Increased photoproduction of hydrogen by non-autotrophic mutants of *Rhodopseudomonas capsulata*

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(Received 19 September 1983/Accepted 13 January 1984)

1. Non-autotrophic (Aut^-) mutants of *Rhodopseudomonas capsulata* B10 were tested for their efficiency of nitrogenase-mediated H_2 production. 2. Three of these mutants (IR3, IR4 and IR5) showed an increased stoichiometry of H_2 production, mediated by nitrogenase, from certain organic substrates. For example, in a medium containing 7 mM-L-glutamate as nitrogen source, strain IR4 produced 10–20% more H_2 than did the wild type with DL-lactate or L-malate as major carbon source, 20–50% more H_2 with DL-malate, and up to 70% more with D-malate. 3. Strain IR4 was deficient in 'uptake' hydrogenase activity as measured by H_2 -dependent reduction of Methylene Blue or Benzyl Viologen. However, this observation did not explain the increased efficiency of H_2 production, since H_2 uptake (H_2 recycling) was undetectable in cells of the wild type. Instead, increased H_2 production by the mutant appeared to be due to an improved conversion of organic substrates to H_2 and CO_2 , presumably due to an altered carbon metabolism. 4. The metabolism of D-malate by different strains was studied. An NAD^+ -dependent D-malic enzyme was synthesized constitutively by the wild type, and showed a K_m for D-malate of 3 mM. The activity of this enzyme was approx. 50% higher in strain IR4 than in the wild type, and the mutant also grew twice as fast as the wild type with D-malate as sole carbon source.

The photosynthetic bacterium *Rhodopseudomonas capsulata* is able to produce H_2 at substantial rates in the light. This photoproduction of H_2 is due to the enzyme nitrogenase, which, in the absence of alternative substrates, is able to reduce protons to H_2 (for reviews, see Gest, 1972; Yoch, 1978; Meyer *et al.*, 1978a). Anaerobic conditions and high light intensities are required, in addition to an effectively metabolized substrate. Hillmer & Gest (1977a) found that the highest rates of H_2 production were shown by cells grown with DL-lactate or pyruvate as carbon and energy source and glutamate as growth-limiting nitrogen source; lactate was converted to H_2 and CO_2 with a yield of 72% of the theoretical maximum. Other organic acids gave slightly lower rates and yields of H_2 production, while sugars were utilized much less efficiently. The rate of H_2 production could be doubled by growth in nitrogen-limited continuous culture (Jouanneau *et al.*, 1982), and yields approaching 80% were obtained (Jouanneau, 1982). However, despite these investigations, little is yet known of the regulatory mechanisms which deter-

mine the efficiency of H_2 production from different substrates.

In addition to nitrogenase, *R. capsulata* possesses a membrane-bound hydrogenase, which appears to function exclusively in H_2 uptake, i.e. H_2 oxidation under physiological conditions (Colbeau *et al.*, 1980). This enzyme enables *R. capsulata* to grow autotrophically on a mixture of H_2 and CO_2 (Klemme, 1968). Hydrogenase is also synthesized under heterotrophic growth conditions and is present in the highest levels in H_2 -evolving cultures, suggesting that its synthesis is induced by H_2 (Colbeau *et al.*, 1980). However, the physiological role of hydrogenase under heterotrophic conditions is not known. In other diazotrophic bacteria, it has been suggested that the role of hydrogenase is to recycle H_2 evolved by nitrogenase, thereby increasing the energetic efficiency of the nitrogen fixation reaction; or, alternatively, to facilitate the protection of nitrogenase against oxygen by allowing H_2 to serve as a substrate for the aerobic respiratory chain (see Dixon, 1972; Robson & Postgate, 1980). In *R. capsulata*, H_2 can

serve as a substrate for the aerobic respiratory chain (Klemme, 1968; Paul *et al.*, 1979), participate in the photoreduction of CO₂ (Hillmer & Gest, 1977b; Kelley *et al.*, 1977; Jouanneau *et al.*, 1980), or act as an electron donor to nitrogenase (Meyer *et al.*, 1978a,b; Hallenbeck & Vignais, 1981). However, recycling of H₂ evolved by nitrogenase has been observed only in cells depleted of organic substrates (Kelley *et al.*, 1977; Jouanneau *et al.*, 1980) suggesting that H₂ recycling is negligible in cultures actively evolving H₂ (see also Willison & Vignais, 1982).

In order to investigate the factors influencing the production and utilization of H₂, we have isolated mutants of *R. capsulata* unable to grow photoautotrophically on H₂ and CO₂. Some of these mutants were found to produce increased amounts of H₂ from various organic substrates. This increased H₂ production was found to be unrelated to specific defects in the enzymes of autotrophic metabolism, and appeared instead to be due to an altered carbon metabolism, which affects the flow of reducing equivalents from organic substrates to nitrogenase.

Experimental

Bacterial strain and growth conditions

Rhodospseudomonas capsulata wild type strain B10 was kindly supplied by the Photosynthetic Bacteria Group, Bloomington, IN, U.S.A. Pre-cultures were grown photosynthetically at 30–34°C in a mineral salts (RCV) medium (Weaver *et al.*, 1975; Hillmer & Gest, 1977a) supplemented with 30 mM-DL-malate and 7.5 mM-(NH₄)₂SO₄ as described previously (Willison & Vignais, 1982). For enzyme assays and measurements of H₂ production, the growth medium contained 7 mM-L-glutamate as sole nitrogen source, in place of (NH₄)₂SO₄. 'Carbon-free medium' was prepared by the omission of the carbon source.

Isolation of non-autotrophic mutants

Non-autotrophic (Aut⁻) mutants were isolated after mutagenesis of strain B10 with either ethyl methanesulphonate (Meynell & Meynell, 1970) or *N*-methyl-*N'*-nitrosoguanidine (Willison & Vignais, 1982). The mutagenized culture was grown photosynthetically to early stationary phase in RCV medium, diluted 10-fold in RCV medium and incubated for 2 h in the light. The cells were then washed three times by centrifugation and resuspension in carbon-free RCV medium and incubated aerobically in the dark for 4 h to deplete the cells of endogenous substrates. The cell suspension (approx. 2×10^8 cells/ml) was then diluted 10-fold into carbon-free RCV medium containing 100 units of penicillin G/ml and

incubated in a Gas-Pak anaerobic jar (BBL, Cockeysville, MD, U.S.A.) in the light for 24 h. The Gas-Pak H₂+CO₂ generator was activated with 25% (w/v) KH₂PO₄ in order to maximize evolution of CO₂ (Madigan & Gest, 1979). Penicillin was next removed by washing twice as described above, and 0.1 ml aliquots of a 10- to 100-fold dilution of the suspension were plated on rich (YPS) agar medium (Weaver *et al.*, 1975). After 48 h aerobic incubation in the dark, colonies were replica-plated onto carbon-free RCV agar medium, and incubated anaerobically in the light for a further 48 h. Colonies which grew poorly on the replicate plates were picked from the YPS plates, purified, and re-tested for lack of autotrophic growth.

Isolation of spontaneous autotrophic revertants

Spontaneous autotrophic revertants of non-autotrophic strains were isolated by an enrichment procedure. An exponential phase culture of the Aut⁻ strain [(2–3) × 10⁸ cells/ml] was washed and starved of carbon substrates as described above and then diluted 10-fold into carbon-free RCV medium. When the culture became turbid, usually after 5–6 days incubation in a Gas-Pak anaerobic jar, appropriate dilutions were plated onto YPS agar medium, and Aut⁺ revertants were identified by replica-plating of colonies.

Assay of hydrogenase activity

Hydrogenase activity with Methylene Blue as electron acceptor was assayed in whole cells as described by Colbeau *et al.* (1980).

H₂-dependent Benzyl Viologen reduction was assayed qualitatively by using a filter paper assay. Colonies were grown photosynthetically on RCV agar medium containing 7 mM-glutamate as nitrogen source to ensure maximal hydrogenase synthesis (Colbeau *et al.*, 1980), and transferred to a piece of Whatman 3MM filter paper soaked in a solution containing 20 mM-Tris/HCl, pH 8.0, 5 mM-Benzyl Viologen and 0.1% Triton X-100. The filter paper was placed on top of several layers of moist filter paper in a Petri dish containing the same medium, and incubated under an atmosphere of H₂ for 1 h. Benzyl Viologen reduction was indicated by the appearance of a purple-red colouration. The stain could be fixed by spraying the filter paper with an anaerobic solution of 1% triphenyl tetrazolium chloride, after which positively reacting cells appeared red and negatively reacting cells became green.

Measurement of H₂ and CO₂ production by bacterial cultures

Cultures were grown in 55 ml-capacity glass syringe bodies filled completely with medium and

sealed at the top with a rubber serum cap and at the bottom with a syringe needle inserted into a rubber stopper. The cultures were incubated at 34°C in a glass-sided water bath illuminated laterally by three 100 W incandescent lamps placed at a distance of 15 cm. The gas produced by the cultures was collected, via short lengths of gas-tight Teflon tubing, in inverted measuring cylinders filled with water, which was acidified to minimize absorption of CO₂. All joints were well sealed with vacuum grease to minimize loss of H₂. The volume of CO₂ in the gas phase was determined by absorption with NaOH and the residual gas (85–90% of the total) was confirmed to be H₂ by g.l.c. The amount of dissolved CO₂ and bicarbonate in the medium was estimated by withdrawing samples of culture with a gas-tight syringe (after injection of an equivalent volume of argon), injecting the sample into a sealed container containing H₂SO₄ (1 M final concentration) and measuring the volume of gas produced. This volume was decreased by about 10% if dissolved CO₂ was first removed from the culture by agitation, suggesting that most of the dissolved CO₂ was present in the form of bicarbonate. Cultures at the end of H₂ production contained 40–50 mM-HCO₃⁻, at pH 7.3.

Measurement of H₂ uptake

H₂ uptake by cell suspensions was measured as described previously (Willison & Vignais, 1982). When starved cells were required, these were obtained by washing the cells twice by centrifugation and resuspension in a mineral salts medium devoid of carbon and nitrogen source (Willison & Vignais, 1982) and incubating them under argon, at 30°C for 3 h in the light. Samples were then injected into argon-filled vials, and H₂ was added to the gas phase to 1% (v/v) final concentration.

Other enzyme assays

Nitrogenase activity was assayed by acetylene reduction or H₂ production as described previously (Willison & Vignais, 1982) except that samples of culture were injected directly into argon-filled vials, without washing.

Ribulose biphosphate carboxylase activity was assayed in toluenized cells as described by Maier (1981). The radioactivity incorporated from NaH¹⁴CO₃ into the acid-stable fraction was counted in an Intertechnique liquid-scintillation counter, with a 90% counting efficiency. The radioactivity (c.p.m.) differed by less than 10% in duplicate assays, and the radioactivity in controls from which ribulose biphosphate was omitted was less than 0.5% of the assay values.

NAD⁺-dependent D-malic enzyme was assayed in the soluble fraction of cell-free extracts after sonification and differential centrifugation as

described by Scrutton (1973). Cell extracts were prepared by sonification and differential centrifugation as described by Hallenbeck *et al.* (1982).

Miscellaneous determinations

Bacterial growth was followed turbidometrically by measuring the absorbance of cultures at 660 nm (Meyer *et al.*, 1978b). Cell dry weights were determined as described by Willison & Haddock (1981) and the amount of cell carbon was calculated by assuming it to represent 50% by weight of dry cell material (van Gemerden, 1968; Göbel, 1978). Poly-(β-hydroxybutyrate) was assayed as described by Law & Slepecky (1961), while total cellular polysaccharide and glycogen were determined by the anthrone method (Herbert *et al.*, 1971). The concentration of L-malate in cell-free culture supernatants was determined enzymically (Gutmann & Wahlefeld, 1974). Protein in whole cells and extracts was assayed as described by Maier (1981).

Chemicals

NaH¹⁴CO₃ was obtained from the Service des Molécules Marquées, Saclay, France. Ultra-pure H₂ and argon (<1 p.p.m. O₂) and acetylene were obtained from l'Air Liquide-Grenoble. Ribulose biphosphate, D-malate, L-malate and penicillin G were obtained from Sigma Chemical Co., while enzymes and coenzymes were purchased from Boehringer. All other reagents were of research-grade quality and were purchased from either Sigma or Prolabo.

Results and discussion

Activities of autotrophic enzymes in non-autotrophic mutants

The non-autotrophic (Aut⁻) mutants IR1 and IR2 were obtained after mutagenesis with ethyl methanesulphonate, while strains IR3, IR4, IR5 and IR6 were isolated after mutagenesis with *N*-methyl-*N'*-nitrosoguanidine. Two strains, IR2 and IR4, were found to be deficient in hydrogenase activity, when this was assayed with Methylene Blue as electron acceptor (Table 1). These strains also gave a negative reaction for H₂-dependent Benzyl Viologen reduction which was tested using a filter paper assay. This procedure could presumably be used to screen colonies for hydrogenase-deficient mutants without prior selection for lack of autotrophic growth. Rocket immunoelectrophoresis of cell-free extracts, followed by activity staining, showed that strains IR2 and IR4 contain decreased amounts of catalytically active hydrogenase protein compared with the wild-type strain, suggesting that they are affected in the synthesis rather than in the specific activity of the hydrogenase protein (Colbeau & Vignais, 1983).

Table 1. *Activities of autotrophic enzymes and stoichiometry of H₂ production in wild type and mutant strains*

The wild type strain, B10, non-autotrophic (Aut⁻) mutants and strain MDO1, a spontaneous Aut⁺ revertant of IR4, were grown photosynthetically to stationary phase in RCV medium supplemented with 30 mM-DL-malate and 7 mM-L-glutamate. Enzyme activities and H₂ production were measured as described in the Experimental section. The theoretical yield of H₂ was calculated after allowing for the assimilation of carbon substrates into cell material, assuming a formula for cell composition of C₅H₈O₂N (van Gernerden, 1968). The values shown are the means of determinations on at least two (enzyme activities) or four (H₂ production) separate cultures, and where appropriate are given $\pm$ S.E.M., with the number of determinations in parentheses. Abbreviation: n.d., not determined.

Strain	Phenotype	Hydrogenase activity (μ mol of Methylene Blue/h per mg of protein)	Ribulose biphosphate carboxylase activity (μ mol of CO ₂ reduced/h per mg of protein)	Yield of H ₂ (% of theoretical maximum)
B10	Aut ⁺	13.4 $\pm$ 5.6 (6)	2.21 $\pm$ 0.33 (6)	51.2
IR1	Aut ⁻	9.7	1.09	50.0
IR2	Aut ⁻	3.1 $\pm$ 1.4 (5)	1.88	49.7
IR3	Aut ⁻	11.0	1.21	61.6
IR4	Aut ⁻	0.8 $\pm$ 0.6 (6)	1.45	64.0
IR5	Aut ⁻	9.4	2.03	63.7
IR6	Aut ⁻	13.2	2.07	32.0
MDO1	Aut ⁺	11.3	n.d.	44.6

During photo-autotrophic growth of *R. capsulata*, CO₂ is assimilated via the Calvin cycle (Stoppani *et al.*, 1955). Ribulose biphosphate carboxylase, one of the key enzymes of this cycle, was found in high levels in *R. capsulata* strain B10 grown photoheterotrophically in malate/glutamate or malate/NH₄⁺ (Table 1), suggesting that synthesis of this enzyme is at least partially constitutive. Furthermore, no increase in activity was detected if cells were washed free of carbon source and incubated in the light for 48 h under an atmosphere of H₂/CO₂/Ar (10:1:89). All the Aut⁻ mutants isolated in this study contained at least 50% of the wild type level of ribulose biphosphate carboxylase activity when grown photoheterotrophically, so the Aut⁻ phenotype of these mutants could not be attributed to a deficiency in this enzyme. Other lesions which might result in an Aut⁻ phenotype are the loss of an electron-transport component involved in coupling hydrogenase to the photosynthetic electron transfer chain, or the loss of phosphoribulokinase.

The Aut⁻ mutants showed significant, but limited, growth on carbon-free RCV agar medium. The difference in growth rate between the mutant strains and the wild type was most marked when 2-day-old heterotrophically grown colonies were replicated onto carbon-free medium and examined after a further 2 days incubation. Experimental cultures were routinely screened for revertants using this method, and Aut⁺ cells were found at a level of <0.1%. The apparent photoautotrophic growth of Aut⁻ mutants on carbon-free agar medium could have been due either to leakiness of the Aut⁻ phenotype, or to use of impurities in the

agar medium as carbon source. That the former was the case was suggested by the observation that, during the enrichment process used to isolate Aut⁺ revertants, only about 1% of the cells which grew in liquid carbon-free medium were phenotypically Aut⁺.

Comparison of H₂ photoproduction by wild type and mutant strains

Three mutants, IR3, IR4 and IR5, produced on average 30% more H₂ than did the wild-type strain in a medium containing 30 mM-DL-malate and 7 mM-L-glutamate; the other mutants, and MD01, a spontaneous Aut⁺ revertant of IR4, produced amounts similar to, or lower than, that of the wild type (Table 1). It is clear from Table 1 that there was no strict correlation between the activity of hydrogenase with artificial electron acceptors and whether or not increased H₂ production was observed. The yield of H₂ was calculated as a percentage of the theoretical maximum for the complete conversion of malate and glutamate to H₂ and CO₂, after allowing for the amount of substrate assimilated into cell material (see below). The yield of 58.8% calculated for strain B10 was in good agreement with the value of 56% obtained in a previous study (Hillmer & Gest, 1977a); the improved yield calculated for strain IR4 was 75.2%.

The effects of varying either the carbon or nitrogen source on the stoichiometry of H₂ production by strain IR4 and the wild type are shown in Table 2. With 7 mM-L-glutamate as nitrogen source, strain IR4 produced between 16% and 20% more H₂ than did the wild type when DL-lactate or

Table 2. Effect of carbon and nitrogen source on the stoichiometry of H_2 production and the activity of NAD^+ -dependent D-malic enzyme in strains B10 and IR4

Strains were grown photosynthetically in RCV medium supplemented with 30 mM carbon source and the nitrogen source shown. The values given are the means of determinations made in duplicate in at least two separate experiments, and where appropriate are given $\pm$ S.E.M., with the number of determinations in parentheses. Abbreviation: n.d., not determined.

Carbon source	Nitrogen source	H ₂ production (ml/ml of culture)		Activity of NAD^+ -dependent D-malic enzyme (nmol of NADH formed/min per mg of protein)	
		B10	IR4	B10	IR4
DL-Malate	Glutamate (7 mM)	1.84 ± 0.27 (9)	2.55 ± 0.62 (9)	376	509
DL-Malate	Glutamate (5 mM)	1.47	2.03	n.d.	n.d.
DL-Malate	Glutamate (3 mM)	1.49 ± 0.06 (3)	1.53 ± 0.01 (3)	n.d.	n.d.
DL-Malate	(NH ₄) ₂ SO ₄ (1.12 mM)	1.35	1.38	n.d.	n.d.
L-Malate	Glutamate (7 mM)	2.56	3.14	402	644
D-Malate	Glutamate (7 mM)	1.27	2.09	382	633
DL-Lactate	Glutamate (7 mM)	2.54 ± 0.22 (4)	2.93 ± 0.29 (4)	n.d.	n.d.

L-malate were provided as major carbon source and approx. 70% more H_2 with D-malate as carbon source. With DL-malate as carbon source, strain IR4 produced between 20% and 50% more H_2 in the presence of high concentrations of L-glutamate (≥ 5 mM) as nitrogen source, but produced similar amounts of H_2 to the wild type when lower concentration of L-glutamate, or 2.25 mM-NH₄⁺, were given as limiting nitrogen source.

The kinetics of H_2 production from DL-malate by strains IR4 and B10 are shown in Fig. 1. The time course of H_2 production was biphasic in both strains, but both the rate and the extent of the second (slower) phase of H_2 production were much greater in strain IR4 than in B10. Since the decrease in rate of H_2 production coincided with the disappearance of L-malate from the medium, it seems probable that the rapid phase of H_2 production was due to the utilization of L-malate, while the slower phase corresponded to the utilization of the D-isomer. In support of this, the rate of H_2 production by strain IR4 in D-malate/glutamate medium was more than twice as high as that of the wild type ($32.8 \mu\text{l/h}$ per mg cell dry wt. versus $14.6 \mu\text{l/h}$ per mg cell dry wt.).

In the experiment shown in Fig. 1, the initial rate of H_2 production by strain IR4 ($127 \mu\text{l/h}$ per mg cell dry wt.) was approx. 40% higher than that of the wild type ($95 \mu\text{l/h}$ per mg cell dry wt.), the latter value being in good agreement with previously published data (Hillmer & Gest, 1977a). However, the nitrogenase activity measured by the rate of acetylene reduction was similar in the two strains, varying from $4.0 \mu\text{mol/h}$ per mg dry wt. during the initial phase, to $1.0 \mu\text{mol/h}$ per mg dry wt. at the end of H_2 production. These apparently conflicting results may reflect differences in the relative rates of H^+ reduction and acetylene

Fig. 1. Kinetics of H_2 production by the wild type strain B10 and the Aut^- mutant IR4

Strains were grown as described in the Materials and methods section, in RCV medium supplemented with 30 mM-DL-malate and 7 mM-glutamate. The percentage of H_2 in the gas phase (85–90%) was determined at the end of the experiment, and the volume of H_2 evolved at earlier times was calculated assuming that the composition of the gas phase remained constant throughout the experiment.

reduction by nitrogenase in the two strains (cf. Willison & Vignais, 1982). Surprisingly, cells withdrawn from cultures that had ceased to produce H_2 and incubated under argon produced H_2 at rates between 400 and 800 nmol/h per mg cell dry wt. for up to 3 h. Washing the cells decreased the rate of H_2 production 3–10-fold, unless 50 mM-NaHCO₃ (pH 7.3) was added to the incubation medium. These results show that the cessation of H_2 production by bacterial cultures is not due to exhaus-

tion of endogenous substrates or to the irreversible inactivation of nitrogenase.

Utilization of D-malate by *R. capsulata*

NAD⁺-dependent D-malic enzyme, the enzyme responsible for the oxidation of D-malate (Stern & Hegre, 1966) was assayed in the soluble fraction of cell-free extracts, and was found to be present in strain IR4 at level 50% higher than in strain B10 (Table 2). This enzyme appears to be synthesized constitutively, as previously reported for *Rhodospirillum rubrum* (Stern & Hegre, 1966). The specific activity of the enzyme was 25-fold lower with NADP⁺ as cofactor than with NAD⁺. The K_m for D-malate of the enzyme was approx. 3mM in both the mutant and the wild-type strain. Strain IR4 also grew more rapidly than strain B10 with D-malate as sole carbon source (Fig. 2). Growth of the wild type was biphasic, with the growth rate increasing to a value similar to that of the mutant after about 60h.

The increased activity of NAD⁺-dependent D-malic enzyme in strain IR4 might explain the higher rate of H₂ production from D-malate, and the higher growth rate on D-malate, but it does not explain the increased yield of H₂ shown by this strain. Furthermore, there appeared to be no strict correlation between increased growth rate on D-malate and increased H₂ production among different strains. Thus, strain IR3, which showed increased H₂ production, grew at the same rate as B10 on D-malate, while the Aut⁺ revertant, MD01, showed wild type levels of H₂ production, but grew

at the same rate on D-malate as its parent strain, IR4. The latter observation suggests that the increased growth rate on D-malate of strain IR4 is due to a different mutation from that which confers increased H₂ production and the Aut⁻ phenotype.

H₂ uptake and carbon balance in H₂-producing cultures

At least one of the mutants which showed increased H₂ production, strain IR4, was deficient in uptake hydrogenase activity. The possibility must therefore be considered that the increase in H₂ production was due to a decreased reutilization (recycling) of evolved H₂. Indeed, the recent observation that H₂ production from cellulose was greater in bacterial co-cultures of *Cellulomonas* and a hydrogenase-deficient mutant of *R. capsulata* than in co-cultures of *Cellulomonas* and the wild type was attributed to the absence of H₂ recycling in the mutant (Odom & Wall, 1983). In previous studies, however, H₂ recycling, either for the photoreduction of CO₂ (Kelley *et al.*, 1977; Jouanneau *et al.*, 1980) or for the reduction of nitrogenase (Meyer *et al.*, 1978a; Hallenbeck & Vignais, 1981), has been observed only in cells depleted of endogenous carbon substrates. In the present study, we failed to observe H₂ uptake, either in starved cells, or in unstarved cells treated with 5% (v/v) acetylene to inhibit H₂ production by nitrogenase (cf. Willison & Vignais, 1982). [Acetylene at 5% (v/v) concentration has no effect on uptake hydrogenase activity in *R. capsulata* (Colbeau *et*

Fig. 2. Growth of strains B10 and IR4 with D-malate as sole carbon source

Strains were grown photosynthetically in 17-ml capacity stoppered tubes in RCV medium supplemented with 20mM-D-malate and 7.5mM-(NH₄)₂SO₄. The turbidity of cultures at 660nm was measured directly and converted to a value proportional to cell density by using a calibration curve. The doubling times calculated from the linear portion(s) of each growth curve were 13.0h for IR4, and 26.8h and 13.6h for B10.

al., 1980).] Moreover, no stimulation of acetylene reduction by H_2 was observed in cells taken from cultures before, during or after the phase of H_2 production.

Another explanation for the increased H_2 production by certain Aut^- mutants was suggested by consideration of the carbon balance in cultures grown on malate/glutamate medium. The amount of organic carbon remaining in the culture supernatant, i.e. neither incorporated into cell material nor converted to CO_2/HCO_3^- , was calculated to be 37.4% for strain B10 and 31.0% for the mutant strain IR4, indicating that the mutant is more efficient in converting organic substrates to H_2 and CO_2 . This difference was not due to an increased utilization of storage materials by the mutant, since at the end of H_2 production the levels of total cell polysaccharide (approx. 250 mg/g cell dry wt.), glycogen (40 mg/g cell dry wt.) and poly(β -hydroxybutyrate) (0.15 mg/g cell dry wt.) were similar in both strains.

It has been suggested that the physiological role of nitrogenase-mediated H_2 production is to dissipate excess ATP and reducing equivalents which are synthesized when growth is limited by factors other than energy source (Gest, 1972). It has also been reported that a strain of *R. capsulata* that does not photoproduce H_2 excretes relatively large amounts of substances absorbing at 330 nm at pH 12, characteristic of the α -oxo-acid precursors of aromatic amino acids (Chakrabarti & Smith, 1981). These authors proposed that the formation and excretion of metabolic end-products might serve as an alternative electron-sink to nitrogenase for the regulation of the intracellular redox state. The absorbance at 330 nm at pH 12 was measured in cell-free supernatants from malate/glutamate cultures that had ceased to produce H_2 . On average, in determinations on five separate cultures, the absorbance for strain IR4 was 2.36 ± 0.77 compared with 0.87 ± 0.42 for the wild type. Since D-malate shows no significant absorbance at this wavelength, these results suggest that strains IR4 and B10 differ in the quantity of metabolites excreted into the growth medium.

The results presented in this report show that, as previously described for a glucose-utilizing mutant of *Rhodopseudomonas sphaeroides* (Macler *et al.*, 1979), the efficiency of H_2 photoproduction from organic substrates can be increased by genetic mutation. Our experiments with mutants indicate that there is an important relationship between photoproduction of H_2 by nitrogenase and the pathways of carbon metabolism in the cell. In these mutants, both the photoproduction of H_2 from organic substrates and autotrophic growth are affected, suggesting a regulatory link between autotrophic and heterotrophic metabolism. Fur-

ther study of these mutants, and, in particular, identification of the end-products excreted and the enzymes involved in their formation, should provide insight into these regulatory processes.

We should like to thank Isabelle Roudil and Patrice Allibert for assistance, Dr. A. Colbeau for performing the hydrogenase assays and Dr. J. M. Odom for many helpful discussions and suggestions. The assistance of Jacqueline Roux in the preparation of this manuscript is gratefully acknowledged. This work was supported by grants from the Centre National de la Recherche Scientifique (APP PIRSEM) and the EEC Solar Energy Research and Development Program (ESD 18-F).

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