred cultures can easily be measured in a few hours with the method described, we considered that the chance of detecting mutants with defects in nitrogen fixing ability was reasonable.

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Methods

Organism, media and growth. Rhizobium japonicum 61-A-101 was maintained and grown in liquid culture in medium 20E [4]. Medium for the assessment of nitrogenase activity contained (per litre): $MgSO_4 \cdot 7\,H_2O$, 370 mg; $CaCl_2 \cdot 2\,H_2O$, 73.5 mg; $Na_2MoO_4 \cdot 2\,H_2O$, 4.84 mg and $FeSO_4 \cdot 7\,H_2O$, 6.96 mg as an EDTA complex with 9.3 mg Na_2EDTA. In addition, the medium which was solidified with 1.5% agar contained 50 mM D-arabinose, 20 mM-Na_2 succinate and 1 mM-L-glutamine. The medium was buffered by the addition of one tenth volume of 0.5 M phosphate buffer (0.5 M-NaH_2PO_4 plus 0.5 M-

K_2HPO_4 at pH 5.8). Salts and arabinose were autoclaved separately as 10-fold concentrated solutions. L-glutamine sterilized by autoclaving as an 0.2 M solution was found to be the best fixed N-source for nitrogenase activity [5] despite the fact that it undergoes extensive decomposition on autoclaving [6]. Na_2 succinate was autoclaved as a 1 M solution. Independently sterilized components were added to hot 3% agar and the medium (which had a final pH of 6.05–6.15) dispensed to give 28 ml per plate.

Mutagenizing and subsequent procedure. Logarithmic phase bacteria grown in medium 20E were pelleted by centrifugation, washed once with water and resuspended at $1-2 \times 10^8$ cells · ml^{-1} in water. A 20 ml aliquot of this suspension was exposed to ultra-violet radiation (low pressure mercury vapour lamp; dose rate 0.35 µW · mm^{-2}) and appropriate dilutions after increasing exposure to the radiation were plated on medium 20E agar. The plates were incubated in the dark at 28 °C for 10 days. Plates with colonies from the survivors of 7 and 10 min exposure (corresponding to 5% and 0.2% survival respectively) were carefully examined. Using sterile wooden toothpicks 200 average sized colonies were picked off the plates and used to inoculate 200 small liquid medium 20E cultures. The small cultures were grown into stationary phase and 0.05 ml used to inoculate the agar surface of the medium for assessment of nitrogenase activity.

Assessment of nitrogenase activity. The construction of the small chambers and the inoculation of the agar surface to give up to seven small spots of growth per plate has been described previously [1]. Nitrogenase activity was measured by the acetylene reduction test in 0.5 ml gas samples taken from the chambers after incubation at 28 °C for 2–4 h as described by Wilcockson and Werner [2].

Nodulation and symbiotic nitrogen fixation. Seeds of *Glycine max* var. Caloria were surface sterilized and germinated as described by Werner and Stripf [7]. The root system of each seedling was infected for 10 min in a suspension of 5 ml bacteria in medium 20E (2×10^7 cells · ml^{-1}) to which had been added 5 ml of 0.15% agar. Each seedling was planted in a clay pot (∅ 80 mm) containing sterilized "Superlite 01" and grown as described by Werner *et al.* [4]. After 21 d, 4 h after the beginning of the 14 h light period. plants were uprooted and the root systems only incubated in test tubes (60 ml) under an atmosphere of 10% acetylene in air and closed with a serum cap. After 1 h at 28 °C in the dark, 0.5 ml gas samples were removed and tested gas chromatographically for nitrogenase activity as described by Wilcockson and Werner [2]. Finally the nodules were picked from the plants, counted and weighed.

Results and Discussion

After 10 d of growth, the approximate time of peak activity for the wild-type strain, nitrogenase activity by the acetylene reduction test was measured on 195 spots of growth, each derived from a single survivor of the mutagenizing treatment. It was arbitrarily decided to retain for further investigation the 10% of the spots with the lowest activity. These were used to inoculate liquid medium 20E and were once again spotted on to the glutamine containing solid medium. Activity was mesured in eight parallel spots of each culture, four on day 8 and four on day 12. As a criterion it was decided to retain only spots which showed 50% or less of wild-type activity on both days. Only three spots remained, which were again grown up in liquid medium and each culture used to inoculate seven plates, each with

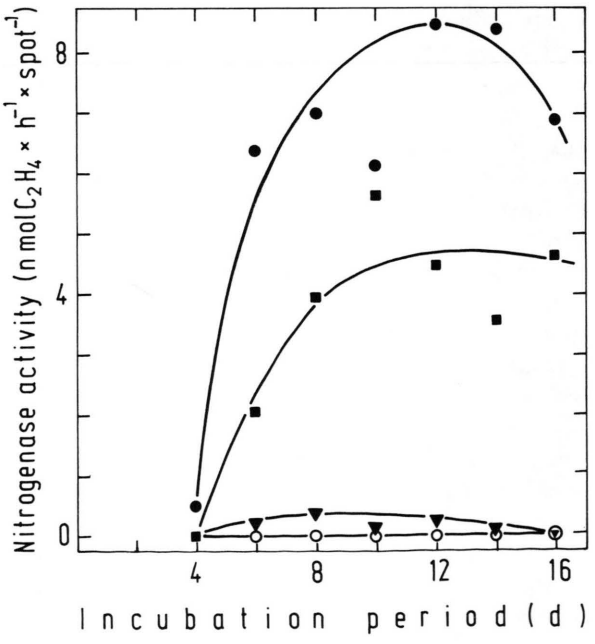

Fig. 1. *Ex planta* nitrogenase activity of *Rhizobium japonicum* on agar surfaces exposed to air. Wild-type strain 61-A-101 (●), mutant RH61 (■), mutant RH93 (▼), mutant RH31 (○).

Table I. Nodulation and nitrogenase activity of *Glycine max* var. Caloria infected with *Rhizobium japonicum* 61-A-101 (wild-type) and mutants RH 61, RH 93 and RH 31.Data averaged from two experiments with 15–20 plants per strain.

	Nodulation		Nitrogenase activity	
	Fresh weight of nodules per plant [mg]	Average fresh weight of single nodule [mg]	nmol $C_2H_4 \cdot h^{-1}$ $plant^{-1}$	nmol $C_2H_4 \cdot h^{-1}$ mg fresh $weight^{-1}$ of nodules
WT	112.3	4.6	1170	8.9
RH 61	108.2	3.68	1317	11.7
RH 93	131.5	2.5	660	4.7
RH 31	75.1	3.82	0	0

four spots. The nitrogenase activity of these three strains over the period 4–16 days of growth (activity in four parallel spots on one plate for each strain being measured on every second day from day four) can be seen in Fig. 1.

One mutant only, RH31, gave no activity throughout the whole screening procedure. RH93 had very low activity, only detectable between 8 d and 12 d of growth. The variation experienced in the measurement of the nitrogenase activity, suggests that other mutants of similar activity to RH61 with 50–60% of wild-type activity could easily be passed over in the arbitrary rejection of 90% of the spots in the first stage of the identification procedure and even in the second step where 50% of wild-type activity was set as the upper limit for retention of the spots for further characterization.

The growth of the mutants was not distinguishable from that of wild-type. All had a doubling time of about 11 h in medium 20E at 28 °C. Of the mutants isolated, only RH31 emerges as a "classical" ineffective strain when tested for nitrogenase activity after symbiosis development (Table I). Nodulation was almost as efficient as with the parental strain but on sectioning it was immediately obvious to the eye that the nodules found after infection of soybeans with RH31 lacked leghaemoglobin pigment. Equivalent size and age nodules from infection by the wild-type are orange-pink in section. Interestingly the mutant RH93 which had less than 2% of the total nitrogenase activity of the wild-type strain *ex planta* (over the period 3–17 d), has half the activity of the wild-type in symbiosis. It was also noted that RH93 seemed to be slightly changed in its nodulation characteristics, making a larger number of somewhat smaller nodules than the wild-type. RH61 was indistinguishable from wild-type in symbiosis.

It is perhaps not surprising that the frequency with which mutants with reduced ability to fix nitrogen in the *ex planta* system used here is so high since, in addition to the relatively large target size of the *nif* gene region, it is clear that "peripheral" defects could impair the ability of air exposed cells to fix nitrogen. RH61 could for example have some small change in respiratory activity relatively unimportant for growth. In a separate experiment a mutant (J 1) apparently similar to RH61 was isolated from 110 spots after ultra-violet mutagenizing. Like RH61 it was normal in symbiotic association but from Table II it can be seen that its *ex planta* nitrogenase activity varied with glutamine concentration in the medium in a quite different way from the wild-type.

Thus although mutants with altered *ex planta* nitrogenase activity can be easily screened for by direct measurement of the activity on plates with the small chamber method, it is clear that not all of these will have defects in the *nif* gene region. Some may have respiratory or substrate permeability changes which could drastically affect *ex planta* nitrogen fixation but give nearly normal symbiotic activity. Others such as RH31 have no nitrogenase activity under any conditions and illustrate that

Table II. Ex planta nitrogenase activity of *Rhizobium japonicum* (wild-type) and mutant J1 growing on media with different glutamine concentrations. Peak specific activity (nmol $C_2H_4 \cdot h^{-1}$ mg $protein^{-1}$).

Glutamine in medium [mM]	Wild-type	Mutant J1
0.5	0.047	3.290
0.75	6.497	3.653
1.00	7.618	3.581
1.25	5.689	2.493
1.50	4.825	2.260
2.0	4.242	0.513

direct screening for such mutants in *Rhizobium*, which it is hoped will lead to a better understanding of the differentiation of the *Rhizobium*-legume symbiosis, is not nearly so tedious as might be supposed.

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