SHORT COMMUNICATION

The Effects of Carbon Dioxide on Nitrogenase-related Activities of *Rhodopseudomonas capsulata*

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*N₂* fixation was investigated in suspensions of washed cells of the photosynthetic bacterium *Rhodopseudomonas capsulata* grown on various carbon and nitrogen sources. Maximum nitrogenase activities were common to cultures supplied with glutamate. Cells grown with nitrate contained appreciable nitrogenase activity and produced *H₂* in the light. Nitrogenase activity in washed cells derived from various nutritional conditions was markedly increased when NaHCO₃ was included in the reaction mixtures.

INTRODUCTION

The ability to fix *N₂* is now well established among purple non-sulphur photosynthetic bacteria (Meyer *et al.*, 1978a). These bacteria also grow on various forms of combined nitrogen, including ammonia. While growth with ammonia is rapid, nitrogenase biosynthesis and activity are repressed in the presence of this nitrogen source (Hillmer & Gest, 1977a; Meyer *et al.*, 1978a). Most purple non-sulphur bacteria also utilize amino acids, e.g. glutamate, although *Rhodopseudomonas acidophila* does not grow on this amino acid (Herbert *et al.*, 1978). In *Rhodopseudomonas capsulata*, nitrogenase biosynthesis and activity is appreciable in the presence of amino acids, except when ammonia accumulates in the culture medium as a result of a high nitrogen/carbon ratio (Hillmer & Gest, 1977a). There appear to be no reports on nitrogenase biosynthesis and activity in cultures grown with nitrate. While a variety of purple bacteria actively reduce nitrate, the ability to grow entirely on this nitrogen source is limited to a few species (Malofeeva *et al.*, 1974). An assimilatory nitrate reductase has been characterized in *Rhodopseudomonas capsulata* strain AD2 (Alef & Klemme, 1977).

In the present communication we compare nitrogenase activities, measured by *N₂* incorporation, acetylene reduction and light-dependent *H₂* production, in suspensions of washed *R. capsulata* derived from cultures grown with a variety of carbon and nitrogen sources. We demonstrate that nitrogenase activity is present in cells cultured with nitrate and that these cells produce *H₂* in the light. In addition, we report on a stimulation by CO₂ of nitrogenase-related activities in *R. capsulata* grown under a variety of nutritional conditions.

METHODS

*Bacteria and growth conditions.* *Rhodopseudomonas capsulata* strain B10 was kindly supplied by the Photosynthetic Bacteria Group, Department of Microbiology, Indiana University, Bloomington, Ind., U.S.A. Cultures were grown at 30 °C in a mineral salt medium (Hillmer & Gest, 1977a; Weaver *et al.*, 1975) supplemented with either sodium DL-lactate or sodium DL-malate (30 mM) as a carbon source and either *N₂*,
1-glutamate or sodium nitrate (7 mM) as a nitrogen source. Cultures were grown under anaerobic conditions in 120 ml bottles illuminated with five 100 W incandescent lamps (about 5000 lx).

**Harvesting bacteria.** Cultures were harvested during the late-exponential growth phase and centrifuged at 27000 g at 4 °C. The cells were then washed with mineral salt medium devoid of carbon and nitrogen compounds (Hillmer & Gest, 1977a; Weaver et al., 1975), recentrifuged and resuspended in fresh mineral salt solution. The washed cells were stored in the dark under argon at 4 °C.

Bacterial growth was measured turbidimetrically at 660 nm in a Shimadzu spectrophotometer. Dry weights were determined as described by Hillmer & Gest (1977a).

**Acetylene reduction by suspensions of washed cells.** The reduction of acetylene to ethylene was followed in 6.5 ml glass vials containing 1.8 ml washed cell suspension (5.4 to 7.6 mg dry wt). Reaction mixtures contained either lactate or malate (22 mM) as well as chloramphenicol (20 μg ml⁻¹). Vials were fitted with Subaseals and thoroughly gassed with argon for 10 min before shaking at 30 °C in a water bath uniformly illuminated with 100 W incandescent lamps (about 5000 lx). After a 15 min equilibration, 0.45 ml acetylene was injected into the gas phase. Samples (50 μl) withdrawn at 5 min intervals from the gas phase of the vials via a gas-tight syringe were injected into a gas chromatograph unit equipped with a flame ionization detector. The 2 m stainless steel column (2 mm diam.) was filled with Porapak R (80 to 100 mesh). The carrier gas was N₂ (30 ml min⁻¹) and the oven temperature was 50 °C. The amount of ethylene produced was calculated by the method of Meyer et al. (1978b).

**Hydrogen production by suspensions of washed cells.** Reaction vials (6.5 ml) containing 1.8 ml washed cell suspension with either lactate or malate (22 mM) were gassed thoroughly with argon for 10 min before incubating in a water bath at 30 °C uniformly illuminated with 100 W incandescent lamps (about 5000 lx). After a 15 min equilibration, H₂ in the gas phase was monitored by withdrawing 100 μl samples and injecting them into a gas chromatograph unit fitted with a thermal conductivity detector. The 1 m glass column (3 mm diam.) was filled with Chromosorb 102 (80 to 100 mesh). The carrier gas was N₂ and the oven temperature was maintained at 51 °C. H₂ production was related to the peak heights of the H₂ standard curve.

**Experiments with ¹⁵N₂.** Malate (22 mM), lactate (22 mM) and NaHCO₃ (0.3 mM), as indicated in Results, were added to Warburg flasks which were rigorously evacuated to 10⁻¹ mmHg. The flasks were then gassed with 25% (v/v) ¹⁵N₂ (33.5% atom excess) in argon before adding 1.8 ml washed cell suspension (6.8 to 7.8 mg dry wt). The flasks were shaken in a water bath at 30 °C, illuminated with 100 W incandescent lamps. After 30 min incubation the contents of each flask were transferred into microKjeldahl flasks containing 2 ml 18 M-H₂SO₄ and 2 g HgO/Na₂SO₄ mixture (7:93, w/w) and digested. The ammonia produced was distilled under alkaline conditions into boric acid and concentrated to 2 ml after adding 0.1 ml 0.5 M-H₂SO₄. The samples were transferred into one limb of a Rittenburg tube and alkaline hypobromite was added to the other (Sims & Cocking, 1958). The Rittenburg tube was evacuated to 10⁻¹ mmHg and the contents were mixed to generate N₂ from ammonia. The gas was then introduced via an evacuated expansion flask into the AEI mass spectrometer (Nicholas & Fisher, 1960).

**Analysis of glutamate and glutamine.** To 1.8 ml washed cell suspension (9-72 mg protein) in 6.5 ml glass vials fitted with Subaseals was added 0.3 mM-NaHCO₃. The vials were gassed with argon for 10 min in the dark on ice, before placing them in an illuminated water bath at 30 °C (about 5000 lx). After 30 min the reaction was stopped by adding 95% (v/v) ethanol. The suspension was then centrifuged at 10000 g for 10 min at 5 °C, and the pellet was resuspended in ethanol and again centrifuged. The supernatant fractions were pooled and dried under N₂ gas. The dried extracts were suspended in 1 ml 0.1 M-lithium citrate buffer (pH 2) and a 0.25 ml sample was then loaded on to a Beckman amino acid analyser unit for the determination of glutamine and glutamate.

**RESULTS AND DISCUSSION**

**Effects of carbon and nitrogen source in culture medium on nitrogenase activity**

Three parameters, namely light-dependent H₂ production, acetylene reduction and ¹⁵N₂ incorporation, were used to estimate nitrogenase activity in washed, resting cell suspensions of *R. capsulata* prepared from cultures grown on various carbon and nitrogen sources. Endogenous rates associated with these functions in washed cells grown as specified were low (Table 1). Higher rates of nitrogenase activity were observed when washed cells were incubated with an excess of the organic acid (22 mM) used for growth. The choice of organic acid as an accessory electron donor is strict since the carbon source is taken up via a specific inducible transport system (Gibson, 1975; Hillmer & Gest, 1977b). Maximum rates for H₂ production, acetylene reduction and ¹⁵N₂ incorporation were observed in cells grown with glutamate irrespective of the type of carbon supply (Table 1). It was of interest that *R.*
Table 1. \( H_2 \) production, acetylene reduction and \( ^{15}N_2 \) incorporation by washed suspensions of *Rhodopseudomonas capsulata* strain B10 grown with various carbon and nitrogen sources

Washed cells from a culture grown anaerobically for 18 h in light were incubated in 6.5 ml flasks fitted with Subaseals and gassed with argon and shaken in an illuminated water bath at 30 °C (about 5000 lux) as described in Methods. Reaction mixtures (2 ml) contained 6 mg dry wt cells, 22 mM-organic acid (malate or lactate) and 0.3 mM-N\( _2 \)HCO\(_3\). The reduction of acetylene to ethylene was followed every 5 min over a 60 min period, after injecting 10 % (v/v) acetylene in argon into the flasks via a gas-tight syringe. The production of \( H_2 \) was determined in other flasks over the same period by sampling the gas phase, and the incorporation of \( ^{15}N_2 \) was determined after exposing the washed cells in similar reaction mixtures to \( ^{15}N_2 \) (33.5 atom % excess; 0.25 atom in 0.75 atom Ar) for 30 min, as described in Methods.

<table>
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<th>Carbon source</th>
<th>Nitrogen source</th>
<th>Endogenous activity</th>
<th>Organic acid</th>
<th>NaHCO(_3)</th>
<th>Organic acid + NaHCO(_3)</th>
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<td></td>
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* Expressed as nmol \( H_2 \) min\(^{-1}\) (mg dry wt cells\(^{-1}\)).
† Expressed as nmol ethylene produced min\(^{-1}\) (mg dry wt cells\(^{-1}\)).
‡ Expressed as \( \mu g \) \(^{15}N \) uptake (mg total N\(^{-1}\)).

* *capsulata* strain B10 assimilated nitrate and these cells had a relatively high nitrogenase activity compared with cells assimilating N\(_2\) (Table 1). In addition, nitrate-grown cells produced \( H_2 \) via the light-dependent activity of nitrogenase (Table 1). Under our experimental conditions, we were unable to demonstrate \( H_2 \) production by suspensions of washed cells prepared from cultures assimilating N\(_2\). While some \( H_2 \) production might be expected under these conditions of growth (Hadfield & Bulen, 1969; Hwang & Burris, 1972), efficient hydrogenase-mediated \( H_2 \) recycling (Kelley et al., 1977) probably explains the absence of gas production. A similar occurrence has recently been observed in the marine purple non-sulphur bacterium *Rhodopseudomonas sulfidophila* (Kelley et al., 1979).

**Stimulation of nitrogenase activity by sodium bicarbonate**

Nitrogenase activity in resting cells of *R. capsulata* is usually determined in the presence of an excess of the carbon substrate used for growth. e.g. malate or lactate, as an accessory electron donor. In the present study, supplementing the reaction mixture containing the organic acid with low concentrations of NaHCO\(_3\) (0-3 mM) significantly increased nitrogenase activity over that of cells supplied with the organic acid only (Table 1). The effect was common to all three parameters used to estimate nitrogenase activity, and was independent of the carbon and nitrogen source used for growth. Maximum nitrogenase activities were observed when the cells were incubated with NaHCO\(_3\) (0-3 mM) alone. As before, stimulation by NaHCO\(_3\) occurred irrespective of the carbon and nitrogen sources used for
growth (Table 1). A possible explanation for the stimulation by CO₂ is as follows: photo-
heterotrophically grown cells still retain a capacity to photoreduce CO₂ (Hillmer & Gest, 1977b) and a major product of CO₂ assimilation in light is glutamic acid (Stoppani et al., 1955). Indeed, we observed that the glutamate content of ethanol extracts of washed heterotrophically grown cells increased significantly upon exposure to NaHCO₃ (0.3 mM) over a 30 min incubation period. This production of additional substrate for glutamine synthetase, which is involved in regulating nitrogenase activity (Johansson & Gest, 1976; Hillmer & Fahlbusch, 1979), may be responsible for the increased nitrogenase activity observed in the present study. The short incubation periods used would exclude any effect on the biosynthesis of nitrogenase. It is also of interest that CO₂ stimulates the reduction of the following substrates by nitrogenase: N₂, acetylene and protons. The precise mechanism of this stimulation remains to be determined.

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REFERENCES


