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Physiological Characterization of the Hydrogen Bacterium *Aquaspirillum autotrophicum*

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Abstract. Aquaspirillum autrotrophicum, an aerobic hydrogen bacterium recently isolated from an eutrophic freshwater lake, was characterized physiologically. It grew autotrophically in a fermenter with a doubling time of 4 h. Heterotrophic growth was faster. pH-Optimum ranged from 5.0–7.5, temperature optimum was about 28° C. During autotrophic growth about 10 moles hydrogen were consumed per 1 mole carbon dioxide fixed.

Hydrogenase activity is inducible. CO_2 did not enhance the oxy-hydrogen reaction by intact cells. The hydrogenase activity was localized in the particulate fraction. The hydrogenase reduced methylene blue and phenazine methosulfate; pyridine nucleotides were not reduced. In cell-free extracts, hydrogenase was sensitive to oxygen. Ribulosebisphosphate carboxylase was present in autotrophically-grown cells and absent from heterotrophically grown cells.

Hydrogenase induction in heterotrophically-grown cells followed parabolic kinetics. Oxygen and D-gluconate repressed hydrogenase synthesis, whereas citrate, DL-lactate and pyruvate stimulated its formation. The repressive effect was delayed. The results suggest that the control of hydrogenase synthesis occurred at the transcriptional level, and that mRNA coding for the hydrogenase had a relatively long life span.

D-Gluconate was degraded via the Entner-Doudoroff pathway, the enzymes of which were constitutively formed. Enzymes of the pentosephosphate and Embden-Meyerhof pathways (except phosphofructokinase) were present, too. Hydrogen did not inhibit heterotrophic growth.

The possible competitive advantage of the physiological properties described with regard to the natural habitat was discussed.

Key words: Aquaspirillum autotrophicum – Hydrogen bacterium – Growth – Chemolithoautotrophy – Particulate hydrogenase – Induction – Repression – Natural habitats.

Although aerobic hydrogen bacteria possess the property of growing autotrophically with hydrogen as electron and energy donor, they differ profoundly, not only with respect to their taxonomical position (Davis et al., 1969, 1970; Aragno et al., 1977; Walther-Mauruschat et al., 1977) but also with respect to the properties of the autotrophic system and their regulation patterns (Schlegel and Eberhardt, 1972; Schlegel, 1976; Schneider and Schlegel, 1977).

A hydrogen-oxidizing spirillum was recently isolated from the water of an eutrophic lake in Switzerland and described as the new species *Aquaspirillum autotrophicum* (Aragno and Schlegel, 1978). Members of the genus *Aquaspirillum* are in general freshwater organisms (Krieg, 1976), thus *A. autotrophicum* may be considered as a typical freshwater chemoautotroph. The study of the physiological properties of such a bacterium, mainly of the regulation of auto- and heterotrophic metabolism, was of interest especially with regard to its peculiar habitat and its taxonomical position.

MATERIALS AND METHODS

Organism. Aquaspirillum autotrophicum strain SA 32 was isolated as previously described (Aragno and Schlegel, 1978) and was deposited with the German Collection of Microorganisms (Göttingen) under the number DSM 732. *Alcaligenes eutrophus* strain H 16 (ATCC 17699, DSM 428) was obtained from the institute's culture collection.

Non-Standard Abbreviations. TEA = triethanolamine; GDH = glyceraldehyde-3-phosphate dehydrogenase; OD = optical density; DCPIP = dichlorophenolindophenol; TIM = triosephosphate isomerase

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Culture Media. The following basic mineral medium, after Schlegel et al. (1961) was used: $Na_2HPO_4 \cdot 12\,H_2O$ 9 g; KH_2PO_4 1.5 g; NH_4Cl 1.0 g; $MgSO_4 \cdot 7\,H_2O$ 0.2 g; $Fe(NH_4)$ citrate 0.005 g; $CaCl_2 \cdot 2\,H_2O$ 0.01 g; trace elements solution SL 4, according to Pfennig and Lippert (1966), however, without EDTA and ferrous salt: 5.0 ml; distilled water: 1000 ml. For autotrophic growth, 0.05% (w/v) $NaHCO_3$ was added. For heterotrophic growth, 0.1 – 0.5% (w/v) organic substrate was added. Fermenter cultures were run in a Biostat S fermenter (Braun, Melsungen) with a 10 l culture vessel containing 8 l medium. Gas mixtures were prepared by means of gas mixing pumps (Wösthoff, Bochum). Gas flow was measured with a gas flow meter (Elster, Mainz) with measuring capacity of 2 – 200 l/h. For following growth, cell densities were determined turbidimetrically with 1 cm cuvettes in a Zeiss PL 4 photometer at 436 nm. An absorbance of 1.0 corresponded to a cell density of 244 mg dry weight per litre when measured with log phase autotrophic cultures ($t_g = 4$ h).

Dry Weight. The dry weight of cells was measured with constant weight membrane filters (Sartorius, Göttingen) of 47 mm diameter and 0.2 µm pore size. Cell suspensions containing about 10 – 20 mg dry weight were filtered, washed twice to remove salts. The membranes were then dried to constant weight at 80° C.

Protein Determination. The Biuret methode as modified by Schmidt et al. (1963), was used with bovine serum albumin as a standard.

Manometric Techniques. The manometric measurements were made in a round Warburg apparatus (Braun, Melsungen) as described by Aggag and Schlegel (1973), except that 50 mM potassium phosphate buffer, pH 7.0, was used. Some induction experiments were made with the help of a Longator recording device (Braun, Melsungen).

Preparation of Cell-Free Extracts. Cells were washed and suspended in 50 mM phosphate buffer, pH 7.0, to give a final density of about 30 mg protein/ml. The cells were disintegrated by means of a sonicator (Schoeller, Frankfurt a. M.) at 20 kHz and 600 W, at intervals of 30 s, the sonication lasting 30 s/ml, or through a French pressure cell (Aminco, Silverspring, U.S.A.) at 800 kg · cm^{-2}. Intact cells and debris were removed by centrifugation at 10000 g for 30 min. The supernatant was the cell-free extract subjected to further assays.

Ultracentrifugation. Ultracentrifugation was made with an Omikron vacuum centrifuge (Christ, Osterode) with a maximal speed of 40000 rpm.

Enzyme Assays. The following enzyme activities were determined photometrically in a Zeiss PL 4 photometer, measuring NAD(P) reduction or NAD(P)H oxidation at 365 nm, with 1 cm cuvettes and 3 ml reaction mixture at room temperature: malate dehydrogenase (1 ml 100 mM Tris-HCl pH 9.2, 1.5 ml bidistilled water, 0.2 ml 4.5 mM NADH, 0.2 ml 23 mM potassium oxaloacetate, 0.1 ml extract); NADH oxidase (1 ml 100 mM Tris-HCl pH 9.2, 1.7 ml bidistilled water, 0.2 ml 4.5 mM NADH, 0.1 ml extract); hydrogen dehydrogenase (2.8 ml 50 mM potassium phosphate buffer pH 7.0 saturated with H_2, 0.1 ml 24 mM NAD, 0.1 ml extract); hexokinase [1.79 ml 50 mM TEA buffer pH 7.6, 0.6 ml 200 mg/ml glucose, 0.2 ml 100 mM $MgCl_2$, 0.1 ml 15 mM ATP, 0.2 ml 15 mM NADP, 0.01 ml glucose-6-phosphate dehydrogenase (1 mg/ml), 0.1 ml extract]; phosphoglucose isomerase [2.54 ml 50 mM TEA buffer pH 7.6, 0.2 ml 100 mM $MgCl_2$, 0.1 ml 15 mM NADP, 0.01 ml glucose-6-phosphate dehydrogenase (1 mg/ml), 0.1 ml 100 mM fructose-6-phosphate, 0.05 ml extract]; phosphofructokinase [2.24 ml 50 mM TEA buffer pH 8.0, 0.1 ml 20 mM fructose-6-phosphate, 0.15 ml 15 mM ATP, 0.03 ml 100 mM $MgCl_2$, 0.20 ml 100 mM glutathione, 0.05 ml 15 mM NADH, 0.01 ml aldolase (2 mg/ml), 0.02 ml GDH/TIM (2 mg/ml), 0.1 ml extract]; fructose-bisphosphate aldolase [2.73 ml 50 mM TEA buffer pH 7.6, 0.05 ml 15 mM NADH, 0.02 ml GDH/TIM (2 mg/ml), 0.05 ml extract, 0.15 ml 2 mM fructose-bisphosphate]; fructose-bisphosphatase [2.56 ml 100 mM Tris buffer pH 8.8, 0.1 ml 20 mM EDTA, 0.1 ml 100 mM $MgCl_2$, 0.1 ml 15 mM NADP, 0.01 ml glucose-6-phosphate dehydrogenase (1 mg/ml), 0.01 ml phosphoglucose isomerase (1 mg/ml), 0.02 ml extract, 0.1 ml 20 mM fructose-bisphosphate]; gluconokinase [2.28 ml 50 mM TEA buffer pH 7.6, 0.2 ml 100 mM $MgCl_2$, 0.1 ml 15 mM NADP, 0.1 ml 15 mM ATP, 0.02 ml 6-phosphogluconate dehydrogenase (2 mg/ml), 0.1 ml extract, 0.2 ml 120 mM gluconate]; 6-phosphogluconate dehydratase [2.13 ml 50 mM TEA buffer pH 7.6, 0.09 ml 100 mM $MgCl_2$, 0.09 ml 13 mM NADH, 0.03 ml lactate dehydrogenase (5 mg/ml), 0.12 ml 2-keto-3-deoxy-6-phosphogluconate aldolase (60 U/ml), 0.06 ml extract, 0.48 ml 25 mM 6-phosphogluconate]; 2-keto-3-deoxy-6-phosphogluconate aldolase [1.38 ml 50 mM TEA buffer pH 7.6, 0.09 ml 13 mM NADH, 0.03 ml lactate dehydrogenase (5 mg/ml), 0.06 ml extract, 1.44 ml 35 mM 2-keto-3-deoxy-6-phosphogluconate pH 7.0]; glucose-6-phosphate dehydrogenase (2.72 ml 50 mM TEA buffer pH 7.6, 0.05 ml 100 mM $MgCl_2$, 0.1 ml 15 mM NADP, 0.03 ml extract, 0.1 ml 120 mM glucose-6-phosphate); 6-phosphogluconate-dehydrogenase [2.5 ml 50 mM TEA buffer pH 7.6, 0.2 ml 100 mM $MgCl_2$, 0.1 ml 15 mM NAD(P) 0.1 ml extract, 0.1 ml 25 mM 6-phosphogluconate pH 7.0]. In all cases, control was made that the substrate concentration used was saturating. Ribulose-bisphosphate carboxylase was tested as described by Bowien et al. (1976). Hydrogenase activities with different electron acceptors were tested manometrically (see above).

Chemicals. Biochemicals were purchased from Boehringer, Mannheim. Ketodeoxygluconate was kindly supplied by Dr. B. Bowien (Göttingen). Other chemicals came from Merck, Darmstadt, and were of analytical grade.

RESULTS

Growth Rates, pH and Temperature Optima

The velocity of cell growth under autotrophic conditions has been measured in the fermenter gassed with $H_2 + O_2 + CO_2$ mixtures (see below). Cultures for measuring the growth rates under heterotrophic conditions were inoculated with a culture shaken overnight grown on the same substrate; they were then shaken in a water bath at 28° C under air. The cells grew exponentially without a lag-phase for 6 h and more. The results are shown in Table 1. High growth rates were observed with succinate, D-gluconate and L-malate. On all the organic substrates tested the growth rates were higher than under autotrophic conditions.

The pH-dependence and temperature-dependence of growth was studied under heterotrophic conditions. The pH optimum (Fig. 1) was relatively broad, ranging from pH 5.0 to pH 7.5. Surprisingly, the cells at pH 6.0 and lower were almost immotile. The temperature optimum (Fig. 2) was about 28° C; growth was scant at 10° C and 35° C, no growth was observed at 37° C.

Autotrophic Growth in Fermenter

Whereas heterotrophic growth is not affected by oxygen at partial pressures up to 20.2 kPa (0.2 atm)

autotrophic growth is sensitive to oxygen. For this reason, the gas of autotrophic cultures in fermenters has to be carefully adjusted. A 10 l fermenter containing 8 l mineral medium was inoculated with an actively growing autotrophic culture. The initial OD (436 nm) was 0.2–0.3. At this density, oxygen concentration in the gas mixture had to be kept low. During the first hours of growth, the culture was gassed with a mixture of 86.5% H_2 + 3.5% O_2 + 10% CO_2 at a rate of 500 ml · min^{-1}, corresponding to 62.5 ml · min^{-1} · l^{-1}. Then, during growth, oxygen supply had to be adapted to the increasing demand, either by raising its concentration in the mixture or by increasing the flow rate of the mixture. Results of a typical growth experiment are shown in Figure 3. The highest growth rates were obtained above OD 0.5, as long as the oxygen supply was periodically adapted to the actual cell density by increasing its concentration in the mixture. The generation time under these conditions was about 4 h, which corresponds to a growth rate $\mu = 0.173$ and to a mass increase of 2.88 mg · min^{-1} · g^{-1} cells (dry weight). The cell density reached an OD (436 nm) of 7.3 while the cells were growing exponentially; this OD corresponded to 1.78 g dry cells (dry weight) per litre. The gas mixture contained 65% H_2, 25% O_2 and 10% CO_2, and was added at a rate of 120 ml · min^{-1} · l^{-1} culture. The gas uptake rate of the culture was then 75 ml · min^{-1} · l^{-1} culture.

Table 1. Growth rates of *Aquaspirillum autotrophicum* on different substrates

Substrate	Growth rate $\mu(h^{-1})$	Doubling time t_g (h)
$H_2 + CO_2$	0.17	4.0
Succinate	0.36	1.9
D-Gluconate	0.35	2.0
L-Malate	0.33	2.1
Pyruvate	0.28	2.5
L-Glutamate	0.25	2.8
Citrate	0.24	2.9
Acetate	0.23	3.0
DL-Lactate	0.22	3.1

The basic equations of gas consumption during autotrophic growth are:

$$2\,H_2 + O_2 \rightarrow 2\,H_2O \quad (1)$$

$$2\,H_2 + CO_2 \rightarrow CH_2O. \quad (2)$$

Equation (1) represents the energy-yielding oxy-hydrogen reaction; Eq. (2) represents the CO_2 fixation reaction.

The ratio between both reactions:

$$n = \frac{O_2 \text{ uptake}}{CO_2 \text{ uptake}} = \frac{H_2 \text{ used for the oxy-hydrogen reaction}}{H_2 \text{ used for generating reducing power}}$$

expresses the efficiency of the hydrogen-chemolitho-autotrophic metabolism.

From the growth rate and an assumed carbon content of the dry cell matter of 50%, we can estimate the rate of CO_2 fixation at about 2.7 ml CO_2 · min^{-1} · g^{-1} cells (excretion of metabolites not considered). Whatever the n-value is, hydrogen uptake is two thirds of the total gas uptake. From the above results the oxygen uptake rate is calculated.

O_2 uptake = 1/3 (total gas uptake) − CO_2 uptake

O_2 uptake rate = 11.3 ml · min^{-1} · g^{-1} cells.

Thus, $n \cong \frac{11.3}{2.7} = 4.2$.

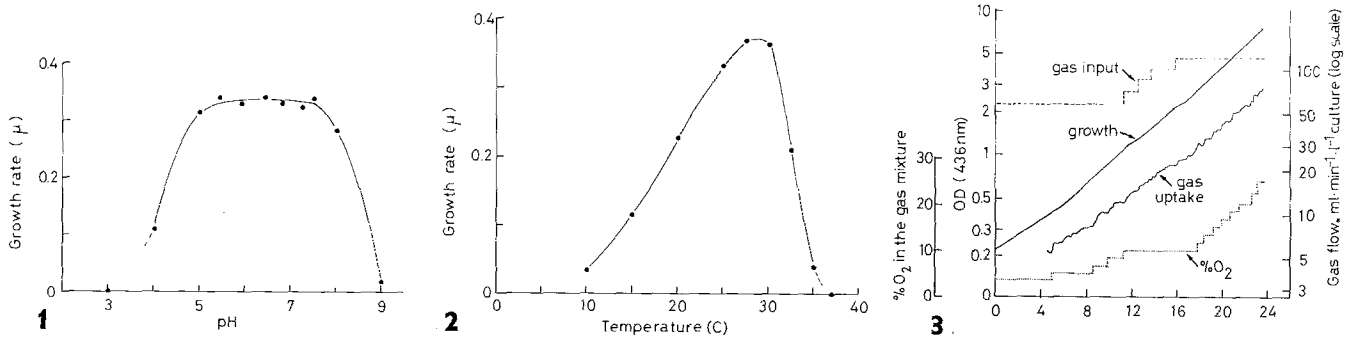

Fig. 1. pH-Dependence of growth rate during growth in a succinate medium. Cells were grown in 200-ml-Erlenmeyer flasks containing 50 ml 0.2% (w/v) succinate mineral medium shaken in a water bath at 30° C. pH was adjusted with eitherHCl or NaOH. Turbidity and pH were measured at intervals, and the growth rate μ was calculated

Fig. 2. Temperature-dependence of growth rate during growth in a succinate mineral medium. Cells were grown as described in Figure 1, however, agitated by magnetic stirring

Fig. 3. Autotrophic growth in a fermenter. Explanations: see text

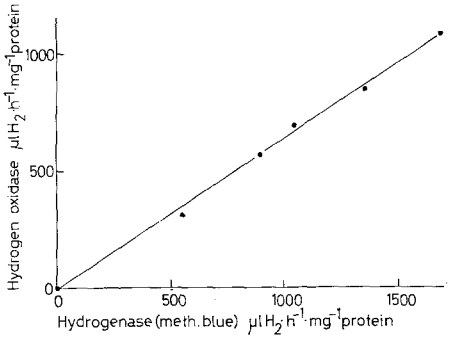

Fig. 4. Correlation between hydrogenase (tested with methylene blue as electron acceptor) and hydrogen-oxidase activities. Activities were measured at intervals during induction of previously citrate-grown cells, under autotrophic conditions, under 5% O_2, 10% CO_2 and 85% H_2

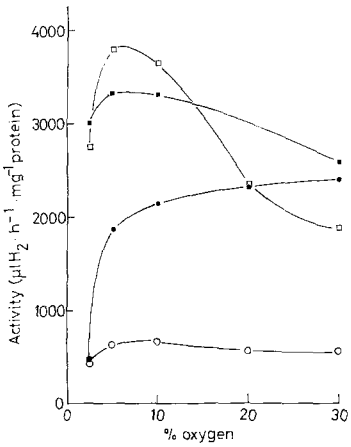

Fig. 5
Effect of oxygen concentration on hydrogenase (tested with methylene blue as electron acceptor) and hydrogen-oxidase activities.
● hydrogen-oxidase activity in intact cells; ■ hydrogenase activity in intact cells;
○ hydrogen-oxidase activity in cell-free extract;
□ hydrogenase activity in cell-free extract

Activity of "Autotrophic" Enzymes

Hydrogenase in Intact Cells. Hydrogenase activity was measured in intact cells either using methylene blue (direct measurement) or oxygen (indirect measurement) as electron acceptors. The second reaction involved the respiratory chain in addition to hydrogenase; this activity is called "hydrogen oxidase".

Hydrogenase activity is inducible in *Aquaspirillum autotrophicum* SA 32. Cells grown on mineral media supplemented with either pyruvate, succinate, D-malate, DL-lactate, D-gluconate or citrate under air did not exhibit any detectable activity.

Hydrogen-oxidase activity in intact cells grown autotrophically was not enhanced by the addition of carbon dioxide to the oxy-hydrogen atmosphere; *A. autotrophicum* did not exhibit a "Bartha effect" as was observed in *Alcaligenes eutrophus* H 16 (Bartha, 1962). During induction, hydrogenase (measured with methylene blue) and hydrogen-oxidase activities varied in linear correlation (Fig. 4). Therefore, hydrogenase is the limiting factor in the electron flow, and hydrogenase activity is well represented by the activity of hydrogen oxidase.

Hydrogenase in Cell-Free Extracts. The crude cell-free extract of autotrophically-grown cells contained hydrogenase activity comparable to that of intact cells. The activity was localized almost exclusively in the particulate fraction, i.e. the sediment obtained after centrifugation at 140000 g for 3 h (Schneider and Schlegel, 1977).

Different compounds were tested as electron acceptors (Table 2). Among them only phenazine methosulfate and methylene blue were reduced at a high rate. Oxygen and ferricyanide were reduced at a lower rate. The other compounds tested were very slowly reduced, and NAD(P) not at all.

Table 2. Hydrogenase activity in cell-free extracts with different electron acceptors

Acceptor	Relative activity (methylene blue = 100)
Phenazine methosulfate[a]	179
Methylene blue[a]	100
Oxygen[b]	32
$K_3Fe(CN)_6$[a]	20
DCPIP[a]	6
FAD[a]	3
FMN[a]	2
Benzylviologen[a]	2
Methylviologen[a]	<1
NAD[c]	0
NADP[c]	0

[a] Tested manometrically at 4 mM acceptor concentration under 100% H_2
[b] Tested manometrically under 95% H_2 + 5% O_2
[c] Tested photometrically

Effect of Oxygen on Hydrogenase Activity. The effect of oxygen on hydrogenase activity was measured manometrically, either with methylene blue or with O_2 as electron acceptors, in atmospheres containing 50% H_2 and variable amounts of O_2 and N_2 (Fig. 5). In intact cells, oxygen in concentrations above 5% had almost no effect on hydrogen-oxidase activity and diminished the activity measured in the presence of methylene blue only slightly. In cell-free extracts the hydrogen-oxidase activity was much lower (about 30% of the activity measured in whole cells) and was not affected by increasing oxygen concentrations. However, the activity measured in the presence of methylene blue was high at an oxygen concentration of 10% and decreased significantly when the oxygen concentration reached 20 or 30%; however, hydrogenase activity still remained above the level of hydrogen oxidase. Hydrogenase was partially sensi-

tive to oxygen, and in intact cells, was protected from the influence of oxygen.

Ribulosebisphosphate Carboxylase. Ribulosebisphosphate carboxylase was tested in extracts from autotrophically and heterotrophically-grown cells (Table 3). The specific activity measured in autotrophic cells indicated that CO_2 is fixed via the Calvin cycle. No detectable activity was measured in heterotrophically grown cells; RuDPCase was apparently entirely inducible.

Induction of Hydrogenase. As already mentioned hydrogenase activity was absent from heterotrophically grown cells; these cells were used to test the induction of this enzyme. Cells growing under air in mineral medium containing 0.2% (w/v) organic substrate were harvested during the log-phase, washed twice with phosphate buffer and resuspended in the autotrophic basal mineral medium. 2.2 ml of suspension, containing about 0.12 mg protein, were introduced into the main compartment of a Warburg flask.

Table 3. Activity of ribulosebisphosphate carboxylase in cell-free extracts

Growth substrate	Activity (U/g)
$H_2 + CO_2$	2120
D-Gluconate	0
Succinate	0

One enzyme unit (U) convers 1 μmole substrate per min

The flasks were then flushed with a $H_2 + O_2 + CO_2$ mixture. A control was made with N_2 instead of H_2. Gas uptake was measured at intervals of 20–30 min, and hydrogen uptake was calculated as the 2/3 of the total gas uptake. Under these conditions, no significant growth occurred until hydrogenase activity reached about 1000 μl $H_2 \cdot h^{-1} \cdot mg^{-1}$ protein. The induction followed parabolic kinetics (Fig. 6) which may be expressed by: $y = m(t-l)^2$, where y: specific gas uptake rate (μl $\cdot h^{-1} \cdot mg^{-1}$ protein); m: parabolic induction rate; t: time; l: lag time or by the linear plot:

$\sqrt{y} = \sqrt{m}\,(t-l)$ (see Fig. 7).

Supposing that 1) the control of hydrogenase synthesis occurs at the transcriptional level; 2) transcription and translation vary linearly with respect to DNA and mRNA concentration during the first hours of induction; and 3) the mRNA has a relatively long life (see below), parabolic induction kinetics should be expected.

Effect of Oxygen on Hydrogenase Induction. Succinate-grown cells were exposed to gas mixtures containing 50% H_2, 10% CO_2, variable concentrations of O_2 and the rest N_2. The results are shown in Figure 7. Oxygen repressed hydrogenase induction, the repression being complete in the presence of 30% O_2. In the following experiment (Fig. 8A), the induction was initiated in the presence of 5% oxygen. In the parabolic phase of induction, oxygen concentration was increased to 30%. Unexpectedly, the activity continued to increase although with diminishing rate. The

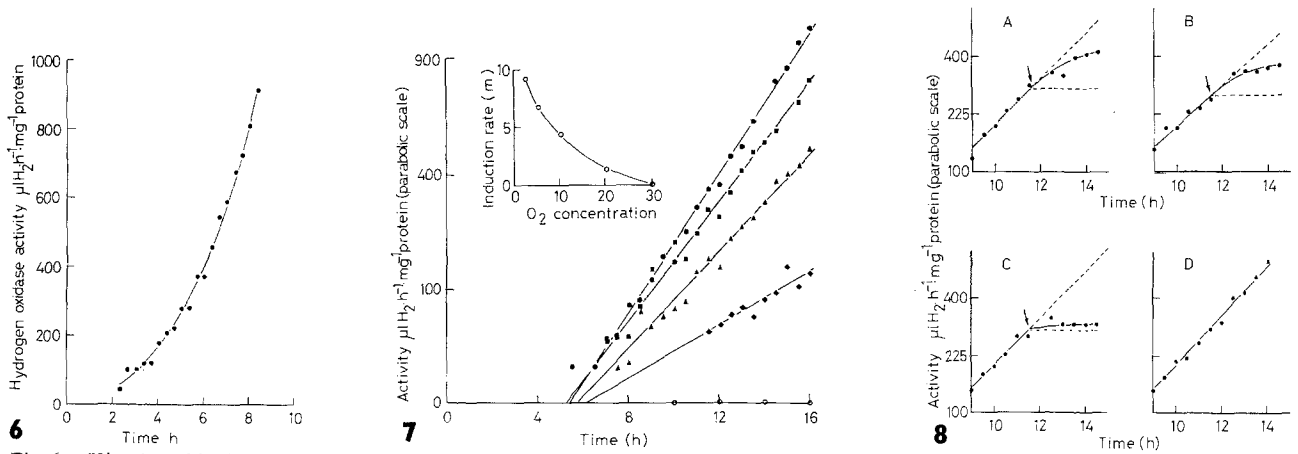

Fig. 6. Kinetics of hydrogenase (hydrogen-oxidase) induction. Cells previously grown on citrate were used. Warburg flasks were flushed with 5% O_2, 10% CO_2 and 85% H_2

Fig. 7. Effect of oxygen concentration upon the induction rate of hydrogenase (hydrogen-oxidase). Cells previously grown on succinate were used. Warburg flasks were flushed with a mixture of 50% H_2, 10% CO_2, x% O_2 and rest N_2. ● 2.5% O_2; ■ 5% O_2; ▲ 10% O_2; ◆ 20% O_2; ○ 30% O_2. *Inset:* dependance of parabolic induction rate m from O_2 concentration. Values were calculated from the above results

Fig. 8A–D. Repression of hydrogenase synthesis. Cells previously grown on succinate medium were used. Warburg flasks were flushed with 5% O_2 + 10% CO_2 + 85% H_2. The repressive treatment was applied (*arrows*) as soon as an activity of about 300 μl $H_2 \cdot h^{-1} \cdot mg^{-1}$ protein had been reached. (A) Repression by raising the O_2 concentration to 30%. (B) Addition of 50 μg $\cdot$ ml^{-1} rifampicin. (C) Addition of 200 μg $\cdot$ ml^{-1} streptomycin. (D) Control without increasing the oxygen concentration in the gas mixture

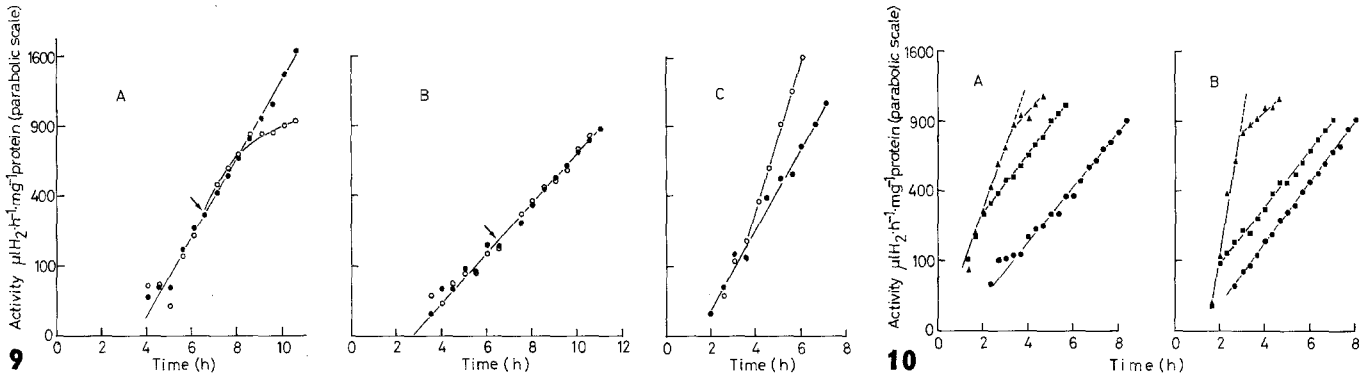

Fig. 9 A–C. Effect of organic substrates on hydrogenase induction. Cells previously grown on the compounds to be tested were used. Warburg flasks were flushed with 5% O_2 + 10% CO_2 + 85% H_2. The organic substrate was added during the induction process (*arrows*). ○ experiment with added organic substrates; ● control without added organic substrate. (A) Effect of gluconate. (B) Effect of succinate. (C) Effect of citrate

Fig. 10 A and B. Stimulation of hydrogenase induction rate by organic substrates. Cells previously grown on the compound to be tested were used. Warburg flasks were flushed with 5% O_2 + 10% CO_2 + 85% H_2. The organic substrate was added at the beginning of the induction process. ▲ 0.01% organic substrate added; ■ 0.001% organic substrate added; ● control without organic substrate. (A) Test with citrate. (B) Test with pyruvate

same phenomenon was observed when induction was stopped at the mRNA synthesis level by rifampicin (Fig. 8 B), whereas streptomycin (acting at the level of protein synthesis) stopped enzyme synthesis immediately (Fig. 8 C). It is, therefore, likely that oxygen (or a compound formed in the presence of oxygen) acts on the transcriptional level. The delay between the addition of the repressing substance and the arrest of enzyme synthesis suggests that the mRNA involved has a relatively long life span.

Effect of Organic Substrates on Hydrogenase Induction. To study the effect of organic substrates on hydrogenase induction cells previously grown on the substrate to be tested were used. In a first experiment, during the induction process in the presence of hydrogen the substrate was added at a concentration similar to that in heterotrophic culture media (15 mM). The results differed remarkably according to the substrate tested (Fig. 9). After addition of gluconate (Fig. 9 A), hydrogenase synthesis continued for about 2 h, before decreasing. With succinate (Fig. 9 B), no change was noted during the time of the experiment, as compared with a control without added substrate. Upon addition of citrate (Fig. 9 C), the rate of hydrogenase formation increased immediately and strongly.

The delayed repressive effect of gluconate is similar to that obtained with oxygen or rifampicin, and tends to confirm the relatively high stability of the mRNA involved. The stimulation of induction by citrate was unexpected. Further tests were performed using lower concentrations (resp. 0.01% and 0.001%) of organic substrate, and the substrate was added at the beginning of the experiment. The results were analogous when either citrate (Fig. 10 A), DL-lactate or pyruvate were used (Fig. 10 B): in the presence of the organic substrate, the initial induction rates were much higher (up to 40 times higher) than in the control without substrate. For a short period the induction rate was high and then decreased to the level of the control without added substrate. The duration of the stimulated induction period, but not the intensity of stimulation, depended on the concentration of the substrate; the return to the "normal" induction rate coincided with the exhaustion of the substrate.

Heterotrophic Growth. The rates of growth on organic substrates under air were, in the best cases, two-fold higher than under autotrophic conditions. The oxidation rates were determined for acetate, gluconate, citrate and succinate, with cells grown either autotrophically or heterotrophically with the same substrate as tested. The cells were harvested during the exponential growth phase, washed twice and suspended in 50 mM phosphate buffer at pH 7.0. Oxygen uptake was measured in Warburg vessels at 30° C. The results (Table 4) showed that autotrophically grown cells oxidized acetate, succinate and gluconate constitutively. The activity was more or less below that in fully adapted cells. Citrate oxidation was, on the other hand, inducible.

Among the sugars and sugar derivatives tested (Aragno and Schlegel, 1978) only gluconate and 2-ketogluconate were utilized as sole carbon and energy sources. The degradative enzymes were tested in cells grown on gluconate, succinate and under autotrophic conditions. Enzymes were tested, which are representative for the Embden-Meyerhof pathway (hexokinase, phosphoglucose isomerase, phosphofructokinase, fructosebisphosphate aldolase, as well

Table 4. Respiratory rates of washed cell suspensions either grown on the corresponding substrate or grown autotrophically

Substrate	Respiratory rates ($\mu l\ O_2 \cdot h^{-1} \cdot mg^{-1}$ protein)		
	Substrate-induced cells	Autotrophically grown cells	
		initial	after 4 h
None (endogenous)	14–22	15	6
Acetate	n.d.[a]	77	79
Gluconate	74	37	37
Citrate	73	18	41
Succinate	157	128	117

[a] n.d. = not determined

Table 5. Activity of enzymes of degradation pathways

Enzyme	Activity (U/g) in extracts of cells grown on		
	Gluconate	Succinate	H_2+CO_2
Hexokinase	20	22	17
Phosphoglucose isomerase	150	46	122
Phosphofructokinase	0	0	0
Fructose bisphosphatase	104	112	157
Fructosebisphosphate aldolase	15	18	9
Gluconate kinase	41	6	4
6-Phosphogluconate dehydratase	689	174	405
2-Keto-3-deoxy-6-phospho-gluconate aldolase	449	88	221
Glucose-6-phosphate dehydrogenase, NADP	105	13	54
Glucose-6-phosphate dehydrogenase, NAD	30	4	18
6-Phosphogluconate dehydrogenase, NADP	0	0	0
6-Phosphogluconate dehydrogenase, NAD	32	14	33

One enzyme unit (U) converts 1 μmole substrate per min

as fructosebisphosphatase), of the Entner-Doudoroff pathway (gluconate kinase, 6-phosphogluconate dehydratase and 2-keto-3-deoxy-6-phosphogluconate aldolase) and of the oxidative pentose-phosphate pathway (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase).

The results (Table 5) indicate that phosphofructokinase activity was absent, indicating that the enzymes of the Embden-Meyerhof pathway serve only a biosynthetic function. This explains also that the activity of the Embden-Meyerhof pathway enzymes are similar

Table 6. Doubling times during growth on gluconate and citrate in the presence and absence of hydrogen

Gas phase	Organic substrate (0.2%)	
	D-Gluconate	Citrate
80% H_2 + 20% O_2	2.3 h	2.5 h
Air	2.4 h	2.4 h

in gluconate and in autotrophically grown cells. The presence of hexokinase should allow *A. autotrophicum* to use hexoses, the failure to grow on hexoses is probably due to the absence of the relevant permeases.

The enzymes of the Entner-Doudoroff pathway were present and constitutively formed, although at a higher level in gluconate-grown cells. In succinate-grown cells, their level was much lower than in autotrophically-grown cells; this indicates that these enzymes are partially repressed by succinate.

Enzymes of the oxidative pentosephosphate pathway were also constitutively formed. A particularity: 6-phosphogluconate dehydrogenase reduced only NAD. It is thus likely that gluconate is degraded mainly through the Entner-Doudoroff pathway, the other pathways being used for biosynthetic purposes.

Since the enzymes of the Entner-Doudoroff pathway were constitutive and were synthesized even under autotrophic conditions, it was expected that hydrogen did not exert a repressive effect on their synthesis. The effect of hydrogen on the growth on D-gluconate and citrate was tested using cells grown initially autotrophically and then with the organic substrate for 4 h. Such cells contained both enzyme systems: hydrogenase and the uptake systems for organic substrates. These cells were then transferred into mineral media with 0.2% (w/v) substrate, and incubated with agitation either under air or under 80% H_2 + 20% O_2. The growth rates under air and under oxy-hydrogen mixture were identical (Table 6). This result indicates that in *A. autotrophicum* hydrogen exerts neither an inhibitory nor a repressive effect on the utilization of organic substrates.

DISCUSSION

Aquaspirillum autotrophicum is so far the only member of the genus which grows autotrophically with hydrogen as electron and energy source (Aragno and Schlegel, 1978). The new species differs remarkably from *Alcaligenes eutrophus*, so far the best studied hydrogen bacterium, with respect to physiological properties and regulation patterns of heterotrophic and autotrophic metabolism (Table 7). The hydrogenase is

Table 7. Comparison of some physiological properties between *Aquaspirillum autotrophicum* SA 32 and *Alcaligenes eutrophus* H 16

	A. autotrophicum	*A. eutrophus*
Particulate hydrogenase, *not* NAD-specific	+	+[a]
Soluble hydrogenase, NAD-specific	−	+[a]
Hydrogenase(s) is (are)	inducible	constitutive[b]
Hydrogenase induction stimulated by citrate	+	−[c]
Ribulosebisphosphate carboxylase is	inducible	constitutive[b]
Entner-Doudoroff pathway enzymes are	constitutive	inducible[b]
6-Phosphogluconate dehydrogenase is	present, constitutive NAD-specific	absent[b]
Bartha effect	−	+[d]

[a] Schneider and Schlegel (1977)
[b] Schlegel and Eberhardt (1972)
[c] Aragno (unpublished)
[d] Bartha (1962)

apparently exclusively membrane-bound, although the presence of an extremely labile NAD-dependent soluble hydrogenase cannot be entirely excluded (Schneider and Schlegel, 1977). It shares this property with most of the known species of hydrogen bateria: *Alcaligenes paradoxus*, *Pseudomonas pseudoflava*, *P. palleronii*, "*Corynebacterium*" *autotrophicum*, *Paracoccus denitrificans* (Schneider and Schlegel, 1977) and a recently isolated thermophilic *Bacillus* sp. (Aragno, in preparation). The hydrogenase appears to be immunologically related to the membrane-bound hydrogenases of the *Alcaligenes* and *Pseudomonas* hydrogen oxidizing species (Schink, 1977). Furthermore all these bacteria have a similar cell wall fine structure (Walther-Mauruschat et al., 1977). The hydrogenase is inducible; even cells grown under air on substrates which do not repress hydrogenase synthesis (citrate, pyruvate, lactate) are completely devoid of hydrogenase. The ribulosebisphosphate carboxylase behaves in a similar manner. In contrast, the enzymes for gluconate degradation (Entner-Doudoroff pathway) are constitutively formed. As a consequence, no repression of heterotrophic growth by hydrogen was observed, even by a substrate the oxidation of which is inducible, such as citrate. The behaviour of *Paracoccus denitrificans* was reported to be similar (Blackkolb and Schlegel, 1968).

Oxygen strongly represses hydrogenase synthesis and does not inhibit the enzyme present in whole cells. Microaerophily observed under autotrophic conditions only, is likely to be a consequence of repression by oxygen. Apparently the control of hydrogenase synthesis operates at the transcriptional level. This conclusion is drawn from the similarities of the repressive effect exerted by oxygen, by gluconate and by rifampicin. The relatively long delay between the addition of the repressor and the arrest of enzyme synthesis suggests that the mRNA involved has a relatively long life span. A long life time was already discussed with respect to the mRNA coding the membrane-bound hydrogenase of "*Corynebacterium*" *autotrophicum* strain 14g (Schneider et al., 1973). Both hypotheses (mRNA with long life span and control of hydrogenase synthesis at the transcriptional level) are supported by the parabolic shape of the induction kinetics.

The relatively high ratio of oxygen consumed to carbon dioxide fixed ($n = 4.2$) is perhaps the consequence of the need for additional energy for the electron transfer from hydrogenase to NAD through an ATP-dependent reverse electron flow. A hydrogen bacterium which possesses both hydrogenases, a membrane bound and a soluble, NAD-dependent one, such as *Alcaligenes eutrophus* needs much less oxygen and hydrogen for its growth; the ratio n may, under especially favourable conditions, even approach unity (Bongers, 1970).

The stimulation of hydrogenase induction by the addition of organic substrates such as citrate, pyruvate or lactate besides hydrogen has up to now received no satisfactory explanation. *Alcaligenes eutrophus* H 16 shows a completely different response. Possibly, the organic substrate serves as carbon and energy source for enzyme synthesis. But other substrates such as succinate, which is quickly metabolised, do not enhance the rate of hydrogenase synthesis. A more sophisticated type of control is thus likely to occur, and needs further investigation.

The constitutivity of the enzymes degrading organic substrates, together with the adaptative nature of autotrophic enzymes indicates that *A. autotrophicum* is predominantly a heterotrophic bacterium. The consideration of its natural habitat results in a different concept. The lake, from which this bacterium was isolated, is a small eutrophic, holomictic lake (Schweizer and Aragno, 1975). In spring, the waterbody becomes stratified and oxygen soon disappears from the bottom layers. In summer, the decomposition of organic matter in the anoxygenic layers gives rise to the production of non-fermentable organic substrates as well as ammonia, sulfide and hydrogen. Thus, when oxygen gets in contact with hydrogen—at the chemocline, or during the autumnal mixing—it gets in contact with oxidizable organic substrates, too. In this habitat, the growth conditions for hydrogen bacteria are, therefore, mixotrophic rather than purely autotrophic. A mechanism resulting in the stimulation of

hydrogenase synthesis by organic substrates is apparently advantageous to the cells in such a system: in the presence of both the substrates, $H_2 + CO_2$ and organic compounds, the growth rate of *A. autotrophicum* may be increased and may support these cells in competing with purely heterotrophic bacteria. Wheter *A. autotrophicum* is particularly well adapted to this environment needs to be studied.

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