Arch. Mikrobiol. 93, 179—193 (1973)

Description and Physiological Characterization of a Coryneform Hydrogen Bacterium, Strain *14 g*

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Received July 12, 1973

Summary. A new hydrogen bacterium has been isolated by enrichment culture on hydrogen, carbon dioxide, and oxygen. It is a strictly aerobic, Gram-positive, immotile rod. Irregular cell shapes, the snapping type of cell division and the partial fragmentation of the rods into coccoid cells in older heterotrophic cultures indicate that strain *14 g* is a coryneform bacterium.

Strain *14 g* grows well in a mineral salts medium under a gas atmosphere of 80% hydrogen, 10% carbon dioxide and 10% oxygen. With increasing oxygen partial pressures, the growth rate decreases. While growth is good or excellent on organic acids, carbohydrates are utilized not at all.

Autotrophically grown cells are able to oxidize organic substrates after a relatively short lag-phase. Growth is retarded when hydrogen is present in the gas atmosphere. Citrate utilization is even inhibited by hydrogen.

In gluconate grown cells gluconokinase, the enzymes of the Entner-Doudoroff system and a NAD-dependent 6-phosphogluconate dehydrogenase are present. All enzymes are inducible.

The uptake rate of the oxygen-hydrogen mixture by resting cells is very high and amounts to 2500 µl gas/mg protein · h. Carbon dioxide does not stimulate the oxyhydrogen-reaction. The hydrogenase activity of intact cells measured with methylene blue as a hydrogen acceptor is limited by the penetration of methylene blue into the cell. In cell-free extracts the hydrogen uptake rate amounts to 2260 µl H_2/mg protein · h with methylene blue as H-acceptor. The hydrogenase of strain *14 g* is a strictly inducible enzyme. It is completely part culate and does not reduce pyridine nucleotides.

Several hydrogen bacteria have been reported to be yellow-pigmented: *Bacillus pantotrophus* (Kaserer, 1906), *Pseudomonas pantotropha* (Romanova and Doman, 1960), *Alcaligenes paradoxus* (Davis *et al.*, 1969), *Hydrogenomonas flava* (Niklewski, 1910), *Pseudomonas palleronii* (Davis *et al.*, 1970) and two coryneform bacteria, strain *14g* (Rudolph, 1968) and strain *7 C* (Siebert, 1969; Tunail and Schlegel, 1972).

Comparative investigations on hydrogen bacteria directed our attention to strain *14 g* since it grows excellently as a hydrogen bacterium and is an immotile, encapsulated bacterium which produces large amounts of slime. Morphological and physiological properties are described in this paper.

Materials and Methods

Enrichment. Strain *14 g* is one of the strains which have been selected from soil samples by liquid enrichment cultures in mineral medium with carbon dioxide, hydrogen and oxygen in the gas atmosphere. Strain *14 g* stood out by its high content of polysaccharides and by its good growth rate under autotrophic conditions (Rudolph, 1968).

Organisms. Hydrogenomonas eutropha strain *H 16* (ATCC 17699) was obtained from the culture collection of the Institute.

Physiological Characteristics. Physiological properties for taxonomical identification were determined as described by Cowan and Steel (1965).

Culture Media and Growth Conditions. The bacteria were grown autotrophically according to Schlegel *et al.* (1961). 0.30 ml trace element solution SL 6 was added to 1 l mineral solution; SL 6 corresponds to solution SL 4 (Pfennig and Lippert, 1966), however, lacks EDTA and iron salt. The complete mineral medium had a pH of 7.0 to 7.2. For growth under autotrophic conditions 0.05% $NaHCO_3$ was added and the gas mixture contained 80% H_2, 10% O_2, and 10% CO_2, if not stated otherwise. For heterotrophic growth sodium bicarbonate was omitted and the medium was supplemented by 0.2 to 0.5% organic substrates, and the gas atmosphere was air. Small volumes of autotrophic cultures (20 to 200 ml) were shaken in Erlenmeyer flasks of different size, placed in desiccator under the required gas mixture. Larger quantities of suspensions were magnetically stirred in 2 or 6 l flat-bottomed round flasks, connected with the gas mixture contained in 10 l gasometer flasks.

Substrate Utilization under Air and Knallgas. The experiments to measure the suppression of substrate utilization by hydrogen were carried out as described by Probst and Schlegel (1973).

Optical Density. The optical density of the cell suspension was measured in cuvettes ($d = 1$ cm) in a Zeiss-photometer (PL 4) at a wave length of 546 nm.

Protein Determinations. The protein content of intact cells was determined by employing a modified method of Schmidt *et al.* (1963). Cell suspensions were heated with 30% NaOH for 15 min in a boiling water bath. The protein content of cell-free fractions was determined by the method of Beisenherz *et al.* (1953).

Dry Weight. 100 ml of the cell suspension were collected by centrifugation, washed twice with a 0.03% NaCl solution, resuspended in 15 ml distilled water and transferred into a preweighed alumina cup. The cup was dried at 90°C and after cooling in the desiccator was weighed again.

Manometric techniques were identical with that described by Aggag and Schlegel (1973).

Preparation of Cell-Free Extracts. Cells harvested at the end of the exponential growth phase were washed and resuspended in 0.067 M phosphate buffer, pH 7.0, to give a final density of 20 mg protein/ml. This suspension was kept frozen at −20°C. After thawing the cell suspension was sonicated (Fa. Schoeller & Co., Frankfurt; 20 kHz and 600 W) at intervals of 30 sec; the sonication lasted 1 min/ml. Intact cells and cell debris were removed by centrifugation at 10000 g for 20 min. The supernatant crude extract was centrifuged at 100000 g for 1 h resulting in the supernatant (soluble fraction) and the sediment (particles), which was resuspended in the same phosphate buffer.

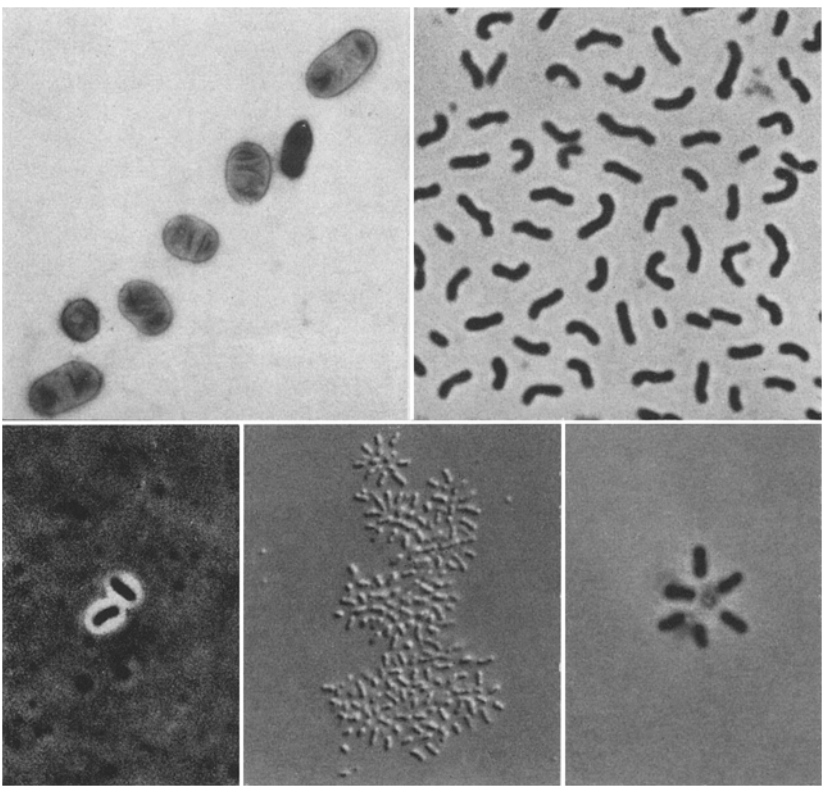
Enzyme Assays. The enzyme activities were determined by optical tests in a Zeiss-photometer (PL 4) at a wave length of 365 nm. One enzyme unit is that which transfers 1 μmole substrate/min. The specific activity is given as unit/g protein. The following enzyme assays were used: Malate dehydrogenase and NADH oxidase (described in detail by Aggag and Schlegel, 1973); gluconokinase (Cohen, 1955); Entner-Doudoroff enzyme system (Gottschalk *et al.*, 1964); 6-phosphogluconate dehydrogenase (Bowien, 1970); gluconate dehydratase (Bender, 1972).

Chemicals. Methylene blue was obtained from Merck, Darmstadt; NAD, $NADH_2$, and oxaloacetic acid from Boehringer Mannheim. All chemicals used were of analytical grade.

Results

Morphological Characterization

The cells of strain *14 g* are Gram-positive, encapsulated (Fig. 1 c), non-flagellated, immotile rods with a diameter of about 0.4—0.7 μm and 0.8—3.0 μm length. The cell ends are round and swollen and contain granules. Shape and size of cells vary with the culture media and the age of the culture. In autotrophic cultures exponentially growing rods have a normal shape, are short and frequently appear almost oval (Fig. 1 a).



c d e

Fig. 1 a—e. Cells of the hydrogen bacterium strain *14 g*. a Electronmicrograph of autotrophically grown cells from the exponential growth phase. b Irregularly shaped cells from a heterotrophically grown (malate) non-agitated liquid-culture. c Demonstration of the bacterial capsuls in an indian ink mount. d Cell aggregates from a nutrient broth medium prepared by interference microscopy. e Cell aggregates ("Starformation") from nutrient broth medium. Magnification: a 5450fold, b 1350fold, c 1000fold, d 480fold, e 1350fold

On solid complex and gluconate media strain *14 g* does not show distinctive pleomorphism but the snapping type of cell division can be observed. This indicates that strain *14 g* is a coryneform bacterium. Significant irregularities of cell shape become visible in liquid cultures, containing certain fatty acids (malate, succinate): Under these conditions the rods become elongated, straight, club-shaped or curved and bent in different ways, often occur in the form of V-shaped pairs (Fig. 1 b). Especially cells grown in succinate media may show thickenings in the middle of the rod and a slight tendency to branch. In aged cultures, after 2–3 days, the long rods partially fragment into cocci or very short rods. Large cocci (cystites), which germinate to produce rod-shaped cells when transferred to fresh media, were not observed.

In liquid media containing nutrient broth, frequently cell aggregates and rosettes are formed (Fig. 1 d and e). Transient formation of such aggregates was observed after inoculation into defined media. Obviously, rosette formation depends on physiological conditions and factors which are present in nutrient broth.

On solid media, round, slimy, glistening, yellow, opaque colonies with entire edge are formed. The intensity of pigmentation is dependent on the growth substrate; colonies appear pale yellow under autotrophic conditions and deep yellow on citrate agar. An influence of light on the intensity of pigmentation has not been observed.

The yellow pigmentation is caused by carotenoids. They were extracted from packed cells by an acetone-methanol mixture (7:2; v/v), and after saponification with 7% KOH in methanol the unsaponifiable fraction was transferred into ether, dried with anhydrous sodium sulfate and separated by chromatography on Kieselgur paper (Schl. & Sch. Nr. 287) employing 50% acetone in petroleum ether as a solvent. Four bands were formed. The main band ($R_f = 0.30$) was eluted and dissolved in different solvents. The carotenoid is insoluble in petroleum ether and ethyl ether, soluble in acetone, ethanol, methanol, chloroform, and pyridine. The absorption maxima are given in Table 1.

Physiological Properties

The cells (autotrophically grown) are Gram-positive and not acid-fast. Nitrate reduction: +; oxidase: +; catalase: + (weak); starch utilization: –; gelatine hydrolysis: –; hydrolysis of poly-β-hydroxybutyrate: –; accumulation of poly-β-hydroxybutyrate: +; arginine dihydrolase: –; indole formation: –; hydrogen sulfide formation: –; Voges-Proskauer reaction: –; anaerobic growth: –; acid or gas formation from carbohydrates: –; purple milk: alcalinization; organotrophic or autotrophic denitrification: –. Penicillin sensitivity + with a minimal inhibitory concentration of approximately 1.2 µg penicillin G/ml.

Substrate utilization was tested on agar and in liquid media contain-

Table 1
Absorption maxima of the main carotenoids of strain *14 g* in different solvents

Solvents	Absorption maxima (nm)		
Petroleum ether	—	—	—
Acetone	(430)	455	480
Ethanol	(430)	452	480
Chloroform	(440)	464	490
Pyridine	(442)	470	498

ing 0.2% of carbon compound. Growth was observed for two weeks. No growth occurs on carbohydrates (D-glucose, D-fructose, D-mannose, D-galactose, L-arabinose, D-ribose, D-xylose, L-rhamnose, sucrose, maltose, starch). However, on D-gluconate growth is moderate. Growth is good or excellent on the following substrates: acetate, succinate, DL-lactate, L-malate, citrate, pyruvate, and β-hydroxybutyrate. The DNA of strain *14 g* has a density of 1:728 and a guanine-cytosine (GS)-content of 69.4% (Mandel, personal comm.).

Heterotrophic Growth and Oxidation Rates

For a few organic substrates the doubling time has been measured. The cells were grown in shaken liquid culture overnight, diluted and used for inoculation of a fresh medium containing the same substrate. The cells grew without a lag-phase and exponential growth continued at least for 8 h. The doubling times (in hours) are listed in Table 2. With glucose, fructose, and galactose as sole energy sources no turbidity increase was observed.

The optimal growth temperature (Fig. 2B) and the pH-optimum for growth (Fig. 2A) were estimated in cultures growing in 0.2% DL-lactate medium. The highest growth rates were found between 30 and 33° C. The pH-curve for growth on DL-lactate exhibits an optimal area between pH 6.7 and 7.2; pH 5.3 is just tolerated, and at pH 5.0 no growth occurs.

The oxidation rates (Q_{O_2}) were determined only for a few substrates. The cells were harvested at the end of the logarithmic growth phase from cultures growing on the particular substrate; the washed cells were suspended in 0.067 M phosphate buffer, pH 7.0, and oxygen uptake was measured in Warburg vessels at 30° C. The highest oxidation rate was that during utilization of succinate with 192 µl oxygen/mg protein per hour (Table 2). Malate, citrate, and lactate are oxidized with a similar rate. Endogeneous respiration is low. Glucose or xylose do not increase oxygen uptake above that of endogeneous respiration. This observation indicates that strain *14 g* is not only unable to grow on these sugars, but is even lacking the corresponding dehydrogenases.

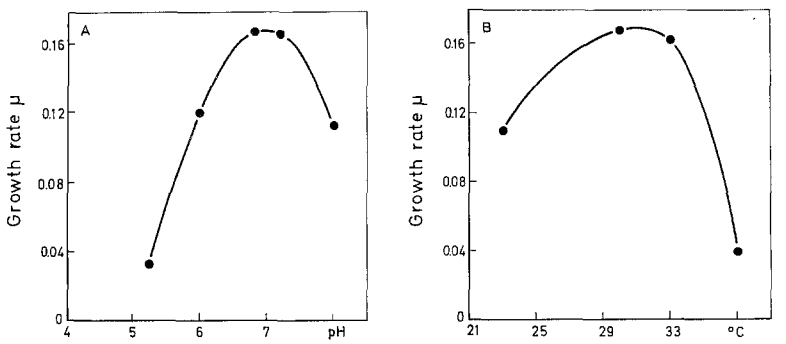


Fig. 2 A and B. Effect of pH (A) and temperature (B) on the growth rate during growth in a lactate medium. 200 ml Erlenmeyer-flasks each containing 50 ml lactate nutrient solution were inoculated by lactate grown cells and magnetically stirred at varied temperatures or pH-values. Turbidity (E_{546}) and the pH were measured at intervals, and the growth rate μ was calculated

Table 2. Growth rates on organic substrates and oxidation rates

Substrate	Growth rate μ (h^{-1})	Doubling time (h)	Oxidation rate Q_{O_2} (μl O_2/mg protein · h)
Succinate	0.228	3.04	192
L-Malate	0.209	3.31	184
β-OH-butyric acid	0.205	3.37	—
Citrate	0.163	4.25	168
DL-Lactate	0.150	4.62	162
Pyruvate	0.133	5.21	—
Acetate	0.108	6.41	—
Gluconate	0.076	9.12	67
Adipic acid	0.063	11.00	—
Glutamate	0.044	15.75	—
Ethanol	0.021	33.65	—
Endogeneous	0		12
Glucose	0		like endogeneous
Fructose	0		—
Galactose	0		—

Chemolithoautotrophic Growth and Oxidation Rates

Strain *14 g* grows very well in a mineral salts medium under a gas atmosphere of 80% hydrogen, 10% carbon dioxide, and 10% oxygen. While the growth rate is not influenced by the partial pressure of hydrogen, it is highly dependent on the oxygen partial pressure. In order to choose an optimal gas mixture, the cells were incubated in the presence of different gas mixtures and the increase of optical density (E_{546}) was measured for eight hours (Fig. 3). While the CO_2 content remained con-

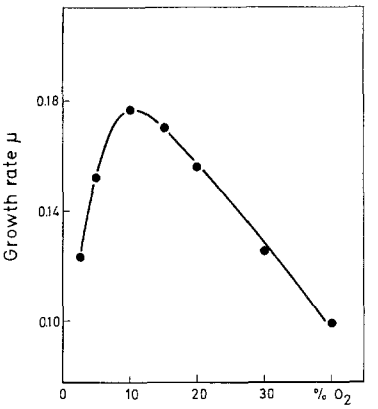


Fig. 3

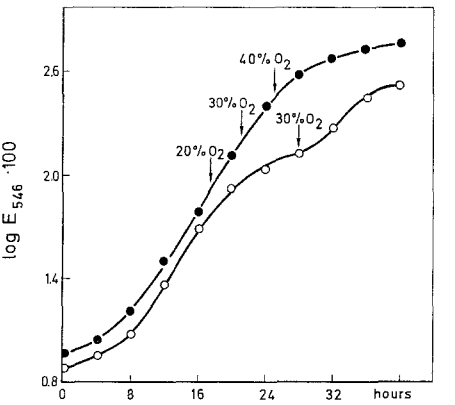


Fig. 4

Fig. 3. Dependence of growth rate on the oxygen contents of the atmosphere. 200 ml Erlenmeyer-flasks each containing 50 ml medium were inoculated with autotrophically grown cells and shaken in a water bath at 30 °C. While the CO_2-concentration was 10% in all flasks the concentration of oxygen and hydrogen were varied. Turbidity was determined at intervals

Fig. 4. Autotrophic growth and the effect of the oxygen concentration in the gas atmosphere. 6 l flat bottomed round flasks containing 3 l mineral medium were inoculated by autotrophically grown cells and magnetically stirred under an atmosphere of 80% H_2, 10% O_2, and 10% CO_2 at 30° C. After 28 h the oxygen concentration was increased to 30% (○). Exponential growth continued when the oxygen concentration was increased stepwise from 10 to 40% (•)

stant, the oxygen content was varied between 2.5 and 40% and correspondingly the hydrogen between 87.5 and 50%. The cell density was kept low in order to maintain the equilibrium between the oxygen concentrations in the gas and liquid phases. Optimal growth rates with doubling times of about 4 h have been measured within a range of 7.5 to 12.5% oxygen. With increasing oxygen partial pressures, the growth rate decreased drastically.

As suggested by these results, exponential growth in magnetically stirred suspensions in 6 l flat-bottomed round flasks or in fermenters is only possible, if the oxygen content of the gas mixture is gradually adapted to the increasing bacterial density of the suspension, i.e. by "gradient gassing". After preliminary experiments we succeeded in establishing exponential growth up to a cell density of $E_{546} = 6.0$ (about 2.5 g dry weight per l) by increasing the oxygen partial pressure from 10 to 40% (Fig. 4).

The rate of gas uptake by resting cells under a hydrogen oxygen gas atmosphere is very high and exceeds the rate measured with *Hydro-*

genomonas eutropha strain *H 16*; it amounts to 2500 µl gas/mg protein · h. The influence on the rate of gas uptake exerted by the presence of carbon dioxide is very small; under $H_2 + O_2 + CO_2$ it is 2816. The percent increase (11%) corresponds to the amount of carbon dioxide which is consumed by the cells when hydrogen is oxidized. This increase of the rate of gas uptake is, therefore, not comparable to that exhibited by strain *H 16*, known as the "Bartha-effect" (Schlegel and v. Bartha, 1961); in this case the increase of the rate of gas uptake amounts to 400% and is hypothetically due to a regulatory effect similar to respiratory control. With respect to the stimulatory effect of CO_2, strain *14 g* belongs to those organisms, like *Pseudomonas saccharophila*, strain *12x*, and strain *12/60/x*, which give almost no response to CO_2 (Eberhardt, 1966, 1969).

If the hydrogenase activity is measured employing intact cells with methylene blue as a hydrogen acceptor, the specific activity is rather low; the highest activity measured amounts to 584 (at a cell density of $E_{546} = 3.0$). Since autotrophically grown cells oxidize hydrogen with a rate of 2508 µl hydrogen oxygen mixture/mg protein · h and since hydrogen and oxygen are consumed in a proportion of 2:1, a Q_{H_2} of approx. 1600 has to be expected. This difference of the H_2-uptake rates with either oxygen or methylene blue as the H-acceptors is indicative for a limitation of the reaction rate by the penetration of methylene blue into the cell. This assumption has been confirmed by experiments using a cell-free extract of autotrophically grown cells. The Q_{H_2} (methylene blue) of a crude extract is 2260 µl H_2/mg protein · h and meets the expectations.

Suppression of Substrate Utilization by Hydrogen

Autotrophically grown cells are able to oxidize organic substrates after a relatively short lag-phase. The enzymes for succinate utilization are obviously constitutive, although not at their maximal concentration. The initial rate is 102 µl O_2/mg protein · h and increases within $1^1/_2$ h to 190. In the presence of lactate the initial rate is 46 and increases within 2 h to 116. The enzymes of citrate utilization are apparently inducible. The initial rate is negligible or zero; without a nitrogen source the rate increases only to 81; that is not half as much as the oxidation rate of maximally adapted citrate grown cells.

In *H. eutropha* strain *H 16* and many other strains growth on organic substrates is suppressed when hydrogen is present in the gas atmosphere (Gottschalk, 1965; Blackkolb and Schlegel, 1968a). This suppression is due to the repression of enzyme formation; if e.g. *H 16* is incubated in a fructose containing medium under a CO_2-free hydrogen-oxygen mixture, the formation of the enzymes of the Entner-Doudoroff system is suppressed. Furthermore, the function of this pathway is inhibited pro-

bably due to the inhibition of glucose-6-phosphate dehydrogenase by $NADH_2$ and ATP (Blackkolb and Schlegel, 1968b).

The potential suppression of organic substrate utilization by hydrogen in strain *14 g* was tested employing gluconate, citrate, or succinate as substrates. When autotrophically grown cells were incubated in a growth medium with each of the organic substrates under an atmosphere of 80% hydrogen and 20% oxygen, growth was retarded compared to the controls shaken under air. The repression of growth was almost complete with citrate as the substrate. This observation is in agreement with the complete adaptive nature of citrate utilization.

When the cells were grown on gluconate, citrate, or succinate under air and were then exposed to the hydrogen-oxygen atmosphere in the presence of their growth substrates, the results were different. With gluconate and succinate as substrates the cells continued to grow unimpaired, while with citrate cell growth was almost completely inhibited. Whether this inhibitory effect is due to inhibition of isocitrate dehydrogenase by "energy charge" indicators has not been tested.

The Degradative Pathway for Gluconate

The only compound related to carbohydrates and used as a growth substrate by strain *14 g* is gluconate. The assumption gluconate to be degraded via the Entner-Doudoroff pathway has been supported by enzyme measurements employing crude extracts of strain *14 g* and as a control of strain *H 16*. From the results summarized in Table 3 the following conclusions may be drawn: In gluconate grown cells gluconokinase as well as the enzymes of the Entner-Doudoroff system (6-phosphogluconate dehydratase + 2-keto-3-deoxy-6-phosphogluconate aldolase) are present.

Table 3
Specific activities of the key enzymes for gluconate degradation in strain *14 g*

Enzyme	Specific activity (μmole/g protein · min) after growth on			
	$H_2 + O_2 + CO_2$ (autotrophically)		Gluconate	
	strain *14 g*	*H 16*	*14 g*	*H 16*
Gluconokinase	0	1.2	5	10.7
Entner-Doudoroff enzyme system	0	1.0	32.4	145
Gluconate-dehydratase	0	0	0	0
6-Phosphogluconate dehydrogenase				
NADP dependent	0	0	0	0
NAD dependent	2	0	41.1	0

Furthermore, the cells contain a NAD-dependent 6-phosphogluconate dehydrogenase. The specific activity of these enzymes is rather low which is in agreement with the low growth rate during growth on gluconate (doubling time about 9 h). Gluconate dehydratase has not been found; this indicates that gluconate is not degraded via a modified Entner-Doudoroff-pathway. The enzymes mentioned are not present in autotrophically grown cells; they are inducible.

Induction of Hydrogenase Formation

Hydrogenase is a constitutive or semi-constitutive enzyme in some hydrogen bacteria *(Hydrogenomonas eutropha* strain *H 16, Pseudomonas saccharophila, Pseudomonas ruhlandii)* and is strictly inducible in other bacteria *(Paracoccus denitrificans)*. In order to test the adaptive behaviour of hydrogenase in strain *14 g*, the cells were grown for 3 transfers in liquid cultures containing different organic acids. Washed cells were used to measure the oxy-hydrogen reaction in Warburg vessels. No organic substrate had supported the formation of hydrogenase. The initial rate of hydrogen oxidation was zero for all samples. During incubation under the H_2-O_2-atmosphere within a few hours all samples reached a specific rate of gas uptake between 650 and 850 µl gas mixture/mg cell protein · h. This means that hydrogenase is not constitutive in strain *14 g* and that the cells are able to synthesize hydrogenase even in the absence of a nitrogen source and under these conditions reach a specific activity corresponding to 25% of that of fully induced cells (Table 4). If the medium contains a nitrogen source and the gas atmosphere contains carbon dioxide, the specific activity of hydrogenase reaches 2062; that is approximately the value of fully induced cells.

If the cells grown heterotrophically as mentioned above are exposed the CO_2 containing hydrogen-oxygen mixture in the presence of their corresponding growth substrates (0.04% organic acids) hydrogenase is induced, too. However, the enzyme is formed at a lower rate. Hydro-

Table 4. Induction of hydrogenase in heterotrophically (malate) grown cells under a hydrogen oxygen atmosphere and in the absence or presence of carbon dioxide and minerals

Specific rate of gas uptake reached after period of time	Gas components (µl/mg protein · h)		
	Resting cells under $H_2 + O_2$	Resting cells under $H_2 + O_2 + CO_2$	Growing cells under $H_2 + O_2 + CO_2$
15 min	0	0	0
1 h	63	95	126
10 h	724	853	2062

genase formation is, therefore, partially repressed by the organic acid present. The repression is never complete. Under these "mixotrophic" conditions, growth is faster than under purely autotrophic conditions indicating that the organic substrate and hydrogen are utilized simultaneously.

If autotrophically grown cells are transferred to a growth medium containing an organic substrate (succinate) and are incubated under air, the specific activity of hydrogenase gradually decreases During the initial 6 h the decrease is slow and than is accelerated. This indicates that during initial hours after transfer from auto- to heterotrophic conditions hydrogenase synthesis still continues and than stops; during the whole incubation period the enzyme is diluted out. Possibly, the life time of m-RNA for hydrogenase is longer than that of other enzymes, as has been recently reported for exoenzyme formation of *Bacillus amylolyticus* (Gould *et al.*, 1973).

Hydrogenase in Cell-Free Extracts

As already mentioned, the crude cell-free extract obtained from autotrophically grown cells by sonic disintegration contains hydrogenase whose specific activity ($Q_{H_2} = 2260$) is in the order of magnitude of that of intact cells ($Q_{H_2} = 1660$). In order to study the localization of hydrogenase in the cell, the crude extract was fractionated by centrifugation at 100000 g for 1 h resulting in a supernatant (soluble) and a sediment (particulate) fraction. Hydrogen uptake was measured using both fractions and oxygen, methylene blue and NAD as H-acceptors (Table 5). Neither the soluble nor the particulate fraction was able to reduce NAD. In both the manometric test and the optical test system NAD failed to function as a H-acceptor. When methylene blue was used as H-acceptor, the specific H_2-uptake rate of the soluble fraction was 672, and the particles reach an eminent rate of 3300 µl H_2/mg protein · h. The activity of NADH oxidase was low. The distribution pattern of both the NADH oxidase and malate dehydrogenase in the supernatant and sediment fractions indicates that the fractionation procedure was satisfactory.

These experiments support the following conclusions: 1. The oxy-hydrogen reaction measured in intact cells does not reflect the maximal activity of hydrogenase. The rate of total gas uptake is limited by the oxygen terminal components of the respiratory chain. The highest specific rate of the oxy-hydrogen reaction amounts to 2500 corresponding to an uptake rate of hydrogen of 1650. This is less than the rate measured with the crude extract and with methylene blue as a hydrogen acceptor (2260). 2. Experiments employing intact cells do not permit the measurement of hydrogenase activity with methylene blue as H-acceptor. Since methylene blue neither exerts a notable inactivation of the enzyme nor

Table 5. Hydrogenase activity of the crude cell-free extract of strain *14 g* and fractions derived therefrom with several hydrogen-acceptors

H-acceptor	Specific hydrogenase activity (μl H_2/mg protein · h) at 30°C		
	Crude extract	Soluble fraction	Particle fraction
Oxygen[a]	312	108	540
Methylene blue[b]	2260	672	3300
NAD (NADP)[c]	0	0	0
Enzyme	**Specific enzyme activity (units/g protein) at 25°C**		
NADH oxidase	29	12	64
Malate dehydrogenase	790	1087	49

[a] Manometrically determined under 95% hydrogen + 5% oxygen.
[b] Manometrically determined under 100% hydrogen.
[c] Manometrically determined in the presence of 2 mM NAD and 25 mM pyruvate + lactate dehydrogenase as trapping system under 100% hydrogen.

inhibits the rate of the oxy-hydrogen reactions of intact cells or cell-free extracts, the low rate of H_2-uptake by intact cells with methylene blue as H-acceptor (584) is probably due to slow penetration rates. 3. From the specific activities of the NADH oxidase in the soluble and particle fraction one has to conclude that the fractionation procedure was not quite effective. The proportion of the specific activities in both fractions is 1:5. This is almost identical to the proportion of the specific hydrogenase activities determined for both fractions with oxygen or methylene blue as the H-acceptor. On the basis of the identity of this rate one can be certain that the hydrogenase activity of the soluble fraction is entirely caused by residual particles in this supernatant. The hydrogenase in strain *14 g* is, therefore completely particulate, and the supernatant (soluble) fraction contains neither a hydrogen dehydrogenase nor a hydrogenase which reacts directly with methylene blue or oxygen.

Discussion

On the basis of morphological observations strain *14 g* has to be assigned to the coryneform bacteria. Irregular cell shapes, the division of cells by snapping and the tendency to fragment into coccoid cells in aging cultures are indicative for this group. The difficulties in delineating the genera within the group of coryneform bacteria has recently been emphasized by Veldkamp (1970) and Yamada and Komagata (1972), who divided the strains tested into seven groups on the basis of the combination of

different morphological, cultural, biochemical and physiological characteristics. Several properties of strain *14 g* agree with those, found in bacteria of *group 1*, which contains the genus *Corynebacterium* (Yamada and Komagata, 1972): The snapping type of cell division, the positive Gram-stain, the immotility, the inability to hydrolyze gelatine and the utilization of organic acids. Furthermore, the cells do not show distinctive pleomorphism in mineral and complex culture media. The irregular shape of cells, grown on acids of the tricarboxylic acid cycle seem to correspond to the observed "citrate effect" of *Corynebacterium* strains (appearance of abnormally formed, elongated and branching cells). The GC content of strain *14 g* is 69.4% and lies within range of the widely distributed GC content of the genus *Corynebacterium* (51 to 70%). Properties characteristic for the genus *Arthrobacter*, such as variable Gram-stain and large spherical bodies (cystites) were not observed.

Besides strain *14 g* two other isolates called attention in our laboratory, mainly due to their morphology, Gram-stain and life-cycle: *Nocardia opaca 1b*, a Gram-positive hydrogen bacterium characterized by hyphal growth (Aggag and Schlegel, 1973) and another yellow-pigmented, coryneform hydrogen bacterium, strain *7 C* (Siebert, 1969) which is very similar to strain *14 g* and probably belongs to the same genus (Baumgarten, pers. comm.). In spite of existing similarities and agreements of strain *14 g* with the genus *Corynebacterium*, a final assignment to a genus and diagnosis of the species will be given after a comparative study has been completed. These studies particularly will include morphological behaviour, the analysis of the cell wall, the determination of DNase activity and the identification of granules found in polar regions of the cells of strain *14 g*.

Strain *14 g* stands out against all other hydrogen bacteria known by its incapability to use carbohydrates as a growth or respiratory substrate. Among the sugar acids tested only gluconate is used for growth; it is degraded via the Entner-Doudoroff pathway. Besides a NAD-dependent 6-phosphogluconate dehydrogenase is present. With some other hydrogen bacteria strain *14 g* shares the property not to contain a (NAD-dependent) hydrogen dehydrogenase. Oxygen and methylene blue are reduced by cell-free extracts, and the ability to activate hydrogen is localized exclusively in the particulate fraction of the cell. The hydrogenase activity is completely inducible. The hydrogenase system is, therefore, completely different from that of *Nocardia opaca 1b*, which contains a soluble and loosely membrane-bound hydrogen dehydrogenase, and from that of *Hydrogenomonas eutropha H 16*, which contains a soluble hydrogen dehydrogenase as well as a membrane-bound hydrogenase not reducing NAD. Strain *14 g* lends itself, therefore, to study NAD reduction by H_2 in the absence of a hydrogen dehydrogenase.

Acknowledgements. The determination of the GC content by Dr. M. Mandel is gratefully acknowledged. Dr. Reh (Fig. 1 d and e) and Dr. Mendgen (Fig. 1 a) provided photographs and electron micrographs, and Dr. Baumgarten lent his advice with respect to the taxonomy of coryneform hydrogen-bacteria.

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