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Diazotrophic Growth of *Rhodopseudomonas acidophila* and *Rhodopseudomonas capsulata* Under Microaerobic Conditions in the Dark

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Abstract. Diazotrophy of *Rhodopseudomonas acidophila* and *Rhodopseudomonas capsulata* was not obligatorily linked to photosynthesis. In the dark *R. acidophila* grew with dinitrogen as sole nitrogen source at a dissolved oxygen tension of 15 Torr (= 2.0 kPa); the doubling time was 8 h. Acetylene reduction by whole cells was more sensitive to oxygen in the light than in the dark. 16.5 mg N_2 were fixed per g lactic acid consumed. *R. capsulata* synthesized nitrogenase and fixed dinitrogen in the dark at a dissolved oxygen tension of less than one Torr (= 0.13 kPa). The doubling time of this bacterium was 16 h and 10.5 mg N_2 were fixed per g lactic acid consumed.

Key words: *Rhodopseudomonas acidophila* – *Rhodopseudomonas capsulata* – Diazotrophic growth in the dark – Oxygen sensitivity of diazotrophic growth – Oxygen sensitivity of nitrogenase activity – Efficiency of microaerobic N_2-fixation.

Two essential prerequisites for dinitrogen fixation are energy in the form of ATP and electron carriers having an electronegative redox couple. Both factors are provided by the metabolism of a nitrogen fixing bacterium. In facultatively aerobic purple nonsulfur bacteria ATP can be regenerated either by a photosynthetic or a respiratory energy metabolism. The reductants for nitrogen fixation can certainly be provided during phototrophic growth under anaerobic to microaerobic conditions. It is, however, not yet established whether the electronegative electron carriers can also be regenerated by the respiratory metabolism in the dark.

Phototrophic bacteria grow well with dinitrogen as sole nitrogen source under anaerobic conditions in the light (e.g. 3.7 h generation time with N_2 compared to 2.9 h with NH_4^+, Herbert et al., 1978). The attempts, however, to show nitrogen fixation in the dark with respiratory energy generation have failed so far (Gest et al., 1950; Lindstrom et al., 1949; Pratt and Frenkel, 1959). Even under conditions taking into consideration the extreme oxygen sensitivity of nitrogenase, neither growth nor acetylene reduction could be obtained with *R. capsulata* under microaerobic conditions in the dark (Meyer et al., 1978).

Based on experiments with other purple bacteria it was concluded that an intimate link may exist between photosynthesis and nitrogen fixation (Arnon and Yoch, 1974). This view contrasts with the experimental results described in the present paper. *R. acidophila* was able to fix nitrogen with reasonable rates both anaerobically in the light and microaerobically in the dark. That this capacity is not unique to *R. acidophila* was shown by positive growth results with several species of purple nonsulfur bacteria in agar shake cultures, and by a growth experiment with *R. capsulata* under controlled oxygen tension in liquid culture.

Material and Methods

Organisms and Media. Rhodopseudomonas acidophila 7050 (DSM 137) and *R. capsulata* Kb1 (DSM 155) were grown at pH values of 5.6 and 6.9 respectively. The medium described by Siefert and Pfennig (1979) was used without NH_4Cl. Na-L-lactate (0.1 % or more) served as substrate.

Test for Oxygen Sensitivity. Preliminary growth tests were carried out in deep agar shake tubes containing lactate medium with and without NH_4Cl and 1.2 % agar. 10 ml portions of the agar medium were pipetted into test tubes, which were closed with cotton plugs and autoclaved. After autoclaving, the test tubes were kept in a waterbath at 42° C. Each tube was inoculated with one drop of a culture grown anaerobically in the light with a growth limiting amount of 0.2 g NH_4Cl/l. The inoculum was homogeneously distributed in the agar by gently turning each tube upside down, regardless of wetting the cotton plug. The tubes were cooled in tap water until the agar solidified. Parallel tubes were incubated under air at 30° C in the dark and in the light.

Abbreviation. kPa = kilopascal

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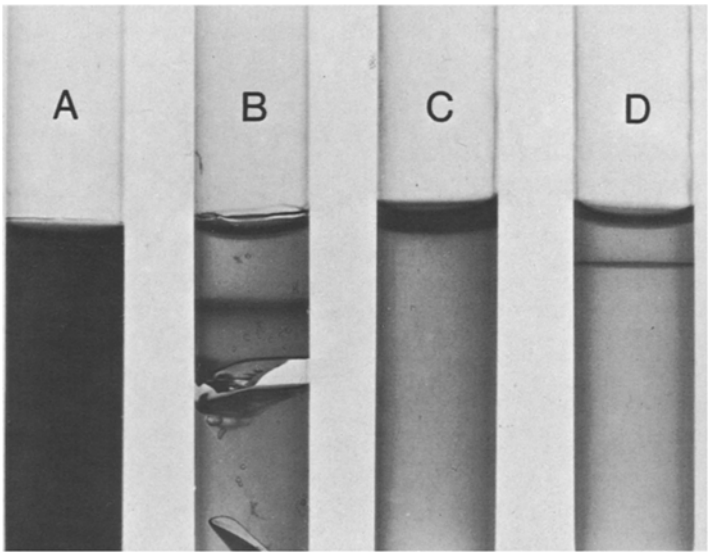


Fig. 1A–D
Effect of atmospheric oxygen on the growth position of *Rhodopseudomonas capsulata* in agar shake tubes when grown under the following conditions:
A Growth with NH_4^+ in the presence of light;
B diazotrophic growth in the light; hydrogen gas is produced in the anaerobic part of the culture in the absence of N_2; **C** aerobic growth in the dark with NH_4^+ as nitrogen source; **D** microaerophilic growth in the dark at the expense of dinitrogen of air as sole nitrogen source

Growth Experiments with Controlled Oxygen Concentration. A 2 l fermenter with control devices for O_2- and CO_2-concentration as described by Siefert and Pfennig (1979) was used to determine microaerobic growth of *R. acidophila* and *R. capsulata* in the dark.

Gaschromatographic Determination of Oxygen. A Perkin Elmer gaschromatograph type 900 was used. N_2 and O_2 were separated on a steel column (3.2 mm in diameter, 3 m in length) packed with molecular sieve 5 Å (type OSO.91, Perkin Elmer) 30–60 mesh. The gases were detected by a thermal conductivity cell under the following operation conditions: injector 100° C; oven 70° C; manifold 100° C; detector 150° C; current 125 mA; flow rate of the carrier gas (argon) 40 ml · min^{-1}.

Nitrogenase. Nitrogenase activity was determined by the acetylene reduction method as described by Siefert and Pfennig (1978).

Bacteriochlorophyll a. The amount of bacteriochlorophyll *a* per ml of culture was determined as described by Siefert et al. (1978).

Nitrogen and Dry Weight Determination. Total nitrogen per ml of culture was determined using a modified Kjeldahl method according to Humphries (1956). For dry weight determinations cellulose nitrate membrane filters (Sartorius, SM 11306) were used.

Ammonium. NH_4^+ was determined using Nessler's reagent according to the Deutsche Einheitsverfahren, 1960 (modified).

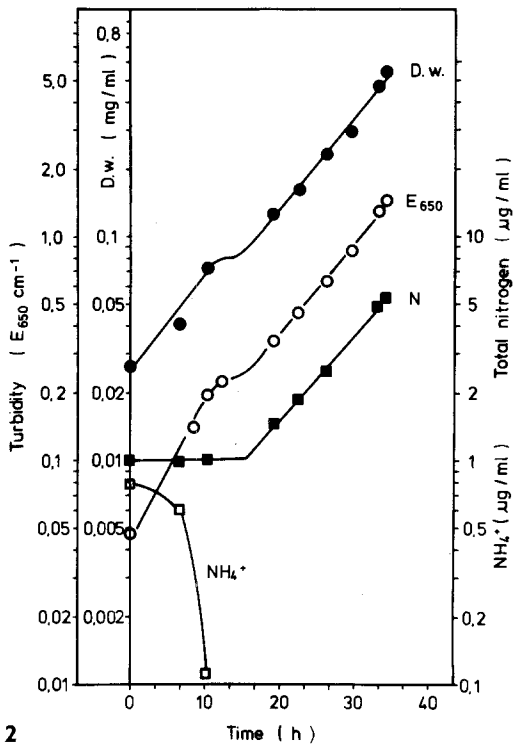
Results and Discussion

Effect of Atmospheric Oxygen on Growth of Purple Nonsulfur Bacteria in Deep Agar Shake Cultures

The deep agar shake culture method has been used widely to examine toxicity of oxygen to bacterial growth (Cohn, 1962; Fredette et al., 1967; de Vries and Stouthamer, 1969; Pfennig, 1970; Kämpf, 1978). We used this auxanographic method to examine inhibition of diazotrophic growth of phototrophic bacteria by oxygen. Deep agar shake cultures inoculated homogeneously with *Rhodopseudomonas capsulata* cells were incubated in the light and in the dark under air. The resulting growth is shown in Fig. 1. In controls supplied with NH_4^+ as nitrogen source, oxygen in equilibrium with the air was neither inhibitory to growth in the light, where the bacteria developed throughout the agar (Fig. 1, A), nor to growth in the dark where the bacteria grew aerobically near the agar surface (Fig. 1, C). In contrast, when the bacteria were dependent on dinitrogen from the air as sole nitrogen source, growth was inhibited by the oxygen concentration in equilibrium with air. In the light the bacteria grew in a zone located 8 mm below the agar surface (Fig. 1, B). *R. capsulata* strain Kb1 was also capable of growing in the dark under microaerobic conditions at the expense of N_2 (Fig. 1, D). The position of growth was 5 mm below the agar surface which indicated a higher oxygen tolerance of N_2-fixation in the dark than in the light.

The colour of the two cultures incubated with NH_4^+ changed from brown to red in the presence of oxygen due to the conversion of spheroidene to its corresponding ketocarotenoid spheroidenone (Eimhjellen and Jensen, 1963). In comparison the N_2-fixing bacteria in tubes B and D (Fig. 1) grew exclusively at an oxygen concentration so low that no major colour change of the culture was caused.

The same auxanographic method was applied to *Rhodospirillum rubrum* strain Ha (DSM 107), *Rhodopseudomonas sphaeroides* 17023 (DSM 158), *R. gelatinosa* 2150 (DSM 149), *R. acidophila* 7050 (DSM 137), and *R. palustris* 17001 (DSM 123). All these strains of the facultatively aerobic species of the



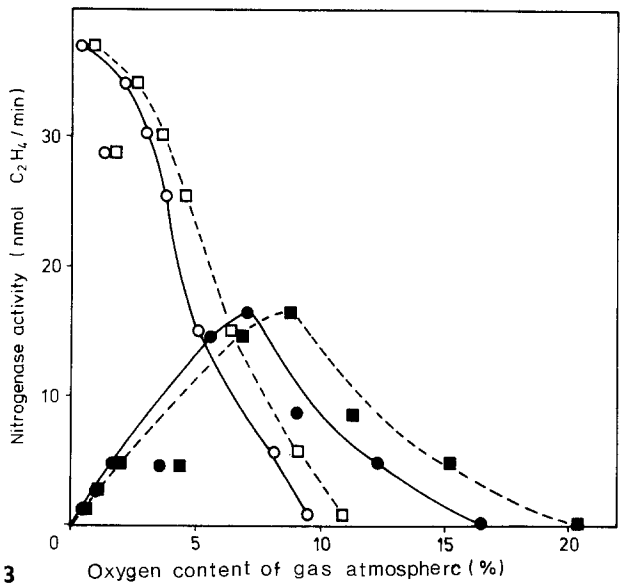


Fig. 2. Diazotrophic growth of *Rhodopseudomonas acidophila* under microaerobic conditions in the dark. The 10 l fermenter was inoculated with 500 ml of a culture grown anaerobically in the light with NH_4^+. Throughout the experiment the dissolved oxygen concentration was constantly kept at 15 Torr (= 2 kPa). Optical density (○——○); dry weight (●——●); total nitrogen of the culture (■——■); NH_4^+ (□——□)

Fig. 3. Effect of oxygen concentration on C_2H_2 reduction of *Rhodopseudomonas acidophila*. The bacteria grown anaerobically in the light with N_2 were harvested by centrifugation and suspended in growth medium at a final concentration of 2.7 mg protein per ml. 1 ml portions of the cell suspension were pipetted into reaction vessels of 20 ml volume. The vessels were closed with serum stoppers and evacuated and flushed with argon several times through a hypodermic needle. Different amounts of oxygen were injected with a gastight syringe into the reaction vessels after an equivalent amount of argon was withdrawn. The vessels were incubated in a water bath at 30° C and the reaction was started by injection of 10 % acetylene. C_2H_2 reduction was stopped after 30 min with 0.1 ml of 1 M trichloro-acetic acid. C_2H_4 formed as well as the remaining oxygen content of the gas mixture was determined gaschromatographically. Nitrogenase activities of illuminated cells (7,000 lx) related to the initial oxygen concentrations (□----□) and to the lower oxygen concentrations at the end of the experiment (○——○). Nitrogenase activities of cells incubated in the dark related to the oxygen concentrations at the beginning (■-----■) and at the end of the experiment (●——●)

Rhodospirillaceae showed, in principle, a growth behaviour similar to that demonstrated for *R. capsulata*. Even *R. palustris*, a microaerophilic strain, showed some growth with molecular nitrogen in the dark after prolonged incubation.

Diazotrophic Growth in the Dark of Rhodopseudomonas acidophila in Liquid Culture

Figure 2 shows microaerobic growth (2.0 kPa) of *R. acidophila* in the dark. The curve has a normal diauxic growth character which has also been shown for this bacterium when grown under anaerobic conditions in the light (Siefert, 1976). NH_4^+ was used up first, then during a growth lag nitrogenase synthesis became derepressed, which enabled the bacterium to grow further at the expense of N_2 as sole nitrogen source. The exponential increase in cell mass of N_2-fixing cells was paralleled by the increase in total nitrogen of the culture medium as measured with the Kjeldahl method. Under the specified growth conditions (Fig. 2), the doubling time of N_2-fixing cells was about 8 h, which is longer than the doubling time of cells supplied with NH_4^+; similarly, the rate of N_2-fixation limited the growth rate of cells grown anaerobically in the light (Herbert et al., 1978).

Dependence of Nitrogenase Activity on O_2 Concentration

The effects of oxygen concentration on acetylene reduction by resting cells of *R. acidophila* is depicted in Fig. 3. In the dark nitrogenase activity was strictly dependent on the presence of oxygen, however, high oxygen concentrations were strongly inhibitory. At optimal oxygen concentration nitrogenase activity reached about 50 % of the maximal activity displayed by cells incubated anaerobically in the light (Fig. 3).

This ratio of 1:2 is in good agreement with the ratio of specific growth rates of $0.09\,h^{-1}$:$0.19\,h^{-1}$ determined for N_2-fixing cells growing microaerobically in the dark or anaerobically in the light (Herbert et al., 1978) respectively. Obviously the rate of N_2-fixation in the dark is limited by the supply of ATP and/or reductants rather than by the amount of nitrogenase.

From the upper limit of oxygen concentration, which totally inhibited nitrogenase (Fig. 3) it is evident that cells kept in the dark tolerated higher concentrations than illuminated cells. This is in accordance with the observed growth inhibition of *R. capsulata* in agar shake cultures: N_2-fixing cells incubated in the dark grew closer to the surface than cells supplied with light (Fig. 1). Since respiration of pigmented phototrophic bacteria is strongly inhibited by light (Clayton, 1955), it is reasonable to conclude that the rate of respiration is one important factor which determines oxygen tolerance of phototrophic N_2-fixing bacteria. The effectiveness of respiratory protection of nitrogenase in phototrophic bacteria has clearly been demonstrated by Meyer et al. (1978) using a respiration deficient mutant of *R. capsulata*.

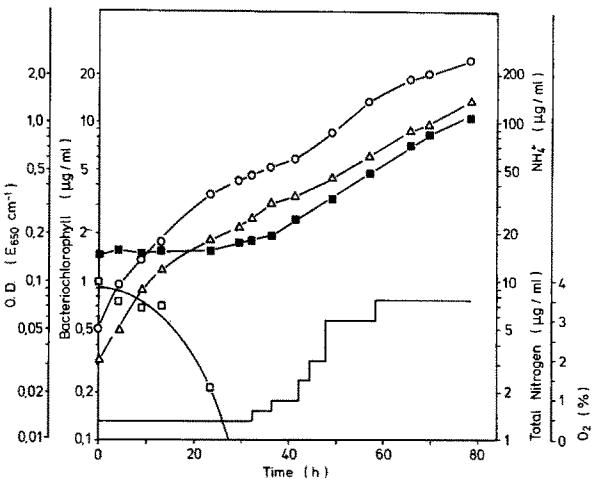


Fig. 4. Diazotrophic growth of *Rhodopseudomonas capsulata* under microaerobic conditions in the dark. The 2 l fermenter was inoculated with 100 ml of a culture grown anaerobically in the light with 0.05% NH_4Cl and 0.1% yeast extract as N source. The oxygen of the gas phase was regulated as specified in the figure. Optical density (○——○); bacteriochlorophyll/ml (△——△); total nitrogen content of the culture (■——■); NH_4^+ concentration (□——□); oxygen concentration of the gas phase (*solid line*). The dissolved oxygen concentration was below the sensitivity of the oxygen electrode used

Diazotrophic Growth of Rhodopseudomonas capsulata Microaerobically in the Dark

In order to test whether in addition to *R. acidophila* other members of the Rhodospirillaceae are also capable of N_2-fixation in the dark, *R. capsulata* was grown in liquid culture. In response to the observation that *R. capsulata* in agar shake culture fixed N_2 at an oxygen concentration so low (Fig. 1) that carotenoids were not converted from spheroidene to spheroidenone, a growth experiment was performed at a dissolved oxygen concentration of 1 Torr (= 0.13 kPa). This is the lowermost oxygen concentration which can be detected by the oxygen electrode used. In the beginning the culture grew on NH_4^+. After depletion of NH_4^+ cells suddenly lost 50% of their respiratory activity and induction of nitrogenase could not be obtained in a period of 6 h during which respiration rate remained constantly low.

Obviously the cells were most oxygen sensitive during the transition period when they converted from NH_4^+-assimilation to N_2-fixation. The experiment illustrated in Fig. 4, therefore, was carried out at an oxygen concentration in the gas phase so low, that dissolved oxygen concentration eluded detection by the oxygen electrode used throughout the experiment. Under this condition, when growth rate was limited by oxygen, *R. capsulata* both derepressed nitrogenase synthesis and grew at the expense of N_2 as sole nitrogen source in the dark with a doubling time of 16 h. *R. capsulata* fixed 10.5 mg N_2 per g lactic acid consumed. This is about one third less than the value obtained with *R. acidophila*, which amounted to 16.5 mg N_2 per g lactic acid.

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