AEROBIC NITROGEN FIXATION BY *RHODOPSEUDOMONAS CAPSULATA*

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1. Introduction

Nitrogen fixation is essentially an anaerobic process, due to the high oxygen lability of nitrogenase. Some facultative anaerobes (*Klebsiella pneumoniae*) and some aerobes (*Azotobacter chroococcum*) do fix nitrogen actively, but the former only under anaerobiosis, whereas the latter possess elaborate mechanisms protecting the nitrogenase from oxygen damage reviewed [1,2].

It is generally agreed that nitrogen fixation in photosynthetic bacteria occurs only under anaerobic conditions ([1] pp. 285, 291–292, [2] p. 20). The purple non-sulfur photosynthetic bacterium *Rhodopseudomonas capsulata* has been shown to contain an active nitrogenase when grown photoheterotrophically in the absence of oxygen [3,4]. *Rps. capsulata* has also been reported to fix nitrogen aerobically, but only in symbiosis with *Bacillus megaterium* [5]. However, since *Rps. capsulata* can switch rapidly from aerobic to anaerobic metabolism [6], it was interesting to investigate in more detail the effect of aerobic conditions on nitrogen fixation in this bacterium.

The results presented here show that *Rps. capsulata* can grow photosynthetically with dinitrogen from air as the sole nitrogen source. Furthermore, the nitrogenase activity in semi-aerobically grown cells does not differ significantly from the activity found in cells grown anaerobically. It has also been found that in vivo nitrogenase activity in *Rps. capsulata* shows similar resistance to inhibition by oxygen as the nitrogenase in *A. chroococcum* [7]. The physiological implications of these findings are discussed.

2. Materials and methods

2.1. *Cells and cultures*

Rhodopseudomonas capsulata strain B10 was a generous gift from the Photosynthetic Bacteria Group, Dept Microbiol., Indiana University, Bloomington IN 47401, USA.

Cultures (32–35°C) were grown in an RCV medium [3,8] supplemented with DL-lactate (38 mM) when dinitrogen was the only nitrogen source, or with DL-lactate (30 mM) and L-glutamate (7 mM) in non-nitrogen fixing conditions. Anaerobic cultures were grown in modified 50 ml syringes [3] or in 500 ml Erlenmeyer flasks sparged with argon (200 ml/min). Semi-arobic cultures (200 ml vol.) were grown in 500 ml flasks stoppered with cotton wool plugs. Aerobic cultures (200 ml vol.) were sparged with air (200 ml/min). All cultures were uniformly illuminated by 100 W incandescent lamps (approx. 10 000 lux).

2.2. *Preparation of resting-cell suspensions*

The following procedures were carried out under argon. Cultures were harvested at late exponential phase and centrifuged (25 000 × *g*, 15 min). The pellets were washed in 50 mM K-phosphate buffer (pH 6.8), recentrifuged as above and resuspended in the phosphate buffer. All solutions were gassed with argon before use. The resting cell suspensions were stored under argon in the dark at 0°C. They were routinely used within 4 h after preparation since no loss of activity was observed during that period.

2.3. *Assay of nitrogenase activity in whole cells*

12 ml vials containing 2 ml resting-cell suspension

and fitted with serum caps were gassed with argon and then shaken at 30°C in a Warburg apparatus (Braun, B., Melsungen, FRG) uniformly illuminated from below by fourteen 40 W incandescent lamps (approx. 5000 lux). After 5 min equilibration, lactate was added (final conc. 20 mM), and after 1 min further incubation, 0.5 ml acetylene was injected. Aliquots (25 μl) were withdrawn from the gas phase of the vials and injected into a Hewlett Packard 5750 gas chromatograph equipped with a flame ionization detector; the 1.5 meter stainless steel column (diam. 3.2 mm) was filled with Porapak R (80 –100 mesh). The carrier gas was nitrogen (60 ml/min) and the oven temp. 70°C. Dry weight determinations were made as in [3].

3. Results

3.1. *Growth and nitrogen fixation under aerobic conditions*

Rhodopseudomonas capsulata does not grow semi-aerobically in the dark on lactate, i.e., it is unable under these conditions to fix dinitrogen from air (fig.1); this result is in good agreement with [4] where it is shown that nitrogenase is not synthesized in the absence of light. Under illumination, *Rhodopseudomonas capsulata* can use atmospheric nitrogen as the only nitrogen source (fig.1): cells grow well in semi-aerobic conditions; however, sparging of air into the culture (200 ml/min) or vigorous stirring have a marked inhibitory effect on growth.

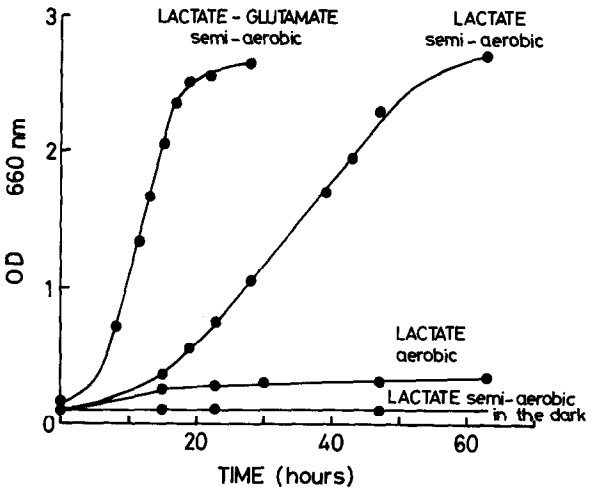

Fig.1. Aerobic growth of *Rps. capsulata* on glutamate or dinitrogen as nitrogen sources. The cells were grown in the light as described in section 2, at 32–35°C. Samples were taken for $A_{660\ nm}$ measurement.

3.2. *Nitrogenase activity in cells grown aerobically*

Nitrogenase activities measured in whole cells grown under different conditions are presented in table 1. It is noteworthy that in cells grown semi-aerobically in lactate–glutamate medium, the activity is not affected by the presence of oxygen during growth. Cells grown under nitrogen-fixing conditions show an increased nitrogenase activity, despite the presence of oxygen. In cells grown aerobically on lactate–glutamate the activity is markedly lower.

Table 1
Nitrogenase activity in resting whole cells of *Rps. capsulata* grown under different conditions

Growth conditions		Nitrogenase activity (nmol C_2H_4/min/mg dry wt)
Substrates	Gas phase	
Lactate–Glutamate	Argon (sparged)	55 ± 8 (6)
Lactate–Glutamate	Air (no stirring)	57 ± 13 (5)
Lactate	Air (no stirring)	72 ± 15 (4)
Lactate–Glutamate	Air (sparged)	22 ± 7 (3)

No. cultures assayed for activity in parentheses

All cultures were illuminated at an intensity of approx. 10 000 lux. The temperature was 32–35°C. For other growth conditions and for the nitrogenase assay, see section 2

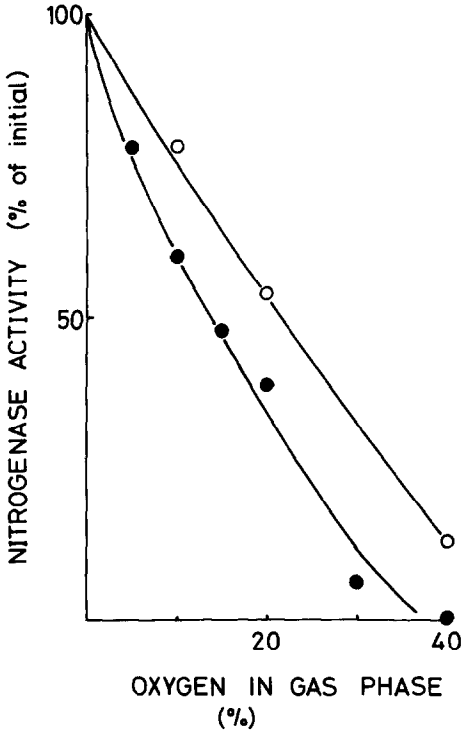

Fig.2. Inhibition of nitrogenase by oxygen in resting cells of *Rps. capsulata*. Cells were grown photosynthetically on lactate–glutamate, and harvested in late exponential phase. Nitrogenase was assayed as described in section 2 under an atmosphere containing various concentrations of oxygen in argon. (○) Semi-aerobically grown cells. (●) Anaerobically grown cells.

3.3. *Inhibition by oxygen of nitrogenase activity in resting cells*

Figure 2 shows that 40% oxygen in the gas phase is required to inhibit completely nitrogenase activity in vivo. Cells grown semi-aerobically are somewhat more resistant to oxygen inhibition than anaerobically grown cells. It is of interest that nitrogenase activity in whole cells of *Rhodopseudomonas capsulata* is about as resistant to inhibition by oxygen as the nitrogenase in the aerobe *Azotobacter chroococcum*: indeed, in vivo nitrogenase activity in *Rps. capsulata* was 50% inhibited by 14% oxygen in the gas phase when the cells were grown anaerobically and by 22% oxygen when the cells were grown semi-aerobically (fig.2); in *A. chroococcum* grown under a gas phase containing 9% oxygen, the nitrogenase in vivo was 50% inhibited in the presence of 8% oxygen, whereas with cells grown under 55% oxygen, a 50% inhibition of nitrogenase was obtained in the presence of 30% oxygen [7]. These figures suggest that the resistance to oxygen inhibition, although similar in both organisms, is more dependent on the growth conditions in A. chroococcum than in Rps. capsulata.

The nitrogenase activity in whole cells is totally inactivated after exposure to pure oxygen for 40 min, in the light (2000–3000 lux), and at 25°C; 50% of the activity is lost after 15 minutes of exposure to oxygen (figure 3). Whether the cells have or have not been exposed to air during growth has no influence on their resistance to oxygen damage. Presumably, under such drastic conditions, other functions than nitrogenase are damaged.

Inactivation of nitrogenase in a mixture of argon

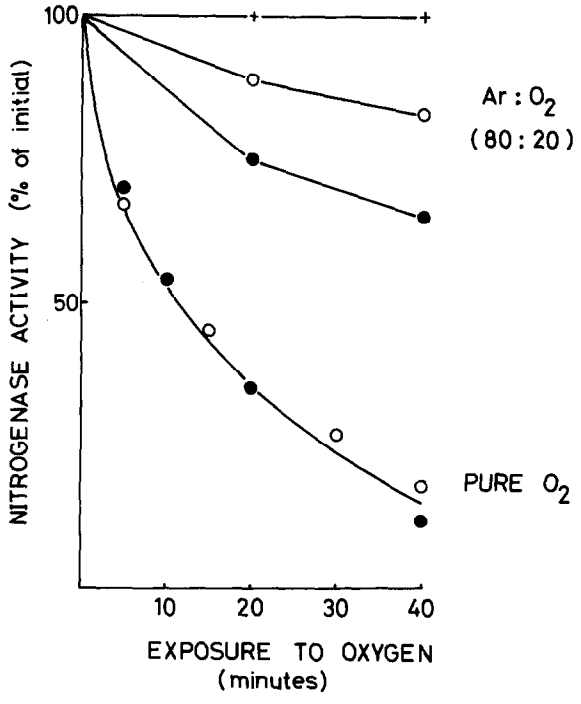

Fig.3. Irreversible inactivation of nitrogenase activity by oxygen in resting cells of *Rps. capsulata*. Resting cells grown on lactate–glutamate were exposed at 25°C and at a light intensity of approx. 3000 lux to pure oxygen of argon:oxygen (80:20) for the periods indicated. The vials were then flushed with argon and the nitrogenase activity was immediately assayed as described in section 2. (+) Cells grown aerobically. (○) Cells grown semi-aerobically. (●) Cells grown anaerobically.

Table 2
Light dependence of nitrogenase activity in resting cells grown semi-aerobically in the light on lactate–glutamate medium

Conditions during nitrogenase assay	Nitrogenase activity (nmol C_2H_4/min/mg dry wt)
Light	48
Light + 10% O_2 in gas phase	38
Dark	0[a]
Dark + 10% O_2 in gas phase	0[a]

[a] The sensitivity limit of the assay was 0.2 nmol/min/mg dry wt

See section 2 for growth conditions and nitrogenase assay. In all cases lactate was present at a final concentration of 10 mM

and oxygen (80 : 20), on the other hand, is dependent on the growth history of the cells (fig.3); presumably, cells grown aerobically develop some kind of oxygen protecting mechanism (see also fig.2).

3.4. *The light dependence of nitrogenase activity in vivo*

Rps. capsulata cells grown semi-aerobically in the light contain nitrogenase (table 1), the photosynthetic membranes and the respiratory chain. Such cell preparations may be employed to establish whether the reductant for the nitrogenase can be generated in the dark. Table 2 summarizes the combined effect of light and oxygen on nitrogenase in resting cells grown semi-aerobically in the light on lactate–glutamate. In the dark and in the presence of oxygen and lactate (table 2, line 4), ATP is generated by oxidative phosphorylation, and nitrogenase activity is potentially present, since it is only 20% inhibited by 10% oxygen (compare lines 1 and 2). Thus, under these conditions, the absence of nitrogenase activity is presumably due to lack of reductant. It is generally agreed that in photosynthetic bacteria this reductant is photoproduced, but there is no definite proof that dark reactions are not involved in vivo ([1] pp. 295–298). In *Rps. capsulata*, at least, as shown here, the production of the reductant for nitrogenase seems to be strictly light dependent.

4. Discussion

We confirmed here that nitrogen fixation in *Rps. capsulata* is strictly light dependent. More interesting is the fact that this bacterium can fix nitrogen under the same conditions as aerobic bacteria: it can grow with dinitrogen as the sole nitrogen source in aerobic conditions, provided the aeration is not too vigourous. The nitrogenase activity in vivo shows similar oxygen sensitivity to that found in *A. chroococcum* [7]. When cells are vigourously aerated, the efficiency of nitrogen fixation is markedly decreased (section 3.1). This is presumably due to inhibition of nitrogenase by oxygen. However, another factor should be considered: the synthesis of photosynthetic membranes in *Rps. capsulata* is repressed by oxygen [9]. Thus, even if nitrogenase is not completely inhibited by oxygen, its activity may not be expressed in vivo due to a lack of reductant which is provided by the photosynthetic machinery (table 2).

Rps. capsulata can fix dinitrogen aerobically only in symbiosis with *Bacillus megaterium* [5]; we have not been able to see foreign colonies on Petri dishes inoculated with samples from *Rps. capsulata* cultures fixing nitrogen aerobically.

The unexpectedly high oxygen tolerance of nitrogenase activity in *Rps. capsulata* suggests that in its natural environment this bacterium fixes dinitrogen aerobically or semi-aerobically in the light. This result permits re-evaluation of some aspects of energy metabolism in *Rps. capsulata*. It evolves large amounts of hydrogen when grown photoheterotrophically in the absence of oxygen [3]. The energy-dependent hydrogen evolution was considered by them to be a means of energy idling in the presence of excess reducing power. However, as pointed out [3,4], H_2

evolution under energy-limiting conditions represents an uncontrolled dissipation of utilizable energy.

It is likely that the large photoproduction of hydrogen observed during anaerobic photoheterotrophic growth is a consequence of specific growth conditions rather than the effect of physiological regulations. In natural habitats, growth conditions favouring such large hydrogen production are unlikely to occur. In addition, the availability of atmospheric nitrogen will divide by 4 the amount of hydrogen evolved in the absence of dinitrogen ([2] pp. 121–126). Furthermore, under conditions allowing aerobic nitrogen fixation, a recycling of hydrogen [10] is likely to be more efficient: during photoheterotrophic anaerobic growth, photoreduction of CO_2 (produced by the oxidation of substrates) is the main if not the only hydrogen-consuming reaction [3,4], whereas in the presence of air, hydrogen can also be an electron donor (via hydrogenase) for the reduction of dinitrogen (nitrogenase) and oxygen (respiratory chain).

Indeed, we have found (unpublished results), that in *Rps. capsulata* as in some blue–green algae [11–13], hydrogen can be recycled directly for the reduction of acetylene, and that *Rps. capsulata* cells, even when grown anaerobically, consume hydrogen rapidly in the presence of traces of oxygen (B. C. K. and A. Colbeau, unpublished results).

We may thus conclude that not only is *Rps. capsulata* potentially an aerobic nitrogen-fixing organism, but even that oxygen may have a beneficial effect as an electron acceptor for the recycling of hydrogen evolved by the nitrogenase reaction. Conversely, hydrogen, as an active electron donor to the respiratory chain, is likely to participate in the scavenging of oxygen and the protection of nitrogenase from oxygen damage.

Acknowledgements

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