JOURNAL OF BACTERIOLOGY, Nov. 1982, p. 706–713
0021-9193/82/110706-08$02.00/0

Vol. 152, No. 2

Control of Nitrogenase in a Photosynthetic Autotrophic Bacterium, *Ectothiorhodospira* sp.

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Received 11 December 1981/Accepted 23 July 1982

An *Ectothiorhodospira* species fixed nitrogen when grown as an autotroph in completely inorganic medium by using a variety of electron donors. The organism also used organic carbon sources; however, this required induction of synthesis of various enzymes, whereas the enzymes needed for autotrophic growth were synthesized constitutively. Nitrogenase induction and function were inhibited by ammonium chloride. Nitrogenase activity was dependent on light and inhibited by oxygen.

Although nitrogen fixation is a property of many procaryotes, most environmental research has concentrated on symbiotic *Rhizobium* species, which provide the bulk of the nitrogen fixed in agricultural soils (21). The contribution of free-living organisms, particularly the photosynthetic cyanobacteria (25) and *Rhodospirillales* (11), has become of interest more recently.

Many of the *Rhodospirillales* fix elemental nitrogen (25). They do so primarily anaerobically in the light (24), under which conditions they grow quite rapidly (7, 24). Such conditions can be found in soils in association with oxygen-utilizing heterotrophic bacteria (11). Evidence of nitrogen fixation in the dark has been obtained for *Rhodopseudomonas acidophila* and *R. capsulata* (24), in both cases with lactate as carbon and energy source. This has also been reported for a *Thiocapsa* sp. and *Ectothiorhodospira shaposnikovii* (29).

The use of organic carbon sources by the *Rhodospirillales* is well known. Many of them can also synthesize their cell material from carbon dioxide if provided with an inorganic electron donor. Most of the *Chromatiaceae* have this ability (27). However, most of the *Rhodospirillaceae* do not use inorganic electron donors other than hydrogen (4, 15, 16). Three species do grow with thiosulfate and bicarbonate, *R. palustris*, (22, 28), *R. sulfidophila*, and *R. sulfoviridis* (27).

The organism studied here, a newly isolated strain of *Ectothiorhodospira*, can fix nitrogen while growing autotrophically or heterotrophically, deriving its reducing power from inorganic sulfur compounds or from organic compounds. It is also quite aerotolerant. These metabolic properties suggest that the organism may contribute ecologically significant amounts of fixed nitrogen to its environment. Here we describe aspects of nitrogenase regulation in this organism.

MATERIALS AND METHODS

Organism. The red photosynthetic organism studied here is a new isolate of the genus *Ectothiorhodospira*, on deposit with the American Type Culture Collection, Rockville, Md., as no. 31751. It was isolated as part of a single-cell protein project and is known for industrial purposes as *E. goldameirae*. The organism grows as short rods 1.0 by 2.0 μm only under anaerobic and microaerophilic conditions. It grows well with sulfide, sulfur, or thiosulfate as photosynthetic electron donor. Several organic compounds can also be used as hydrogen donors. During growth on sulfide, elementary sulfur is deposited outside the cells in the medium and disappears during further growth. This, and the large stacks of intracellular lamellar membranes, classify it among the *Ectothiorhodospira* (3, 26). Its physiology will be reported in detail elsewhere.

Media. The medium of Baalsrud and Baalsrud (1) was used with minor modifications. The main substrates for growth were added at the following concentrations (per liter): sodium thiosulfate, 16 g; sodium sulfide, 2 g; elemental sulfur, 0.1 to 10 g; bicarbonate, 10 g; and acetate and succinate, 10 g. Cultures were incubated under nitrogen or argon at 30°C with 5,380 to 10,760 lx (500 to 1,000 foot candles) of light provided by 40-W incandescent light bulbs. Anaerobic conditions were established either by filling containers with medium or by bubbling nitrogen through the medium and closing the containers with rubber stoppers.

Growth experiments. To assess the ability of cells to adapt from one medium to another, exponential-phase cultures were chilled in ice water, centrifuged at 4°C under argon, washed, and inoculated into medium including 10^{-4} M glutathione through which nitrogen had been bubbled immediately before use. Cells were washed with minimal medium which contained 10^{-4} M glutathione but no electron donor. Turbidity was followed by using flasks fitted with sidearms for direct reading of turbidities in a Klett-Summerson colorimeter with a 640-nm filter.

Dark microaerophilic growth. Deep agar shake tubes

were prepared according to the method of Kampf and Pfennig (9). Media contained the minimal salts described above, including 1 g of ammonium chloride and 5 g of bicarbonate per liter. Depending on the experimental conditions, the medium was supplemented with 2 g of thiosulfate, 5 g of acetate, or 0.2 g of sulfide per liter. Some tubes were inoculated, whereas others were left uninoculated. Tubes were incubated for 3 weeks in the dark at 30°C.

Preparation of resting cells. Cells harvested and washed as above were suspended in the washing medium at 1 to 2 mg of protein per ml and incubated under nitrogen for 24 to 48 h at 30°C with the usual illumination. Such preparations could then be stored for several days in the refrigerator with very little effect on their nitrogenase activity. For nitrogenase assays, these cells were diluted 1:2 with washing solution supplemented with the appropriate substrates.

Nitrogenase assays. Samples, usually 5 ml, of either exponential-phase cells or resting cells were removed with a syringe through the stopper of the culture flask under a flow of argon and injected into assay flasks, usually 30 ml in total volume, through which argon was bubbled for 5 min. After 2 ml of acetylene was injected, the cultures were incubated with illumination and shaking at 30°C. Rates were measured by measuring ethylene content at four intervals of a 20 to 25 min incubation by injecting 10- to 100-μl samples of the gas phase into a Perkin-Elmer F11 gas chromatograph with a stainless steel column (6 ft. by 0.125 in. i.d. [ca. 1.83 m by 0.42 cm]) containing 5% phenylisocyanate on Porasil C.

In vivo control of nitrogenase. For studies of effectors on nitrogenase, an initial rate of nitrogen fixation was determined in the absence of the effector; inhibitors in solutions previously treated with argon were injected into the assay flasks, and a second rate was determined.

Chemical determinations. Protein was measured by the method of Lowry et al. (14) on trichloroacetic acid precipitates redissolved in NaOH.

RESULTS

Growth of *Ectothiorhodospira* sp. strain ATCC 31751 in various Media. *Ectothiorhodospira* sp. strain 31751 ATCC is a particularly efficient photosynthetic autotroph. It grew anaerobically in the light with a doubling time of 4.5 h at 30°C in a completely inorganic medium, using bicarbonate as carbon source, ammonium chloride as nitrogen source, and thiosulfate as electron donor. An organic compound such as acetate or succinate could be used as carbon source or as carbon source and electron donor, but this increased the growth rate only slightly.

Ectothiorhodospira sp. strain ATCC 31751 grew with a variety of inorganic electron donors including thiosulfate, sulfide, and elemental sulfur. Although sulfide was toxic at high levels, it was used as an electron donor at 0.8% or less.

Ectothiorhodospira sp. strain ATCC 31751 derived its nitrogen from inorganic ammonium salts, some amino acids, urea, and dinitrogen, but not from nitrate. In a completely inorganic medium with bicarbonate, thiosulfate, and dinitrogen, it grew with a doubling time of 7.5 to 8 h at 30°C, i.e., at about half the rate of a culture given an ammonium salt.

Ectothiorhodospira sp. strain ATCC 31751 did not grow under fully aerobic conditions on any medium tested, organic or inorganic. It did, however, grow in the dark in microaerophilic conditions as provided by Pfennig deep agar shake cultures (9) in medium supplemented with ammonium chloride and (i) acetate alone, (ii) acetate and either thiosulfate or sulfide, or (iii) bicarbonate and either thiosulfate or sulfide. *Ectothiorhodospira* sp. strain ATCC 31751 also fixed nitrogen when grown in Pfennig tubes with acetate.

Constitutive synthesis of enzymes needed for autotrophic growth. As indicated above, *Ectothiorhodospira* sp. strain ATCC 31751 could grow in many diverse conditions. The enzymes needed for autotrophic growth seemed to be formed in all growth conditions. Cells grown in heterotrophic conditions could transfer to autotrophic conditions without a lag. The reverse shift required a long period of adaptation. Thus, *Ectothiorhodospira* sp. strain ATCC 31751 grown in thiosulfate, bicarbonate, and ammonium chloride transferred to the same medium without a lag (Fig. 1A, curve 1) but had a long lag when transferred to medium with acetate, bicarbonate, and ammonium chloride (Fig. 1A, curve 3). If a small amount of thiosulfate (0.04%) was provided with the acetate, the lag was abolished (Fig. 1A, curve 2). The thiosulfate presumably provided reducing power during the transition to use of acetate, since it did not suffice for any significant growth in the absence of acetate (Fig. 1A, curve 4). Thus, the transfer of *Ecotothiorhodospira* sp. strain ATCC 31751 from thiosulfate to acetate is formally similar to the transfer of *Escherichia coli* from glucose to lactose (17).

In the reciprocal experiment (Fig. 1B), an acetate-grown culture was transferred to medium with acetate (curve 1) and with thiosulfate (curve 3), in both cases without a lag. Synthesis of enzymes for the use of thiosulfate and bicarbonate thus appeared to be constitutive in this strain. It differs in this respect from *R. palustris*, which also grows as an autotroph with inorganic electron donors, but does so only after adaptation (22).

Use of electron donors for acetylene reduction. The ability of the cells to use electron donors was tested, using the whole cell assay of nitrogenase by acetylene reduction. Cells grown with thiosulfate, bicarbonate, and dinitrogen contained a great deal of nitrogenase. Resting cells prepared from cells grown in this medium were unable to reduce acetylene unless an exogenous

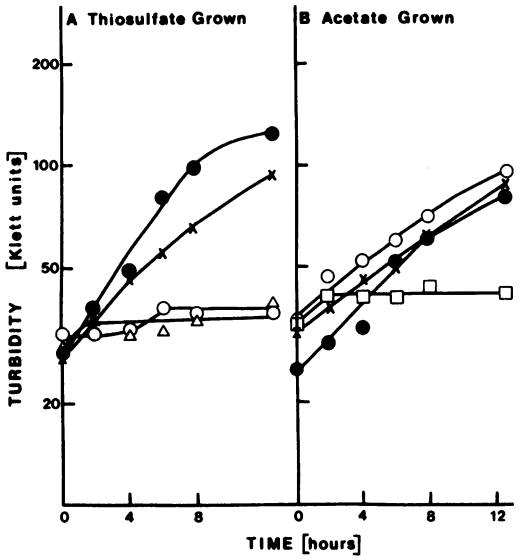

FIG. 1. The growth of *Ectothiorhodospira* sp. strain ATCC 31751 with different electron donors. Cells from exponential phase of cultures grown in two different media, (A) 0.8% thiosulfate, 0.5% bicarbonate, and (B) 0.5% acetate, 0.5% bicarbonate, were chilled, washed, and subcultured into media with the following electron donors. (A) curve 1, 0.8% thiosulfate (●); curve 2, 0.04% thiosulfate and 0.5% acetate (×); curve 3, 0.5% acetate (○); curve 4, 0.04% thiosulfate (△). (B) curve 1, 0.5% acetate (○); curve 2, 0.05% acetate and 0.8% thiosulfate (×); curve 3, 0.8% thiosulfate (●); curve 4, 0.05% acetate (□).

electron donor was added, presumably because their endogenous stores of reducing power were depleted. The activity measured then by using a variety of electron donors would be a function of the resting cells ability to use the particular compound.

Cells grown photoautotrophically (thiosulfate, bicarbonate, dinitrogen) used thiosulfate to reduce acetylene at a rate of 28 nmol/min per mg of protein (Table 1). However, they also used succinate and acetate at significant rates, though they were unable to use α-ketoglutarate or malate.

Cells grown with acetate as carbon and electron source were able to use organic compounds (acetate and succinate), and inorganic compounds (thiosulfate, sulfide, and sulfur) equally well (Table 2). Their rate with thiosulfate (19 μmol/min per mg of protein) was similar to that of thiosulfate-grown cells, and they were able to use sulfide at a much higher rate. This may be due to the increase in redox potential in the presence of sulfide.

Physiological effects of oxygen on growth. *Ectothiorhodospira* sp. strain ATCC 31751 grew photosynthetically only under anaerobic conditions and stopped growing when oxygen was introduced. To test whether the effect of oxygen was reversible, air was bubbled for 1 h into an anaerobic photosynthesizing culture growing with bicarbonate, thiosulfate, and ammonium chloride. The culture immediately ceased to increase in turbidity; however, soon after oxygen was removed by bubbling nitrogen, growth resumed.

The same experiment was repeated with a culture growing in the same medium with dinitrogen rather than ammonium chloride (Fig. 2), air being passed through the culture for 15 min in one case and for 1 h in another. In both cases, growth ceased soon after air was bubbled into the culture and resumed at close to the preceding rate 2 h after the air was replaced by nitrogen. No significant difference was seen with the longer period of aeration. It is clear then that the effect of oxygen was drastic but reversible.

Regulation of nitrogenase synthesis in vivo. Nitrogenase activity was not seen in cells grown

TABLE 1. Nitrogenase activities in thiosulfate-grown cells assayed with various electron donors[a]

Electron donors	Nitrogenase activity (nmol acetylene reduced/ min per mg of protein)
None	5.2
Acetate	14.6
Thiosulfate	28.0
Succinate	31.3
Malate	0
α-Ketoglutarate	5.1

[a] Resting cell suspensions of cells grown with thiosulfate, bicarbonate, and dinitrogen were diluted 1:2 in medium containing the compounds indicated, incubated under argon, and assayed for nitrogenase. The results given are averages of several experiments, except for those for malate and α-ketoglutarate which are the results of single experiments.

TABLE 2. Nitrogenase activities in acetate-grown cells assayed with various electron donors[a]

Electron donors	Nitrogenase activity (nmol acetylene reduced/ min per mg protein)
None	2.1
Acetate	25.7
Thiosulfate	19.0
Succinate	31.2
Sulfur	11.5
Sulfide	61.4

[a] Resting cell suspensions of cells grown with acetate and dinitrogen were diluted 1:2 in medium containing the compounds indicated, incubated under argon, and assayed for nitrogenase. Results are averages of two or more experiments, except for succinate which is the result of a single experiment.

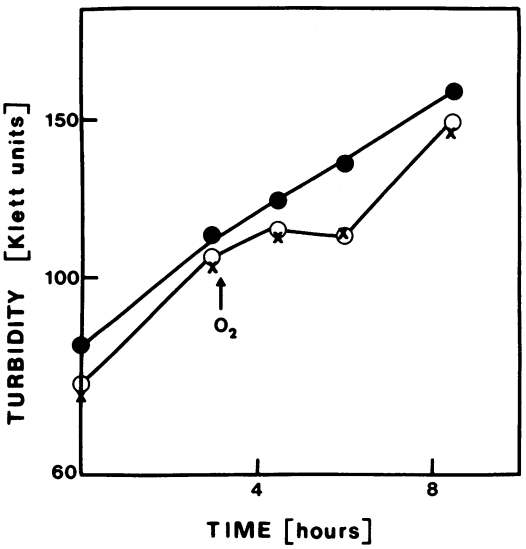

FIG. 2. Effect of aeration on the growth of *Ectothiorhodospira* sp. strain ATCC 31751 with dinitrogen as nitrogen source. At the time indicated by the arrow, air was bubbled through exponential-phase cultures at 30°C in the light for periods of 0 (●), 15 (○), and 60 (×) min. After the aeration period, nitrogen was passed through the culture to remove oxygen, and turbidity followed thereafter.

with ammonium chloride. When cells grown with ammonium chloride were washed and suspended in medium under dinitrogen as sole nitrogen source, nitrogenase activity could be assayed after 3 h of incubation and reached its maximum specific activity by 6 h (Fig. 3). If chloramphenicol was added to the suspended cells, no nitrogenase activity was seen. This seems to indicate that cells grown with ammonium chloride do not contain nitrogenase and that protein synthesis is needed for its appearance.

To investigate the regulation of the induction of nitrogenase activity, cells grown with thiosulfate, bicarbonate, and ammonium chloride were incubated under argon for 24 h with various possible effectors of nitrogen metabolism and then assayed for nitrogenase thereafter (Table 3). Cultures incubated with dinitrogen as sole nitrogen source, under argon alone, or with one of several amino acids all showed nitrogenase activity. Indeed, nitrogenase activity at 24 h was considerably higher than steady-state nitrogenase activity, which may represent an overshoot in synthesis. However, cells incubated with ammonium chloride or urea showed no activity.

Regulation of nitrogenase activity. (i) Inhibition of nitrogenase activity by ammonia. As can be seen in Fig. 4A, nitrogenase activity in *Ectothiorhodospira* sp. strain ATCC 31751 as in many other photosynthetic bacteria (10, 25) was inhibited by ammonium chloride. The effect of ammonium ion was very dependent on the concentration of the cell suspension tested. Nitrogenase activity of a suspension of cells containing 0.2 mg of protein per ml was inhibited transiently by ammonium ion at 10^{-5} M, for about 3 h at 10^{-4} M, and for longer at 10^{-3} M. The same cells in more dilute suspension (0.04 mg of protein per ml) were inhibited strongly even by 10^{-5} M NH_4Cl. The nitrogenase assay is a whole cell assay performed under conditions in which the cells can grow and concomitantly assimilate NH_4Cl. In more dilute suspension, the cells assimilate less NH_4Cl per unit time, and therefore may be inhibited for longer by lower concentrations. A 10^{-5} M concentration is thus an overestimate of the minimum concentration of NH_4Cl needed to inhibit nitrogenase.

(ii) Effect of oxygen on nitrogenase. The nitrogenase of *Ectothiorhodospira* sp. strain ATCC 31751 was inhibited by oxygen added to the gas phase in concentrations as low as 2% (Fig. 4B). The extent to which different concentrations inhibited depended on the concentration of the cell suspension tested, just as in the case of NH_4Cl, more dilute cultures being inhibited by lower amounts of oxygen. This seems to imply that the cells have a considerable ability to remove oxygen metabolically from the medium, thus allowing nitrogenase to function. This could be a factor in the relatively long lag between the

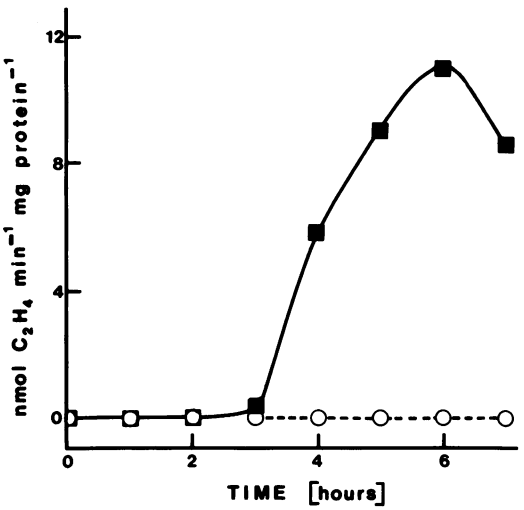

FIG. 3. Induction of nitrogenase. Cells from exponential-phase cultures grown on medium containing 0.1% ammonium chloride were centrifuged and suspended in the same medium without ammonium chloride. One culture also contained 20 μg of chloramphenicol per ml (○); the other did not (■). At the times indicated, samples were removed from each flask and assayed for nitrogenase activity in the presence of chloramphenicol.

TABLE 3. Effect of various compounds on synthesis of nitrogenase[a]

Substance added	Nitrogenase activity (nmol acetylene reduced/ min per mg of protein)
None	57
Nitrogen	79
Alanine	76
Arginine	52
Glutamate	50
Ammonium chloride	0
Urea	0

[a] Cells were grown with thiosulfate, bicarbonate, and ammonium chloride, harvested, and incubated at 30°C under argon in the same medium from which ammonium chloride was omitted. The substances noted were added at 6 mg/ml, except for nitrogen. When nitrogen was added it served as the atmosphere in place of argon. Nitrogenase activity was assayed after 24 h.

addition of oxygen and the onset of inhibition, a matter of some 20 min.

The inhibitory effect of oxygen was readily reversed by the removal of oxygen. When cells whose activity had been totally inhibited by 5% oxygen were gassed with argon and reassayed, a rapid recovery of 100% of the original activity was observed (Fig. 5). This recovery was seen even in the presence of chloramphenicol, though only to the extent of 54%. We have often noticed a decrease in nitrogenase activity in cells incubated with chloramphenicol, perhaps because a nitrogen-containing intermediate accumulated and inhibited the enzyme. This may account for the lower level of activity seen with chloramphenicol here. It seems clear that nitrogenase is inhibited in the presence of oxygen, but it is not permanently inactivated—at least not in the short periods of time (2 to 3 h) examined here.

(iii) Dependence of nitrogenase activity on light. Nitrogenase activity is dependent on a supply of reducing power and energy, the latter provided in this organism by light. When light was removed, nitrogenase activity became unmeasurable within 15 min (data not shown). The enzyme was not inactivated, since restoration of light was followed about 15 min later by a restoration of nitrogenase activity. These delayed effects probably reflect the depletion of ATP and its resynthesis. Since photosynthesis occurs exclusively under anaerobic conditions, this may also explain the delayed inhibition by oxygen.

DISCUSSION

We have described here an *Ectothiorhodospira* species, a photosynthetic bacterium which grew rapidly in totally inorganic media by using bicarbonate as carbon source, thiosulfate as electron donor, and ammonium chloride as nitrogen source. The organism also used organic sources of carbon and electrons such as acetate and succinate. However, cells grown in any medium tested could transfer to growth in inorganic medium without a lag, whereas growth with organic sources required adaptation and was only slightly more rapid than growth with inorganic compounds.

Ectothiorhodospira sp. strain ATCC 31751, like many other photosynthetic bacteria, can fix dinitrogen. It can use organic or inorganic sources of reducing power; these support similar

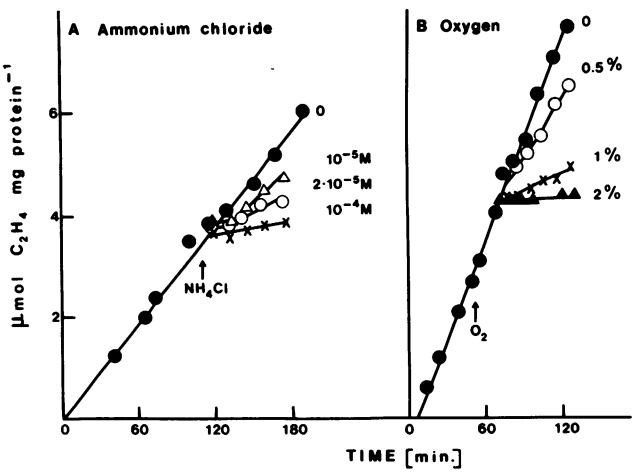

FIG. 4. Inhibitors of nitrogenase activity. Exponential-phase cells grown with dinitrogen as nitrogen source were assayed for nitrogenase as described in the text. Initial activity was determined, inhibitors added at the time indicated by the arrow, and the assay continued. The inhibitors tested were (A) ammonium chloride and (B) oxygen, stated as percent ambient gas.

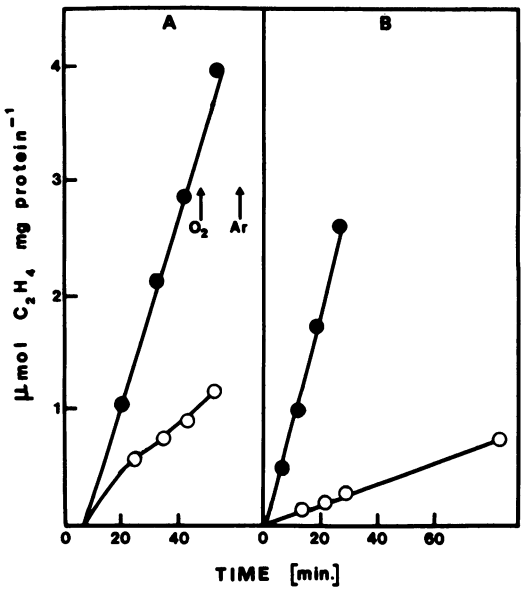

FIG. 5. Reversibility of nitrogenase inhibition by oxygen. (A) Exponential-phase cells grown with dinitrogen as nitrogen source were assayed for nitrogenase activity. At the time indicated by the first arrow, oxygen was injected to 5% of the ambient gas. (B) At the time indicated by the second arrow, the gas phase was flushed with argon, acetylene was reinjected into the flasks (at time 0 in [B]), and nitrogenase was again assayed. The experiment was carried out in the presence (○) and absence (●) of chloramphenicol (20 μg/ml).

rates of nitrogen fixation and growth. Whereas the use of organic compounds for growth required adaptation, their use as electron donors was constitutive. It seems likely then that the organism makes the enzymes for oxidizing these compounds constitutively, but does not make all the enzymes needed to assimilate carbon from them.

Nitrogen fixation in the genus *Ectothiorhodospira* has been reported for both *E. shaposnikovii* (5, 29, 30) and *E. mobilis* (2). These organisms use a similar range of nitrogen sources for growth except that *E. shaposnikovii* uses nitrate (13, 20) and the organism we used does not. We think that *E. shaposnikovii* is very similar to our strain, but differs in aspects of its nitrogen metabolism. *E. shaposnikovii* has only been shown to fix nitrogen when grown on organic carbon sources (5, 29, 30). Under these conditions it will use thiosulfate as an electron donor to nitrogenase when arginine is supplied as nitrogen source, but not with dinitrogen (5). Moreover, it will not use sulfide as an electron donor (29) nor did it grow with sulfide and dinitrogen under autotrophic conditions (30). The *Ectothiorhodospira* sp. strain ATCC 31751 studied here used inorganic sulfur compounds very well, whether grown autotrophically or with organic carbon sources. It appears that *E. shaposnikovii* does not fix nitrogen in a totally inorganic medium, but the authors did not state whether they tested this.

The fixation of nitrogen is intensive in the use of energy and reducing power (18, 25), particularly because the use of electron donors by nitrogenase is inherently inefficient. Much of the reducing power may be diverted to hydrogen formation. Nitrogen-fixing cultures of *Ectothiorhodospira* sp. strain ATCC 31751 are easily identified visually by the copious production of hydrogen bubbles on slight agitation.

Because of the physiological demands of the process, the capacity for nitrogen fixation is extremely sensitive to the energy state of the cell. It can be inhibited not only by inhibitors of the enzyme itself, but also by conditions which decrease the energy stores of the cell (e.g., lack of light) or the reducing power (e.g., less effective electron donor). The electrons of thiosulfate are at too high a potential to act directly as reductants for nitrogenase. Cells grown with thiosulfate and bicarbonate use energy in the form of light-generated ATP to reduce the electrons further (6, 12).

Since the supply of both energy and reducing power is dependent on light, it is not surprising that nitrogen fixation is regulated by light, at least in light-grown cultures. The 10- to 20-min lag found in response to both the shutoff and the return of light suggests that the cell has an exhaustible store of a reduced intermediate, perhaps a ferredoxin (25).

Nitrogen fixation does not depend on light when other sources of energy are available. *Ectothiorhodospira* sp. strain ATCC 31751 grows in the dark with either acetate or sulfide as energy source. In either case it requires enough oxygen to serve as terminal electron acceptor, but it is inhibited by higher levels. The required microaerophilic conditions are easily provided by Pfennig deep agar shake cultures (9). These cultures can fix nitrogen, by using thiosulfate, sulfide, or acetate, in the dark. We have been unable to show this in liquid culture.

Nitrogen fixation is inhibited by oxygen, also with a considerable lag. Oxygen inhibits at least two functions of photosynthetic bacteria: nitrogenase activity and photosynthesis (10, 19, 25). Cells in exponential growth with ammonium chloride as nitrogen source stop growing as soon as air is bubbled into the medium and resume growth as soon as the air is replaced with nitrogen. It would appear that their energy supply is cut off by the addition of oxygen but that no irreversible inactivation occurs.

Nitrogen-fixing cells treated in the same way show a longer, but still comparatively short lag. This suggests that the primary effect of oxygen in the nitrogenase assay must be on availability of energy and not directly on nitrogenase. This is consistent with the fact that the effect of oxygen is reversible, at least after short periods of exposure. It may be that some of the nitrogenase is inactivated, since it took 2 h before an increase in turbidity was seen in our experiment (Fig. 2). However if the nitrogenase enzyme were extensively inactivated, the cell would be starved for nitrogen and could not recover in the short time it actually took to resume growth.

The delay in the onset of inhibition by oxygen may also indicate that the organism can protect itself by metabolizing oxygen, as is done by a wide variety of photosynthetic organisms (8, 15, 16, 23). This delay is seen in cells oxidizing both organic and inorganic compounds. The ability of organisms to grow under microaerophilic conditions shows that the cells have oxygen-handling facilities at least under those conditions. The extent to which these are available in anaerobically grown cells is not known.

The properties we have described for nitrogenase regulation in *Ectothiorhodospira* sp. strain ATCC 31751 are very similar to those of other photosynthetic bacteria. Ammonia represses, and oxygen both inhibits and represses nitrogenase in all organisms tested (10). In photosynthetic bacteria, light also is a regulator of nitrogenase (25). Differences arise from the metabolic processes used by various organisms to obtain energy, reducing power, and protection from oxygen (4). The organism described in this work is of particular interest because its metabolic versatility and rapid growth in autotrophic conditions with light and inorganic substrates may make it useful as a commercial source of biomass and derivatives thereof.

ACKNOWLEDGMENTS

This work was supported by a strategic grant of the National Science and Engineering Research Council of Canada.

The authors wish to recognize the work of several people who have been involved in establishing conditions for growing this organism. Particular thanks are due to James Whittaker, who was the first person to grow it in a dependable manner, and to Ian Krantz, whose devoted work over three summers added a great deal to this project.

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