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Hydrogen Gas Production by an *Ectothiorhodospira vacuolata* Strain

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A hydrogen gas (H_2)-producing strain of *Ectothiorhodospira vacuolata* isolated from Soap Lake, Washington, possessed nitrogenase activity. Increasing evolution of H_2 with decreasing ammonium chloride concentrations provided evidence that nitrogenase was the catalyst in gas production. Cells were grown in a mineral medium plus 0.2% acetate with sodium sulfide as an electron donor. Factors increasing H_2 production included addition of reduced carbon compounds such as propionate and succinate, increased reducing power by increasing sodium sulfide concentrations, and increased energy charge (ATP) by increasing light intensity.

The genus *Ectothiorhodospira* consists of purple phototrophic bacteria belonging to the family *Ectothiorhodospiraceae* (6). The family is characterized by their ability to oxidize reduced inorganic sulfur compounds to sulfate, with elemental sulfur as an extracullar intermediate. Like other phototrophic bacteria, this organism uses the anaerobic process of cyclic photophosphorylation to generate ATP. Reducing equivalents come from hydrogen gas (H_2), reduced sulfur compounds, or certain organic compounds.

The genus *Ectothiorhodospira* has the ability to grow by using atmospheric nitrogen (N_2) as its sole nitrogen source (6), thus providing evidence for the existence of nitrogenase activity. Nitrogenase is the enzyme responsible for the fixation of N_2 into ammonia (NH_3). In the absence of N_2 the enzyme can reduce many compounds, including protons with the production of H_2 gas (14), and some H_2 production even occurs during regular N_2 fixation (13). Since N_2 fixation is energy expensive, the accumulation of NH_3 (13) and ADP (2) acts to inhibit the enzyme complex.

Several factors involved in maximizing and partially characterizing H_2 production were examined. Hydrogen gas production was measured at various light intensities, increased concentrations of ammonium ion (NH_4^+) and $Na_2S \cdot 9H_2O$, and by the use of different carbon sources. The two more reduced carbon sources, propionate and succinate, allowed good gas production. Maximum production was also obtained at low NH_4^+ concentrations, at high light intensities, and with high concentrations of $Na_2S \cdot 9H_2O$. Similar results have previously been shown for the nonsulfur purple bacteria *Rhodopseudomonas* (*Rhodobacter*) *capsulata* (4) and *R. sphaeroides* (12).

The *Ectothiorhodospira* strain used in this study was an isolate from Soap Lake, Washington. This strain (10) closely resembles *Ectothiorhodospira vacuolata* isolated from soda lakes in Egypt (7). Both strains are rod shaped, gram negative, and motile by means of polar flagella and form gas vesicles. Both strains have spirilloxanthine as the major carotenoid and utilize acetate, pyruvate, propionate, malate, and succinate as sources of carbon and electron donors. Optimal growth for both is at 30 to 40°C, at pH 7.5 to 9.5, and with 1.0 to 6.0% NaCl. The Soap Lake strain has a G+C content of 62.8 mol% and that for *E. vacuolata* is in the range of 61.4 to 63.6 mol%.

The following medium was used for experiments unless otherwise specified (in grams per liter in distilled water): $CaCl_2 \cdot 2H_2O$, 0.4; NH_4Cl, 0.1; NaCl, 7.0; $MgSO_4 \cdot 7H_2O$, 0.01; KH_2PO_4, 0.1; sodium acetate $\cdot 3H_2O$, 2.0; iron citrate, 0.002; and trace element solution, 1.0 ml/liter (8). The medium was adjusted to pH 6.0 and autoclaved. Sterile $NaHCO_3$ was added to a concentration of 0.08% (1.6 ml of 5% $NaHCO_3$ per 100 ml of medium). Other carbon sources substituted for sodium acetate were added to obtain a final concentration of 0.353 g (29.4 mmol) of carbon per liter of medium. Experiments involving the effect of NH_4Cl on H_2 gas production were performed with NH_4Cl concentrations ranging from 0 to 0.2 g/liter.

Experiments were carried out in 25 ml of medium, using 27-ml aluminum-seal-type anaerobic culture tubes (Bellco Glass, Inc., Vineland, N.J.). Inoculum, grown overnight with 0.2 g of NH_4Cl per liter of medium, was concentrated to an absorbance of 20, and 0.4 ml was added per tube along with the sulfide. The tubes were stoppered, aluminum caps were added, and 10-ml syringes with 23-gauge, 1-in. (2.54-cm) needles were inserted into the airspace above the medium. Immediate syringe displacement of approximately 1.5 ml was observed. This gas was expelled and the syringes were reinserted. Experiments were performed at 35°C (Fisher Refrigerated Incubator; Fisher Scientific) and at approximately 1,500 lux units (LU). Light was provided continuously from a 60- or 100-W Sylvania light bulb. Recordings of gas collected were taken periodically until production ceased, usually within 48 h.

Light intensity experiments were performed at LU ranging from 0 to 1,500. Light readings were measured with a Li-Cor Light Photometer model LI-185 (Lambda Instrument Corp., Lincoln, Neb.).

Protein was used as a measurement of growth. Cell pellets from 5.0 ml of culture were digested with 1.0 N NaOH at 80°C for 30 min, and the extracted protein was determined by the method of Lowry et al. (11). Ammonium determinations were made with Nessler reagent and followed the protocol in the *Manual of Methods for General Bacteriology* (1).

Evidence for the presence of nitrogenase was determined by growing cultures anaerobically on nitrogen-free medium

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by the thioacetamide protocol of Irgens (9) and by assaying for nitrogenase, using the acetylene reduction procedure of Hardy et al. (3). A Varian Aerograph gas chromatograph (Varian Instrument Group, Walnut Creek, Calif.) was used with a Porapak R column, 80–100 mesh. The carrier gas was N_2, the temperature was 30°C, and the flow rate was 15 to 20 ml/min.

Chromatographic analysis of the gas above the medium was carried out on an HP-5890A gas chromatograph (Hewlett-Packard, Palo Alto, Calif.), using a thermal conductivity detector. A Chromosorb 101 column (80–100 mesh) was used. Helium was the carrier gas, used at 70°C and a flow rate of 17.0 ml/min. Standards of CO_2 and NH_3 were run on the column.

Gas produced by the *Ectothiorhodospira* sp. was chromatographically analyzed to determine whether any component besides H_2 was present. The presence of air (O_2, N_2, and argon) was demonstrated and was probably introduced during inoculation, since inoculation took place under aerobic conditions. Carbon dioxide and ammonia were not found in the gas above the medium. By process of elimination, H_2 was assumed to be the main gas produced by the organism. This assumption was reinforced by the characteristic H_2 "pop" produced by the gas when the culture tube was flamed.

Chromatographic analysis showed that the *Ectothiorhodospira* sp. could reduce acetylene, thereby proving a positive test for the presence of the nitrogenase. A 5-ml amount of culture (ca. 0.4 mg of protein per ml) generated hydrogen gas linearly at the rate of 2 nmol/min over a 55-min period.

The amount of NH_4Cl in the medium affected the amount of H_2 produced. Figure 1A shows a decrease in the amount of gas produced with increasing NH_4Cl concentrations. This graph also reveals a steady rise in cell numbers, as measured by protein, with increasing NH_4Cl concentrations. Figure 1B shows the relationship between the depletion of nitrogen and the evolution of H_2 by the *Ectothiorhodospira* sp. over time in a medium originally containing 0.1 g of NH_4Cl per liter of medium. Therefore, decreasing the amount of nitrogen (NH_4^+) available to the organism stimulated H_2 production.

Ten carbon sources were tested to determine their effects on H_2 evolution. Four compounds, ethanol, butyric acid, lactate, and glycerol, proved to be poor substrates and did not allow growth. Of the other six compounds, namely, acetate, pyruvate, propionate, fumarate, malate, and succinate, the ones with the greater hydrogen/oxygen ratio supported the larger amounts of gas production. Propionate with an H/O ratio of 3 allowed the production of 16 ml of H_2 per 25 ml of culture, whereas pyruvate with an H/O ratio of 1 supported the production of 7.0 ml of H_2 per 25 ml.

The amount of sodium sulfide in the medium affected H_2 production by the *Ectothiorhodospira* sp. Cells receiving large amounts of sodium sulfide in one batch did not produce as much gas as cells receiving the same amount over an extended period of time (Fig. 2). Intermittently fed cells also grew better than cells fed in one batch, probably because of a toxic reducing effect of excessive sulfide concentration on cellular components.

The graphs in Fig. 3 demonstrate the effect of increasing light intensities on H_2 evolution by the *Ectothiorhodospira* sp. Figure 3A shows the amount of H_2 gas collected at the end of 48 h when cultures were incubated at different LU. The graph also shows that the most efficient light harvesting occurs at 400 LU; i.e., at this light intensity the volume of H_2 gas produced per LU provided is the greatest. The maximum rate of growth, possibly associated with saturation of the cyclic electron transport system, occurs at a light intensity of 2,000 LU or greater. Figure 3B shows the effect of light intensity on H_2 gas production when parallel sets of cultures with increasing sulfide concentrations were incubated at different light intensities. As before, the total volume of gas

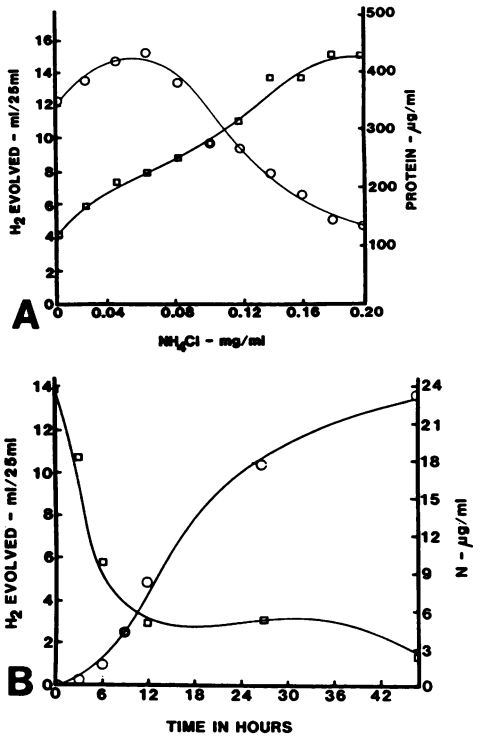

FIG. 1. Hydrogen gas production and protein content of the *Ectothiorhodospira* sp. cultured at increasing ammonium chloride concentrations (A) and hydrogen gas production and nitrogen depletion of *Ectothiorhodospira* cultures over time (B). (A) Symbols: (○) hydrogen gas production; (□) protein content of cultures, as measured by the Lowry method, after the cultures had reached maximal gas production (about 2 days). (B) Symbols: (○) hydrogen gas production; (□) nitrogen content remaining in the cultures as ammonium ions, as measured with Nessler reagent.

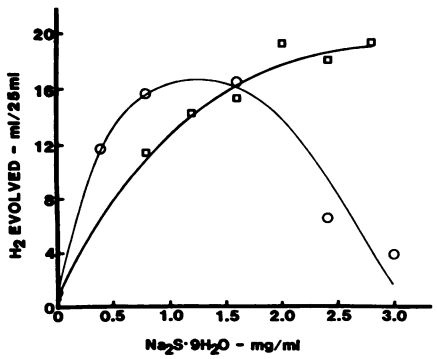

FIG. 2. Hydrogen gas production by the *Ectothiorhodospira* sp. cultured at increasing concentrations of sodium sulfide. Symbols: (○) gas production by cultures fed their respective sodium sulfide amounts at zero time; (□) gas production by continuous cultures intermittently fed small amounts of sodium sulfide until the final concentration reached that of the parallel cultures, which were batch fed at zero time.

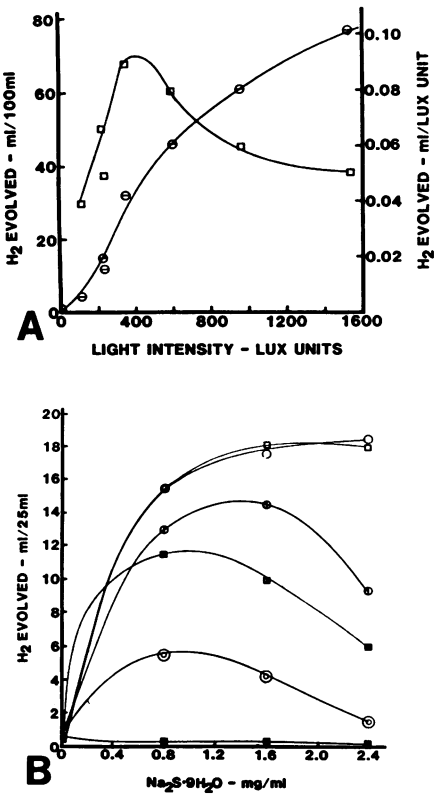

FIG. 3. Hydrogen gas production by the *Ectothiorhodospira* sp. cultured at increasing light intensities (A) and grown at different sodium sulfide concentrations and at different light intensities (B). (A) Expressed as both milliliters of gas per 100 ml of medium (○) and per lux unit (□). (B) Symbols: (■) 0 LU; (◎) 300 LU; (◨) 800 LU; (◐) 1,300 LU; (○) 1,800 LU; (□) 2,300 LU. The total gas volume generated was measured at 48 h.

generated was measured after 48 h. At low light intensities, gas production of one set of tubes first increased and then decreased with increasing sulfide concentrations. At the higher light intensities, there was no decrease in gas production with increasing sulfide concentrations. These data provide strong support for the argument that light (energy) is essential for the oxidation and conversion of excessive reducing power (sulfide) to H_2 gas.

In this communication we have shown that the *Ectothiorhodospira* isolate possesses nitrogenase activity. The experiments as performed have helped us understand the physiology behind this gas production and have given us ideas as to how this output can be maximized.

Hillmer and Gest (4, 5), in 1977, and Macler et al., in 1979 (12), demonstrated that the greatest and fastest production of H_2 gas by *Rhodopseudomonas* species occurred by using anaerobic photoheterotrophic growth on reduced carbon compounds in ammonium-minimal medium (12). The same can be said about the *Ectothiorhodospira* sp.

Because of the physiology of the *Ectothiorhodospira* sp., there is a positive connection among light intensity, sulfide concentration, and H_2 gas production.

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