Effect of Hydrogen, Sucrose and Oxygen on Uptake Hydrogenase in Nitrogen-fixing and Ammonium-grown *Pseudomonas saccharophila* ATCC 15946

By WILFREDO L. BARRAQUIO AND ROGER KNOWLES*

Department of Microbiology, Macdonald College of McGill University, 2111 Lakeshore Road,
Ste Anne de Bellevue, PQ H9X 1C0 Canada

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Pseudomonas saccharophila, grown microaerobically in batch culture, showed much higher uptake hydrogenase activity in N_2 -fixing than in NH_4^+ -grown cultures. Hydrogenase synthesis was induced by H_2 under a low partial pressure of O_2 and under air, in autotrophic as well as in heterotrophic conditions, provided that the sucrose concentration was relatively low. Sucrose at 15 mM repressed hydrogenase formation but did not inhibit preformed activity. Other utilizable carbon substrates repressed, while non-utilizable substrates did not repress, hydrogenase synthesis. The activity, unlike nitrogenase, was not sensitive to O_2 but hydrogenase synthesis was partially repressed by O_2 .

INTRODUCTION

Pseudomonas saccharophila is a facultative chemoautotroph which was isolated by $H_2/CO_2/O_2$ enrichment from the mud of a stagnant pool; so far only one strain of the species is known (Doudoroff, 1940; Palleroni, 1984). Recently, some isolates from surface sediment of freshwater lakes were assigned to *P. saccharophila* (Schink & Zeikus, 1984) but further characterization is needed to verify their identity. The autotrophic and heterotrophic metabolism of *P. saccharophila* has long received attention (e.g. Donawa *et al.*, 1971; Doudoroff, 1940; Palleroni, 1956), but its ability to fix N_2 was not critically examined and it was thus reported as non- N_2 -fixing (Palleroni, 1984). Recently, we found that among the known hydrogen pseudomonads tested only *P. saccharophila* could fix N_2 , and that it does so under both heterotrophic and autotrophic conditions (Barraquio *et al.*, 1986).

Nitrogenase catalyses not only N_2 reduction but also ATP-dependent H_2 evolution. The oxidation of evolved H_2 by a N_2 -fixing culture may recover some of the energy lost through H_2 production, may prevent inhibition of nitrogenase by hydrogen, and may provide protection to nitrogenase by removal of excess oxygen (Dixon, 1972). Such H_2 recycling occurs in *Azotobacter chroococcum* (Walker & Yates, 1978), *Rhizobium japonicum* (Emerich *et al.*, 1979) and *Rhizobium leguminosarum* bacteroids (Salminen & Nelson, 1984). Uptake hydrogenase, the enzyme responsible for the unidirectional conversion of molecular H_2 to protons, is found in several other diazotrophic genera (e.g. *Azospirillum*, *Alcaligenes*, *Derxia* and *Xanthobacter*) (Bowien & Schlegel, 1981). The hydrogenase system of these organisms, however, is regulated differently by O_2 and H_2 , and by carbon and nitrogen sources.

It was suggested that since *P. saccharophila* can utilize H_2 as an electron donor during chemoautotrophic N_2 -fixation, it may have the ability to utilize the H_2 evolved during heterotrophic N_2 -fixation (Barraquio *et al.*, 1986). We report here the presence of an active uptake hydrogenase in *P. saccharophila* during heterotrophic N_2 -fixation, and the regulation of its activity and synthesis by H_2 , sucrose and O_2 .

Abbreviations: CM, chloramphenicol; MB, methylene blue; PMSF, phenylmethylsulphonyl fluoride.

METHODS

Organism and growth. The bacterial strain used in this study, *P. saccharophila* ATCC 15946, was the same culture as used previously (Barraquio *et al.*, 1986). Stock cultures were kept in screw-cap 4 ml vials containing 0.5 ml dense cell suspension and 0.5 ml half-strength Difco nutrient broth plus glycerol (10%, v/v) at -80°C . A new stock culture was used for each experiment.

For inocula and batch cultures we used the mineral salts plus sucrose (0.1%) plus NH_4Cl (0.1%) medium described by Barraquio *et al.* (1986), except that the phosphate concentration was reduced from 50 mM to 16.7 mM and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ was reduced from 0.2 to 0.02 g l^{-1} . The pH of the medium was 6.8. NH_4Cl was omitted in the nitrogen-deficient medium. Yeast extract was routinely added at 0.01% (w/v) to both the nitrogen-deficient and the NH_4^+ -containing media (see Results). CaCl_2 , MgSO_4 and sucrose solutions were autoclaved separately and added to the cooled medium.

A colony from a plate (half-strength Difco nutrient broth solidified with 1.5% w/v, agar) was transferred into 10 ml of culture medium which was incubated on a gyratory shaker (250 r.p.m.) for 24 h at 30°C . A sample (5 ml) was transferred into 50 ml of the same medium in a 250 ml flask, and then shaken for 12 h. The cells were harvested by centrifugation (10000 g for 10 min), washed three times with culture medium and finally suspended in 10–20 ml of the same medium.

Batch culture medium (950 ml) was inoculated with 9.5 ml of the washed cells, then sparged at 450 ml min^{-1} with a cotton-filtered mixture of 0.71 to 1.0% (v/v) O_2 in N_2 . Hydrogen gas was used at 2.2% of the gas phase. The culture was incubated at 30°C and continuously stirred by a Teflon-coated magnetic bar. Culture samples were withdrawn from the culture flask through a Suba-seal-plugged bottom port using a 10 ml syringe. At the end of each experiment, the culture was checked for purity by plating on nutrient agar. Most gases were obtained from Linde (Union Carbide Canada); mixing of H_2 , O_2 and N_2 was done by using flowmeters in the laboratory. Some O_2/N_2 mixtures were purchased from Liquid Carbonic Canada. Compositions of gas mixtures are given throughout as % (v/v).

Uptake hydrogenase assay. Hydrogen uptake was measured by the O_2 -dependent H^3H uptake method as described by Tibelius & Knowles (1983) using 2 ml culture samples in 14 ml vials with silicone-reinforced butyl rubber caps for 30 min at 30°C . H^3H was added at 3% of the gas phase and O_2 was added as indicated. H^3H had a specific activity of 19.9 to 20.6 $\mu\text{Ci ml}^{-1}$ (736 to 762 kBq ml^{-1}) and was obtained from Gollob Analytical Service. Methylene blue (MB) at 5 mM was also used as an electron acceptor. The MB (J. T. Baker Chemicals), dissolved in phosphate buffer (pH 6.8), was kept in vials which were evacuated and backfilled three times with N_2 , and was added by N_2 -flushed syringe into N_2 -prefilled vials containing the culture samples. Assays were started by removing 3% of the gas phase and replacing it with H^3H . Incubation time was 20–30 min.

Acetylene reduction assay. Nitrogenase activity was measured by the acetylene reduction method in 2 ml culture samples contained in 14 ml vials. The assay was done using a gas phase of 5% C_2H_2 and O_2 as indicated in He for 1 h at 30°C on a gyratory shaker (250 r.p.m.).

Analyses. H_2 and O_2 were measured by gas chromatography as described by Chan *et al.* (1980) and C_2H_4 as described by Tibelius & Knowles (1983). Optical density was measured at 430 nm with a Bausch and Lomb Spectronic 20. Protein of washed cells was estimated by the Lowry method as previously described (Barraquio *et al.*, 1986) or by the modified method of Markwell *et al.* (1978). Reducing sugar was determined by the phenol method (Herbert *et al.*, 1971) using sucrose as the standard. Dissolved H_2 and C_2H_4 were calculated using the appropriate Ostwald coefficients (Wilhelm *et al.*, 1977).

RESULTS

K_m for H₂ of uptake hydrogenase

The apparent K_m for H_2 of hydrogenase was determined in whole cells of a stationary phase N_2 -fixing culture. From an Eadie–Hofstee plot, the apparent K_m for H_2 in the O_2 - and MB-dependent assays was 4.3 μM and 1.82 μM in solution, respectively. In the gas phase, these values correspond to 0.56% and 0.24% (v/v) for O_2 - and MB-dependent activities, respectively. These values are lower than that for *Azospirillum brasilense* which was 1.2 to 3% (Pedrosa *et al.*, 1982; Tibelius & Knowles, 1983). The value for MB-dependent activity is similar to that for *R. japonicum* which was 0.23% (Lim & Shanmugam, 1979). The H^3H concentration used in this study (3%) was sufficient to saturate the O_2 - and MB-dependent components of the hydrogenase system; in both cases the uptake of H^3H was linear with time up to about 90 min (data not shown).

Fig. 1

Fig. 2

Fig. 1. Effect of yeast extract on growth of *P. saccharophila* under N₂-fixing conditions. The organism was grown with shaking at 30 °C in 125 ml side-arm flasks each containing 15 ml of nitrogen-deficient medium plus (○) or minus (△) 0.01% (w/v) Difco yeast extract in an atmosphere of 0.80% O₂ in N₂. Data are the average of five replicate flasks.

Fig. 2. Growth (a) and hydrogenase activity (b) of *P. saccharophila* under N₂-fixing (open symbols) and NH₄⁺-grown (closed symbols) conditions. Both cultures were grown under 0.713% O₂ in N₂. MB was used as the electron acceptor in the hydrogenase assay.

Growth and H₂ oxidation under N₂-fixing and non-N₂-fixing conditions

P. saccharophila ATCC 15946 showed negligible growth unless supplemented with Difco yeast extract (Fig. 1), although both supplemented and unsupplemented cultures showed nitrogenase activity (data not shown).

The growth rates in both N₂-fixing and NH₄⁺-grown microaerobic cultures were practically the same in the first 6 h (Fig. 2a), no doubt due to the initial utilization of the yeast extract. Thereafter, the N₂-fixing culture grew relatively slowly, but during this period a marked increase in hydrogenase activity in this culture was observed (Fig. 2b). Activity was very much higher in the N₂-fixing culture than in the NH₄⁺-grown culture.

Hydrogenase induction

The results of the above experiment could indicate that the H₂ evolved during N₂ fixation stimulated synthesis of hydrogenase. We therefore determined if such synthesis could be induced by exogenous H₂. Indeed, the level of hydrogenase under non-N₂-fixing autotrophic conditions increased greatly in the presence of H₂ (Fig. 3). Addition of chloramphenicol (Cm) prevented the increase. The results suggest that the H₂ did not activate preformed enzyme but

Fig. 3. Effect of H₂ on hydrogenase synthesis in *P. saccharophila*. A mid-exponential phase, NH₄⁺-grown heterotrophic culture was washed, and then resuspended in a small quantity of the same medium but without sucrose. Samples (5 ml, OD₄₃₀ = 18, 1.5 mg protein ml⁻¹) were inoculated into 500 ml vacuum flasks each containing 150 ml of the same medium. Zero-time samples were taken, and then the flasks were evacuated and backfilled with N₂; 20% of the headspace was withdrawn then replaced individually with 5% CO₂, 5% O₂ and 10% H₂ or Ar. Cm (100 μg ml⁻¹) was added as indicated (arrow). The flasks were shaken at 30 °C, and samples were taken each hour. Hydrogenase assays were done under air. The treatments were: plus H₂ (□), plus H₂ and Cm (■), plus Ar (△), and plus Ar and Cm (▲). Data are the average of triplicate samples.

Fig. 4. Growth and hydrogenase activity of *P. saccharophila* with (closed symbols) and without (open symbols) H₂ (2.2%) under non-N₂-fixing conditions (aerobically with NH₄Cl). (a) Growth (○, ●) and residual reducing sugar as sucrose (△, ▲). (b) Hydrogenase activity. Activity was measured using 2% O₂ as the electron acceptor. Data are the average of triplicate samples for residual sucrose and hydrogenase activity.

rather caused derepression of synthesis of hydrogenase. Cultures supplied with Ar instead of H₂ had very low or negligible activity (Fig. 3).

We then determined if synthesis of hydrogenase could be induced by H₂ under aerobic heterotrophic conditions. NH₄⁺-grown batch cultures were sparged with air alone or with air containing 2.2% H₂. The growth and sucrose-utilization patterns were nearly identical for both cultures (Fig. 4a). However, the hydrogenase activity remained negligible in the absence of exogenous H₂ (Fig. 4b). In contrast, the H₂-sparged culture had much higher hydrogenase activity and the maximum activity was reached when the concentration of sucrose in the medium was relatively low. In this experiment, MB-dependent hydrogenase was compared with O₂-dependent activity at different growth stages of the H₂-sparged culture. MB-dependent hydrogenase activity was higher than the O₂-dependent activity by 1.6 ± 0.18 times (average $\pm$ SEM of determinations from nine time intervals). A similar ratio was obtained by Berndt &

Table 1. *Effect of different carbon sources on formation of hydrogenase in P. saccharophila previously grown under N₂-fixing conditions*

A mid-exponential phase N₂-fixing culture was washed three times with nitrogen-free medium lacking carbon source. The pellet was resuspended in the same medium, and then 0.2 ml samples of the concentrated cell suspension (OD₄₃₀ = 8, 0.56 mg protein ml⁻¹) were dispensed into 14 ml vials containing 1.8 ml of the same medium plus or minus carbon source (15 mM). The vials were stoppered then evacuated and backfilled three times with N₂. Zero-time samples were then assayed for hydrogenase activity using 2% O₂ as the electron acceptor. The gas phase of the remaining vials contained: 5% O₂, 5% CO₂, 10% H₂, and the balance N₂. The vials were shaken for 13 h at 30 °C after which cultures were assayed for hydrogenase activity. Data are the average of two replicates (except control and no CO₂ treatments which had four replicates each) ± SEM.

Carbon source	Δ Hydrogenase activity*	
	[(activity at 13 h) – (activity at 0 h); nmol H ₂ (mg protein) ⁻¹ h ⁻¹]	Relative activity
None (control)	7414 ± 1891	100
D(+)-Fructose	9589 ± 828	129
D(+)-Mannose	8477 ± 790	114
D(+)-Xylose	4939 ± 569	67
D(+)-Glucose	4630 ± 568	62
Sodium pyruvate	1489 ± 106	20
Sodium citrate	223 ± 44	3
L(+)-Arabinose	133 ± 10	1.8
Sucrose	89 ± 26	1.2
Sodium acetate	53 ± 5	0.7
Sodium succinate	20 ± 20	0.2
D(+)-Galactose	4 ± 1.4	0.05
Sodium malate	0	0
None, no CO ₂ (replaced by Ar)	6063 ± 1450	82

* Zero-time activity in all treatments ranged from 22 to 81 nmol H₂ (mg protein)⁻¹ h⁻¹ with an average of 44 ± 6.

Wölfe (1978) in *Xanthobacter autotrophicus*. The results might suggest that in the O₂-dependent assay, some components of the electron transport chain were limiting. The O₂- and MB-dependent hydrogenase activities at different growth stages were proportional with a correlation coefficient of 0.94.

Effect of carbon sources on hydrogenase activity and synthesis

The results of the previous experiments suggested that the concentration of sucrose may affect hydrogenase formation. Carbon substrates which are used to different extents for growth of *P. saccharophila* (Barraquio *et al.*, 1986) were therefore examined for their effects on hydrogenase formation. Carbon sources which are utilized only slightly or not at all, such as mannose, fructose, xylose and glucose, repressed hydrogenase formation either not at all or at the most by 40% (Table 1). Pyruvate, a better substrate, showed 80% repression. Those carbon sources which support vigorous growth of the organism exhibited 97–100% repression. In the absence of carbon source, and with CO₂ replaced by Ar, there was about a 20% reduction in hydrogenase expression.

The kinetics of repression of hydrogenase synthesis and of inhibition of preformed enzyme by sucrose were then studied. In the presence of sucrose, preformed hydrogenase activity was not affected but synthesis was almost completely repressed throughout the 8 h experiment (Fig. 5). Hydrogenase formation was greatly stimulated in the absence of sucrose. In the absence of sucrose but with Cm there was a rapid decline in activity. Sodium azide prevented this decline whereas phenylmethylsulphonyl fluoride (PMSF) did so only partially.

Effect of O₂ on nitrogenase and hydrogenase activity and synthesis

N₂-fixing cultures showed maximum nitrogenase and hydrogenase activities in the exponential and stationary phases, respectively (Fig. 6). Maximum nitrogenase activities were shown at pO₂ 0.0025 to 0.0050 atm depending on the growth phase (Fig. 6a), and activity was

Fig. 5

Fig. 6

Fig. 5. Effect of sucrose on preformed activity and synthesis of hydrogenase in *P. saccharophila*. A late-exponential phase, N_2 -fixing culture was washed, then resuspended in a small quantity of NH_4Cl -containing (minus sucrose) medium. Samples (5 ml, $OD_{430} = 12.4$, $0.86 \text{ mg protein ml}^{-1}$) were inoculated into 500 ml vacuum flasks each containing 150 ml of the same medium. Zero-time samples were taken, then sucrose (15 mM), sodium azide (1 mM), PMSF (1 mM) and Cm ($100 \mu\text{g ml}^{-1}$) were injected immediately as desired. The flasks were evacuated and backfilled with N_2 ; 20% of the headspace was then withdrawn and replaced individually with 10% H_2 , 5% CO_2 and 5% O_2 . The flasks were shaken at 30°C , and samples were taken each hour. MB was used as the electron acceptor in the hydrogenase assay. The treatments were: minus sucrose ($\circ$), plus sucrose ($\bullet$), minus sucrose plus Cm ($\square$), plus sucrose plus Cm ($\blacksquare$), minus sucrose plus Cm and sodium azide ($\triangle$), and minus sucrose plus Cm and PMSF ($\blacktriangle$). Data are the average of triplicate samples.

Fig. 6. O_2 optima for nitrogenase (a) and hydrogenase (b) activities of *P. saccharophila*. Activities were measured using early exponential ($\circ$), mid-exponential ($\bullet$), late-exponential ($\triangle$) and early stationary ($\blacktriangle$) phase culture samples. Results are the average of triplicates.

Table 2. Effect of O_2 on formation of hydrogenase in *P. saccharophila*

A mid-exponential phase, NH_4^+ -grown aerobic heterotrophic culture was washed then resuspended in a small quantity of NH_4Cl -containing (minus sucrose) medium. Samples (0.2 ml , $OD_{430} = 6$, $0.50 \text{ mg protein ml}^{-1}$) were dispensed into vials containing 1.8 ml of the same medium without or with sucrose (15 mM). One set of vials received 10% H_2 , 5% CO_2 , 2.8% O_2 , and the balance N_2 ; the other set received 10% H_2 and 5% CO_2 in air. The cultures were shaken for 8 h, after which they were analysed for O_2 , then evacuated and backfilled with N_2 . The H_2 -uptake assay was done with MB as electron acceptor. Values in parentheses are percentage O_2 at the end of the derepression period. Data are the average of four replicate samples $\pm \text{SEM}$.

Oxygen (%)	Hydrogenase activity [$\text{nmol H}_2 \text{ (mg protein)}^{-1} \text{ h}^{-1}$]	
	Minus sucrose	Plus sucrose
2.80	2107 ± 288 (2.43 ± 0.18)	12 ± 2 (0.21 ± 0.05)
18.26	443 ± 32 (15.80 ± 0.05)	22 ± 1 (5.80 ± 0.19)

observed at all growth phases tested. The hydrogenase showed wide tolerance to O_2 , maximum activity being observed from pO_2 0.009 to 0.2 atm (Fig. 6a). Early exponential cultures did not show hydrogenase activity. Low pO_2 values became limiting in late-exponential and early stationary phases but not in mid-exponential phase, probably because of the low hydrogenase activity in the latter.

It is possible that O_2 may become limiting when sucrose is available in the medium, thus causing a low level of hydrogenase synthesis (P. van Berkum, personal communication). To test this hypothesis, hydrogenase formation was induced under microaerobic and aerobic conditions in the presence and absence of sucrose. In the absence of sucrose, hydrogenase formation was partially repressed by 18% O_2 , the activity being 80% lower than that under 2.8% O_2 (Table 2). In the presence of sucrose, much lower activity developed. O_2 may have been limiting in the 2.8% O_2 plus sucrose treatment but since 5.8% O_2 remained in the 18% O_2 treatment with little appearance of activity, it is probable that sucrose and not O_2 availability was the important factor.

DISCUSSION

P. saccharophila was reported to have only a membrane-bound hydrogenase, the formation of which was demonstrated under non- N_2 -fixing autotrophic conditions (Podzuweit *et al.*, 1983), and although a soluble hydrogenase was reported (Bone, 1960) the finding has not been confirmed. After demonstrating that *P. saccharophila* can fix N_2 (Barraquio *et al.*, 1986) our present data clearly show the presence of an active uptake hydrogenase in N_2 -fixing cultures of this organism. Hydrogenase was expressed significantly more in N_2 -fixing than in non- N_2 -fixing culture, as in *Azotobacter chroococcum* (Partridge *et al.*, 1980), *Xanthobacter autotrophicus* (Berndt & Wölfe, 1978) and *Azotobacter vinelandii* (Lee & Wilson, 1943) but in contrast to *Azospirillum brasilense* where an NH_4^+ -grown culture had hydrogenase activity similar to, or slightly higher than, that in a N_2 -fixing culture (Tibelius & Knowles, 1983). Our results show that NH_4^+ *per se* does not inhibit hydrogenase activity and, in the presence of H_2 , synthesis of the enzyme is greatly induced. Thus, the inducible hydrogenase system of *P. saccharophila* resembles that of *Aquaspirillum autotrophicum* (Aragno & Schlegel, 1978), *Alcaligenes hydrogenophilus* (Friedrich *et al.*, 1984) and *Rhizobium* ORS 571 (de Vries *et al.*, 1984), but differs from that of *Azospirillum brasilense* (Tibelius & Knowles, 1983) and most strains of *Paracoccus denitrificans* (Nokhal & Schlegel, 1980). In *Rhodopseudomonas capsulata* (Colbeau & Vignais, 1983) the H_2 evolved during N_2 fixation is probably responsible for the stimulated hydrogenase expression in N_2 -fixing culture. In both *R. capsulata* (Colbeau & Vignais, 1983) and *P. saccharophila*, expression of hydrogenase in both autotrophic and heterotrophic NH_4^+ -utilizing cultures provided with exogenous H_2 suggests that hydrogenase is independent of nitrogenase expression.

Under carbon-limited conditions hydrogenase activity of *Azotobacter chroococcum* is consistently high (Partridge *et al.*, 1980; Walker & Yates, 1978). Likewise, we observed maximum hydrogenase activity when residual sucrose was low or nil. In the presence of 15 mM-sucrose, the synthesis of hydrogenase was repressed but the activity of preformed enzyme was not inhibited. Other utilizable carbon substrates tested also repressed hydrogenase synthesis whereas those which were little or not at all utilized showed a negligible repressive effect. Similar behaviour is seen in *Rhizobium japonicum* (Maier *et al.*, 1979). However, in *Aquaspirillum autotrophicum* (Aragno & Schlegel, 1978), *Rhizobium* ORS 571 (de Vries *et al.*, 1984) and *R. japonicum* strain JH (Graham *et al.*, 1984), some utilizable carbon substrates do not repress enzyme formation. In the study of *R. japonicum* mutants, Merberg *et al.* (1983) found that a common element is involved in the regulation of hydrogenase by oxygen and by carbon substrates. In our study, it seems that sucrose repression of hydrogenase formation is independent of repression by O_2 . Catabolite repression involving cyclic AMP and possibly other effector molecules is implicated in malate-mediated repression of H_2 uptake in *R. japonicum* (Lim & Shanmugam, 1979; McGetrick *et al.*, 1985). The discrepancies among these reports may indicate only the presence of different mechanisms by which carbon substrates regulate H_2 -oxidation. Nevertheless, O_2 and carbon substrates may be essential coregulators especially in O_2 -sensitive hydrogenase systems.

The decline in activity in the presence of sucrose plus Cm was prevented by addition of 1 mM-sodium azide, a known inhibitor of ATP production, but not by PMSF, a proteolysis inhibitor. Schlesier & Friedrich (1981) reported inactivation of soluble hydrogenase in *Alcaligenes eutrophus* strain H16 when intact cells were exposed to a $H_2/O_2/CO_2$ atmosphere, and this

inactivation was increased in the presence of Cm; cells exposed to H_2/O_2 with 1 mM-sodium azide retained only about 10% of the activity of cells exposed to either air or H_2 but 27% of the activity was retained in the presence of 5 mM-sodium azide. However, addition of 2 mM-ATP to cell extracts did not enhance inactivation of hydrogenase and it was concluded that inactivation was due to superoxide radical anions produced by hydrogenase itself during catalysis. Our results seem to suggest that inactivation is ATP-supported and, as in *A. eutrophus*, is not due to simple proteolysis. Further study is needed to test this hypothesis.

Hydrogenase activity of *P. saccharophila* was not sensitive to O_2 and in this respect resembles the H_2 uptake system of *Aquaspirillum autotrophicum*, *Alcaligenes eutrophus* and *Azospirillum amazonense* (Aragno & Schlegel, 1978; Fu & Knowles, 1986; Wilde & Schlegel, 1982) but differs from that of other diazotrophic bacteria such as *Azospirillum brasilense* and *Azospirillum lipoferum* (Fu & Knowles, 1986; Pedrosa *et al.*, 1982; Tibelius & Knowles, 1983), and *Rhizobium japonicum* and its bacteroids (Maier *et al.*, 1979; Ruiz-Argüeso *et al.*, 1979). However, 18% O_2 partially repressed hydrogenase formation. Complete repression was reported in *Aquaspirillum autotrophicum* at 30% O_2 whereas enzyme synthesis occurred at 20% O_2 but at a much lower rate than at 2.5% O_2 (Aragno & Schlegel, 1978).

Nitrogenase activity was maximal at a pO_2 of 0.0025 to 0.005 atm and was almost completely inhibited at 0.05 pO_2 in common with many other aerobic N_2 -fixing bacteria (Barraquio *et al.*, 1986; Fu & Knowles, 1986; Tibelius & Knowles, 1983). Unpublished data from late-exponential phase N_2 -fixing cultures showed that the presence or absence of H_2 and thus of O_2 -dependent H_2 uptake had no effect on the O_2 tolerance of the nitrogenase. This is in agreement with data for *Azospirillum brasilense* (Pedrosa *et al.*, 1982) but contrasts with *Azotobacter chroococcum* (Walker & Yates, 1978) where oxidation of exogenous H_2 afforded some protection for nitrogenase.

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