Nitrogenase Activity of *Rhizobium japonicum* Growing on Agar Surfaces in Relation to Slime Production, Growth and Survival

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A reproducible, simple method for assessing nitrogenase activity by the acetylene reduction test on agar plates is described. The effects of phosphate, succinate and arabinose on nitrogenase activity and slime production by *R. japonicum* strain 61-A-101, growing on a solid medium with leucine or glutamine as sole source of fixed nitrogen, were investigated. Slime production varied with different media but there was no simple correlation between slime production and high nitrogenase activity. Arabinose greatly enhanced nitrogenase activity of bacteria growing on glutamine as sole nitrogen source, especially if the amino acid concentration was 1 mM or less. Without arabinose, the optimum concentration of glutamine for nitrogenase activity was 2 to 2.5 mM, but 1 mM-glutamine plus 50 mM-arabinose gave twice the activity [8 to 10 nmol ethylene produced h^{-1} (mg protein) $^{-1}$].

INTRODUCTION

Although all purified nitrogenase preparations are extremely sensitive to oxygen and are irreversibly inhibited by it (Zumft & Mortenson, 1975), many organisms, in addition to the so-called aerobic nitrogen fixers, can fix nitrogen aerobically when growing in a sufficiently thick layer on an agar surface. So-called aerobic N_2 -fixing organisms have special mechanisms for protecting their nitrogenase against the potentially damaging effects of oxygen (Dalton & Postgate, 1969). So-called anaerobic N_2 -fixing organisms, provided that they are able to grow in air, can fix nitrogen when grown aerobically on agar surfaces at rates which are significant fractions of the best anaerobic rates (Wilcockson & Werner, 1976).

Immunological studies (Bishop *et al.*, 1975) indicated that free-living rhizobia were able to synthesize a component of nitrogenase, and nitrogenase activity was subsequently demonstrated in free-living bacteria growing on an air-exposed agar surface (Pagan *et al.*, 1975; Kurz & LaRue, 1975; McComb *et al.*, 1975). Previously, nitrogenase activity had been observed only in what appeared to be loose associations of rhizobia with tissue cultured plant cells (Holsten *et al.*, 1971; Child & LaRue, 1974; Philips, 1974; Child, 1975; Scowcroft & Gibson, 1975). Studies of rhizobia grown in defined liquid media showed that a low concentration of dissolved oxygen is required for development of nitrogenase activity (Keister, 1975; Tjepkema & Evans, 1975). Much of this work used the *Rhizobium* strain 32H1 of the cowpea nodulating group of rhizobia, which is less exacting in its requirements for the development of nitrogenase activity *ex planta* than many other strains. An extensive study of growth and nitrogenase activity of strain 32H1 grown on agar surfaces has been reported (Gibson *et al.*, 1976). With the soybean nodulating *Rhizobium japonicum* strain 311b85, the effect of 14 proteinogenic amino acids as sole nitrogen source on the development of nitrogenase activity in a defined medium has been reported (Werner, 1976).

However, the methods used, in which agar contained in small bottles was surface inoculated with bacteria, gave poor reproducibility. We report here the activity, growth and slime production of the relatively slow-growing *Rhizobium japonicum* strain 61-A-101 growing, not in small containers as in most published work with other strains, but on the surface of agar in disposable Petri dishes. The simple, reproducible method described for assessing nitrogenase activity should be useful for screening for mutants of rhizobia defective in nitrogenase activity.

METHODS

Organism, media and growth. *Rhizobium japonicum* strain 61-A-101, from the Nitragin Company, Milwaukee, U.S.A., was maintained on medium 20E solidified with agar (Werner *et al.*, 1975) and grown in 100 ml of liquid medium 20E at 28 °C in 300 ml Erlenmeyer flasks on a rotary shaker. Solidified medium for induction of nitrogenase activity contained (per litre): $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 370 mg; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 73.5 mg; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 4.84 mg; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 6.96 mg as an EDTA complex with 9.3 mg Na_2EDTA (i.e. the 20E salts without KNO_3 and phosphate); mannitol, 1.82 g, glycerol, 4.6 g; and agar, 15 g. The salts, as a 10-fold concentrated solution, were added to 100 ml of sterilized hot 3 % agar. Mannitol and glycerol were added as 10- or 100-fold concentrated solutions. Other additions for particular experiments were: 0.1 M-L-leucine, 0.2 M-L-glutamine, 0.5 M-D-arabinose and phosphate buffer (0.5 M- NaH_2PO_4 plus 0.5 M- K_2HPO_4 at pH 5.8). All additions to the agar were as sterile solutions, separately autoclaved, including double-distilled water added to give a final volume of 200 ml. Equal portions of the medium were then dispensed into eight Petri dishes. The final pH of the medium was 6.05 to 6.15. Before use, plates were dried either overnight at 28 °C or for 1 to 2 h at 50 °C. They were inoculated with bacteria in early stationary phase in 20E medium by means of an automatic pipette (Eppendorf, Hamburg, W. Germany) using alcohol-sterilized tips to dispense 0.05 ml (1×10^8 to 2.5×10^8 organisms) in a spot (0.7 to 1.0 cm diam.) at four approximately equally spaced positions on the agar surface.

Assessment of nitrogenase activity. Small chambers consisting of a glass ring (internal diam. 2.0 cm, height 2.3 cm) capped with a Suba-Seal serum cap were pushed into the agar around each spot of bacterial growth on a plate. Care was taken not to disturb the cell mass, and a syringe needle which pierced the serum cap to avoid a pressure build-up while the chamber was pushed into the agar surface was subsequently removed. From each chamber, using a 1 ml syringe and needle, 0.3 ml of trapped air was taken out and replaced by 0.3 ml of acetylene. This gave a final acetylene concentration of 10 % (v/v) under which the bacteria were incubated for 2 to 6 h. Nitrogenase activity was measured by the acetylene reduction test on 0.5 ml gas samples as described by Wilcockson & Werner (1976).

Measurement of growth and slime production. After removing the four chambers from one plate, the circles of agar cut out by the glass rings were arranged in a small beaker and 5 ml distilled water was added. Bacteria were removed from the agar surface and evenly suspended by a standardized procedure drawing the liquid in and out of a 5 or 10 ml syringe and needle. The suspension was filtered through nylon gauze with 10 μm pores (Vereinigte Seidenwebereien, Krefeld, W. Germany). The procedure was repeated and the filtrates were pooled. The turbidity of appropriate dilutions was determined using an EEL nephelometer, and a total cell count was made in a counting chamber (Helber, W. Germany). A viable count of the suspensions was estimated from colony-forming ability on 20E agar. The relative viscosities of undiluted suspensions from different stages of growth and on different media were estimated by comparing the fall-time of the meniscus in a fine bore pipette (Wilcockson & Werner, 1976; Wilcockson, 1977). The dry weight of bacteria was determined by drying 5 ml of undiluted suspension at 105 °C (16 to 24 h) in a previously heated and weighed 'boat' of aluminium foil. Results were corrected for any material washed from the agar during resuspension, and other sources of error, by subtracting a value obtained from uninoculated agar circles cut from the same agar and treated in a similar manner to the bacterial suspension procedure. Bacterial protein was measured using the method of Lowry *et al.* (1951).

From each experiment, two colonies from two plates used for viability counts were picked off and the suspensions obtained after growth in 20E medium were used to test the ability of the bacteria to nodulate soybeans. A positive nodulation test was taken as evidence that the effects found in the experiment were due to activities of the original *Rhizobium japonicum* strain.

RESULTS

Both glutamine and leucine as sole sources of fixed nitrogen supported growth of *Rhizobium japonicum* strain 61-A-101, but at different rates (Fig. 1*a, b*). Optimum growth was obtained with 35 mM-leucine and 5 mM-glutamine. At concentrations above 5 mM,

Fig. 1. Growth of *R. japonicum* 61-A-101 with leucine or glutamine as sole source of fixed nitrogen. In addition to the basic medium constituents (see Methods), the agar plates contained 50 mM-phosphate and 10 mM-succinate with either (a) leucine at 5 mM (○), 10 mM (■), 20 mM (●), 35 mM (△) or 50 mM (▲), or (b) glutamine at 0.5 mM (○), 1 mM (●), 2 mM (△), 2.5 mM (▲, two experiments), 5 mM (□), 10 mM (■) or 20 mM (▼).

Fig. 2. Nitrogenase activity of *R. japonicum* 61-A-101 growing on agar containing leucine or glutamine as sole source of fixed nitrogen. In addition to the basic medium constituents (see Methods), the agar plates contained 50 mM-phosphate and 10 mM-succinate with either (a) leucine at 5 mM (○), 10 mM (■), 20 mM (●), 35 mM (△) or 50 mM (▲) or (b) glutamine at 0.5 mM (○), 1 mM (●), 2 mM (△), 2.5 mM (▲), 5 mM (□), 10 mM (■) or 20 mM (▼). Nitrogenase activities are expressed as nmol ethylene produced h^{-1} (mg protein) $^{-1}$.

Fig. 3

Fig. 4

Fig. 3. Colony-forming ability of *R. japonicum* 61-A-101 growing on agar containing 50 mM-phosphate and 10 mM-succinate with either leucine at 10 mM (○) or 20 mM (△) or glutamine at 10 mM (●) or 20 mM (▲). The percentages of the total cells at d 14 which were found to be colony formers at these concentrations of nitrogen source were, respectively, 100% and 74.7% with leucine and 54.4% and 14.4% with glutamine.

Fig. 4. Slime production measured as the relative viscosity (meniscus fall time in s) of suspensions of *R. japonicum* 61-A-101 grown on agar containing 50 mM-phosphate and 10 mM-succinate with either 10 mM-leucine (▼) or glutamine at 0.5 mM (○), 1 mM (●), 2 mM (△), 2.5 mM (▲) or 10 mM (■).

glutamine inhibited growth. Nitrogenase activity at the optimum concentration of glutamine (2.0 to 2.5 mM) was higher than with the optimum concentration of leucine (10 mM) (Fig. 2*a,b*). Higher concentrations of leucine induced nitrogenase activity earlier in the growth cycle, but such early induction was not seen with glutamine at any concentration tested.

At the two highest concentrations of leucine tested, the viability at 14 d was less than 50%; at the other concentrations survival was between 70 and 100%. At the optimum concentration of glutamine for nitrogenase activity, viability never exceeded 75%. Glutamine at 10 and 20 mM resulted in a loss of colony-forming ability in the later stages of the growth cycle (Fig. 3). The relative viscosities (estimated as meniscus fall times) of suspensions of bacteria grown on agar containing more than 2 mM-glutamine were very low throughout growth (Fig. 4). By the time 1 mg protein per spot was formed, the meniscus fall time was between 5 and 6 s (water = 4.5 s). The equivalent value for bacteria grown on 10 mM-leucine was over 13 s (Fig. 4) and approached 20 s (see Fig. 6). Suspensions of bacteria grown on agar containing 0.5 or 1 mM-glutamine gave intermediate values (Fig. 4).

Effect of phosphate and succinate concentrations

With leucine as the sole source of fixed nitrogen, the effect of increasing the phosphate concentration was to delay the onset of nitrogenase activity (Fig. 5). This effect was most marked with 75 and 100 mM-phosphate which also resulted in a lower specific activity of nitrogenase. Inhibition of growth did not occur; indeed, growth increased from 1.4 mg protein per spot (at 12.5 mM-phosphate) to 2.65 mg (at 100 mM-phosphate) after 14 d.

Fig. 5. Effect of phosphate concentration on the nitrogenase activity of *R. japonicum* 61-A-101 growing on agar containing 10 mM-leucine and 10 mM-succinate with phosphate at 12.5 mM (○), 25 mM (■), 50 mM (●), 75 mM (△) or 100 mM (▲). Nitrogenase activities are expressed as nmol ethylene produced h⁻¹ (mg protein)⁻¹.

Table 1. Growth and nitrogenase activity of *R. japonicum* 61-A-101 on agar surfaces at different succinate concentrations with leucine or glutamine as the sole source of fixed nitrogen

Organisms were grown on the basic agar medium (see Methods) plus 50 mM-phosphate, various concentrations of sodium succinate and either 10 mM-leucine or 2 mM-glutamine. Nitrogenase activities are expressed as nmol ethylene produced h⁻¹ (mg protein)⁻¹. The time at which peak activity occurred is given as d after inoculation. Growth is expressed as mg protein per spot after 14 d.

Succinate in medium (mM)	Leucine			Glutamine		
	Peak activity	Time of peak activity (d)	Growth	Peak activity	Time of peak activity (d)	Growth
2.5	0.59	10	2.36	3.53	10	2.00
5	0.75	12	2.02	4.30	14	2.28
10	1.46	12	1.91	4.49	10	2.39
20	2.37	12	1.39	5.05	10	2.10
40	1.91	12	1.12	2.82	6	1.56

In subsequent work, 50 mM-phosphate was used as this was the highest buffer concentration that did not inhibit nitrogenase activity.

When the concentration of succinate was increased from 2.5 to 40 mM, with leucine as the sole source of fixed nitrogen, there was a steady decrease in growth (Table 1). However, with 2.0 mM-glutamine as sole source of fixed nitrogen, the succinate concentration for optimum growth was 10 mM. With either amino acid in the medium, 20 mM-succinate was optimal for nitrogenase activity.

When the phosphate concentration was varied, with 10 mM-leucine in the medium, the fraction of the total organisms in each spot that were able to form colonies after 14 d was always between 70 and 100%. Concentrations of succinate above 20 mM always resulted in a considerable loss of colony-forming ability, which seemed to be additive to the loss caused by a high amino acid concentration. However, the quantification of this effect was difficult especially for 40 mM-succinate (see Discussion).

High concentrations of both succinate and phosphate decreased slime production of organisms grown on a medium which would otherwise render them very slimy. A bacterial growth of 1 mg protein per spot gave suspensions with meniscus fall times in the range 17 to 20 s when growth was on 10 mM-succinate or less (Fig. 6). Higher concentrations of succinate gave lower values of 12.5 s at 20 mM and 7 s at 40 mM. With 10 mM-leucine as fixed nitrogen source and succinate at 10 mM, the corresponding values when the phosphate concentration was varied were about 20 s for 50 mM or less, and 11 and 6.5 s for 75 and 100 mM, respectively.

Fig. 6. Effect of succinate concentration on slime production measured as the relative viscosity (meniscus fall time in s) of suspensions of *R. japonicum* 61-A-101 grown on agar containing 10 mM-leucine and 50 mM-phosphate with succinate at 2.5 mM (○), 5 mM (■), 10 mM (●), 20 mM (△) or 40 mM (▲).

Table 2. *Effect of arabinose on nitrogenase activity and growth of R. japonicum* 61-A-101 on agar surfaces

Organisms were grown on the basic agar medium (see Methods) plus 50 mM-phosphate with the nitrogen source and other additions indicated. Nitrogenase activities are expressed as nmol ethylene produced h^{-1} (mg protein) $^{-1}$. Growth is expressed as mg protein per spot after 14 d.

Fixed nitrogen source	10 mM-succinate		20 mM-succinate + 50 mM-arabinose		2 mM-succinate + 50 mM-arabinose		10 mM-succinate + 50 mM-arabinose	
	Peak activity	Growth	Peak activity	Growth	Peak activity	Growth	Peak activity	Growth
Leucine (10 mM)	2.18	1.57	2.91	1.59				
Glutamine (2 mM)	3.17	2.91	4.58	2.81	1.99	1.80	4.51	2.67
Glutamine (1 mM)	0.86	1.65	9.16	1.33	0.97	1.02	9.17	1.53

Effect of arabinose concentration

With 10 mM-leucine as fixed nitrogen source, arabinose in the range 12.5 to 50 mM barely increased the growth or nitrogenase activity and did not change the relative viscosity of suspensions or colony-forming ability. For each of the amino acids at their optimum concentration for nitrogenase activity, a comparison was made between the conditions under which the optimum was determined (50 mM-phosphate, 10 mM-succinate) and what seemed likely to be the optimum conditions (50 mM-phosphate, 20 mM-succinate, 50 mM-arabinose) (Table 2). A clear increase in nitrogenase activities occurred under the latter regime but the size and the variation encountered rendered it impossible to decide whether or not the effects of 20 mM-succinate and 50 mM-arabinose were additive. However, in the same series of experiments we included plates containing 1 mM-glutamine with 10 mM-succinate (without arabinose) or with 20 mM-succinate and 50 mM-arabinose. The latter combination increased the specific nitrogenase activity approximately 10-fold to about twice that for 2 mM-glutamine with or without additional succinate and arabinose. This unexpected finding has its origin, not in a differential effect of succinate, but in the presence of arabinose itself (Table 2). The pentose had only a minor effect when 2 mM-glutamine was the fixed nitrogen source but a large one when glutamine was present at 1 mM.

DISCUSSION

Using rhizobia growing on the surface of agar in small bottles we found poor reproducibility in our data obtained for nitrogenase activity. The main reason for this became clear when we began to use the methods described here which gave an apparent standard deviation for acetylene reduction between spots on the same agar plate of better than 20%.

Occasionally a spot gave a value very much lower than its companions. Invariably such a spot proved to be disturbed by the glass ring and would, in time, spread around the inside of the ring. This spreading of the cell mass only occurred when the bacteria were growing on a medium which tended to produce slime (i.e. 10 mM-leucine or less and unmodified by the addition of high phosphate or succinate). On such media the spot had a blueish-white, streaked appearance and was often surrounded by a halo of clear slime. Growth under conditions giving low relative viscosities, as measured by the meniscus fall times, formed more compact yellowish cell masses with no halo and had a dry appearance. Clearly, as suggested by Gibson *et al.* (1976), the integrity of the cell mass is very important for the nitrogenase activity of these air-exposed systems. During growth on the surface of agar in small bottles, the extent of wall contact and also the time when it occurs are both quite variable events. The effect of disturbing nitrogen-fixing *Klebsiella* growing on an agar surface is much less serious (Wilcockson, unpublished observations); this probably reflects the difference in the respiration rates of the two organisms since the rapidly respiring, fast-growing *Klebsiella* quickly re-establishes a high rate of nitrogenase activity and gives reproducible results when growing on agar in small bottles.

No cross-contamination of gases from one chamber to another on the agar surfaces was seen and, in a simulated experiment, no transfer of gas from a centrally situated chamber containing 10% ethylene to surrounding air-filled chambers was detected. Loss of gas from chambers during incubation periods of 6 h or less was generally very low and the occasional dramatic loss (by leakage as opposed to diffusion) was obvious since the size of the peak corresponding to acetylene also decreased and the value could be disregarded. The variation between similar samples in different experiments was greater than that between spots on the same plate.

Gibson *et al.* (1976) proposed that the mucoid material produced by rhizobia might aid nitrogenase activity by lowering the speed of transfer of oxygen, as suggested by Postgate (1971) for other bacteria. A possible role for succinate in this process was suggested since it increases the production of polysaccharides (Dudman, 1964). For *R. japonicum* 61-A-101 our data are contrary to these suggestions. High succinate concentrations (above 10 mM) decreased slime production (Fig. 6) and yet, under all conditions tested, 20 mM-succinate was as good as, or better than, 10 mM for nitrogenase activity (Tables 1 and 2).

Some positive correlations between slime production and high nitrogenase activity were apparent in our work: (i) the optimum leucine concentration for nitrogenase activity was the highest concentration of that amino acid which still gave maximum slime production (Figs 2 and 4); (ii) increasing the phosphate concentration of a leucine-supported culture progressively lowered the activity (Fig. 5) and gave a concomitant reduction in slime production – this also occurred when succinate was increased to 40 mM.

However, against these positive correlations must be balanced the data for glutamine-supported systems (Figs 2 and 4) where the optimum concentration for nitrogenase activity corresponds to almost minimum slime production. With the exception of the arabinose-dependent nitrogenase activity with 1 mM-glutamine, the highest nitrogenase activities were obtained using a medium containing 2 mM-glutamine, 50 mM-phosphate, 20 mM-succinate and 50 mM-arabinose (Table 2). This combination gave the lowest slime production and values for protein as a fraction of the dry weight were in the range of 48 to 52%. Comparison of the protein content (as % of dry weight) of organisms grown on different media (Table 3) with the data for nitrogenase activity under the same conditions leads to the conclusion that

Table 3. *Effect of medium constituents on slime production by R. japonicum 61-A-101 growing on agar surfaces*

Dry weight and protein were measured 14 d after inoculation. Results show the concentrations (mM) of the variable medium constituent giving low, intermediate and high slime production. Arabinose at 12.5 to 50 mM had no effect on slime production by cultures grown in the presence of 50 mM-phosphate, 10 mM-leucine and 10 mM-succinate.

Variable medium constituent*	Slime production		
	Low (protein = 40 to 50 % of dry wt)	Intermediate (protein = 25 to 40 % of dry wt)	High (protein = 15 to 25 % of dry wt)
Leucine (50 mM-phosphate, 10 mM-succinate)	50	20 to 35	5 to 10
Glutamine (50 mM-phosphate, 10 mM-succinate)	2 to 20	0.5 to 1	
Phosphate (10 mM-leucine, 10 mM-succinate)	100	75	12.5 to 50
Succinate (50 mM-phosphate, 10 mM-leucine)	40	20	5 to 10

* Other constituents, in addition to the basic medium (see Methods), are shown in parentheses.

slime could have little more than a minor role in protecting nitrogenase from oxygen and that it is probably incidental. Furthermore both high and low slime producing strains of *Klebsiella pneumoniae* had similar ratios of nitrogenase activity under aerobic or anaerobic conditions when grown on agar surfaces and slime did not appear to contribute significantly to the oxygen resistance of the nitrogenase activity (Wilcockson, 1977).

Gibson *et al.* (1976) reported that the pH increased during growth on agar of strain 32H1 of the cowpea group of *Rhizobium*. We also observed this with the *R. japonicum* strain 61-A-101. It is unlikely that this pH rise was the limiting factor for activity, or hastened its decline, since the activity of a leucine-supported system did not change significantly when the initial pH was varied in the range from 5.3 to 6.7. It is thus also unlikely that the lower and later activities obtained with higher concentrations of phosphate buffer (Fig. 5) were due to increasing the resistance to a pH rise caused by the bacterial growth.

Neither arabinose nor phosphate influenced the survival of colony-forming ability during the 2 weeks of the experiments. However, survival decreased steadily with increasing glutamine concentration above 2 mM. No explanation can be offered for this. With succinate at 20 mM and above the apparent loss of colony-forming ability corresponded with changes in the microscopic appearance of the organisms that were detectable with 20 mM and became more pronounced at 40 mM succinate. These suspensions contained some individual highly distorted cells with terminal and medial swellings and also small aggregates containing some distorted organisms. Thus quantification of loss of colony-forming ability under these conditions was not possible. The suspension procedure used, i.e. first removing the bacterial cell mass from the agar with a jet of water and then forcing the suspension in and out of a syringe and needle, gave almost entirely single cells except from bacteria grown with high succinate in the agar. The cell aggregates in these suspensions did not resemble the classical 'star' formations found in rhizobia grown in liquid culture which would, in any case, be broken up by the suspension procedure employed here.

An alternative role for succinate in nitrogen-fixing rhizobia, which is consistent with our results, was suggested by Bergersen (1977). In chemostat cultures of the strain 32H1,

withdrawal of succinate caused the O_2 concentration to rise and nitrogenase activity to decrease. Thus a relatively high succinate concentration in the agar in our system could be necessary to create in the interior of the cell mass a significant region with a low enough O_2 concentration to permit nitrogenase to function.

The large enhancement of nitrogenase activity in a system supported by 1 mM-glutamine when supplemented with 50 mM-arabinose is perhaps the most interesting finding. The increase was not due to increased growth or slime production since both were unaffected. The peak activity of about 10 nmol ethylene produced h^{-1} (mg protein) $^{-1}$ required both arabinose and succinate since changing the latter from 10 or 20 mM (where the activity is essentially the same) to 2 mM virtually eliminated it (Table 2). Perhaps the system supported by 2 mM-glutamine did not show this arabinose effect because the higher concentration of amino acid was partially repressive and what nitrogenase was being made already functioned as well as possible under these limiting conditions. Bergersen (1977) found that removal of glutamine from a nitrogen-fixing continuous culture of strain 32H1 increased nitrogenase activity indicating that the glutamine previously present was perhaps partially repressive. However, the situation could not be maintained since even the new higher rate of N_2 -fixation would not support the growth of the culture alone.

The isolation of mutants of rhizobia defective in nitrogenase activity, whether through damage to the *nif* genes themselves or to peripheral functions, is not simple since even in continuous cultures gassed with the optimum oxygen concentration organisms are able to support only a fraction of their growth by the nitrogen they fix. Thus rhizobia cannot at present be screened for defective mutants by the failure of such mutants to grow after replica-plating on to nitrogen-free media. The reproducibility and the speed of the assessment of nitrogenase activity by the small chamber method described here should enable such mutants to be isolated by the direct screening of large numbers of spots on agar.

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