Arch. Mikrobiol. 88, 299–318 (1973)

Studies on a Gram-Positive Hydrogen Bacterium, *Nocardia opaca* Strain *1 b*

I. Description and Physiological Characterization

M. Aggag and H. G. Schlegel

Institut für Mikrobiologie der Gesellschaft für Strahlen- und Umweltforschung mbH, München, in Göttingen, Federal Republic of Germany

Received October 10, 1972

Summary. A new hydrogen bacterium has been isolated by enrichment culture on propane. It is a strictly aerobic, Gram-positive, non acid-fast bacterium, characterized by filamentous growth, and has been tentatively assigned to *Nocardia opaca* (strain *1 b*).

It grows heterotrophically, on many organic compounds (71 out of 138 tested substrates including organic acids and sugars), on hydrocarbons (C_{11}—C_{18}) as well as under autotrophic conditions (under an atmosphere of hydrogen, oxygen, and carbon dioxide = 8:1:1) In the absence of a nitrogen source storage materials, mainly carbohydrates, are accumulated.

Hydrogenase is an inducible enzyme. Under appropriate growth conditions the specific hydrogenase activity reaches high values: 2700 enzyme units/g cell protein. The formation of hydrogenase is repressed by fructose. With increasing oxygen concentrations during growth the specific hydrogenase activity decreases. In resting cells oxygen progressively inhibits the oxyhydrogen reaction.

Cell-free extracts of autotrophically grown cells are able to reduce oxygen, benzyl- and methylviologen, dichlorphenolindophenol, methylene blue and nicotinamide adeninedinucleotide with hydrogen.

The majority of the chemolithoautotrophic bacteria are Gram-negative, i.e. the nitrifying bacteria, sulfur and iron oxidizing bacteria, and those species of hydrogen-bacteria listed by Davis *et al.* (1969). Only a few Gram-positive chemolithoautotrophic bacteria are known: *Bacillus thermophilica* (Egorova and Deryugina, 1963) and a few hydrogen bacteria, such as *Bacillus pycnoticus* (Ruhland 1922, 1924; Grohmann, 1924), *Nocardia autotrophica* (Takamiya and Tubaki, 1956; Hirsch, 1961), *N. petroleophila*, and related strains (Hirsch, 1961), *Brevibacterium 12-60-x* (Eberhardt, 1969), and *Mycobacterium fortuitum* (Lukins and Foster, 1959, 1963).

Since most of these Gram-positive bacteria grow rather slowly, they have neither been adequately described nor has their physiology been

investigated. A newly isolated strain of *Nocardia opaca* growing autotrophically with a generation time of 7.5 h has now been studied in some detail.

Methods

Enrichment. The "direct plating technique" was used. The mineral medium described by Dworkin and Foster (1958) was used, solidified by 1.8% Bacto-Agar. Soil samples were applied to Petri dishes which were incubated under an atmosphere of 40% propane, 5% carbon dioxide and 55% air within anaerobic jars at 28° C for 6 days. Among 105 strains isolated those were selected which were able to grow under a carbon dioxide-containing knallgas atmosphere. For details see Siebert (1969).

Organisms. Hydrogenomonas eutropha strain *H 16* (ATCC 17699), *Arthrobacter* strain *7 C*, and strain *1 b* identified as *Nocardia opaca* were obtained from the culture collection of the institute. *N. opaca* strain T_{16} was kindly supplied by Dr. Webley, Aberdeen; ATCC strains 17039 and 4276 were supplied by Dr. Lessel from the American Type Culture Collection. *Mycobacterium rhodochrous* strains 560, 765, 768, W 3406, W 3408, ATCC 13556 and *Nocardia petroleophila* 1002 were obtained from Dr. R. Gordon, Institute of Microbiology, New Brunswick, New Jersey. *Nocardia autotrophica* was given by Dr. P. Hirsch, Institut für Mikrobiologie, Kiel.

Physiological Characteristics. Physiological properties for the purpose of taxonomical identification were determined according to the methods described by Cowan and Steel (1965).

Culture Media and Growth Conditions. The bacteria were grown autotrophically according to Schlegel *et al.* (1961). The gas atmosphere was composed of 5—10% oxygen, 10% carbon dioxide, and 80—85% hydrogen. For heterotrophic growth sodium bicarbonate was omitted from the mineral salts medium, and the medium was supplemented by 0.2—1.0% organic substrates; the gas atmosphere was air.

Small volumes of autotrophic cultures (up to 100 ml) were either magnetically stirred in 500 ml Erlenmeyer flasks (connected with the gas mixture contained in 10 l gasometer bottles) or shaken in 1 l-flasks placed in dessicators under the required gas mixture. At intervals of 5—10 h the vessels were evacuated and refilled with a fresh gas mixture.

For cultivating larger amounts of cells, 2—6 l flat-bottomed round flasks were half filled with the nutrient solution and connected to a container with the gas mixture. The culture was stirred magnetically at about 400 rpm. In all the above cultivation methods the cells did not grow beyond 0.75 g dry weight per liter. In order to obtain higher cell densities, a 10 l Biostat fermenter (B. Braun, Melsungen) with a modified Waldhof-type of agitation (400 rpm) was used. Up to 8 liters basal mineral salts medium were passed through a bacterial Seitz-filter into the pre-autoclaved fermenter. The gas mixture (5—10% oxygen + 10% carbon dioxide and 80—85% hydrogen) was allowed to flow continuously through the culture at a rate of 300 ml/min by means of precision gas pumps. Cell yields up to 2—3 g dry weight/l were frequently obtained after about 3 days under these conditions.

Optical Density. The optical density of the cell suspension was measured in cuvettes ($d = 1$ cm) in a Zeiss-PL 4-photometer at a wave length of 436 nm.

Dry weight. According to the cell densities, 25—50 ml of the cell suspensions were harvested on constant weight membrane filters (Sartorius, Göttingen, diameter, 50 mm; pore size 0.2 μm). Traces of salts were washed out with double-destilled water. The membrane filters were then dried to constant weight at 80° C.

Protein Determination. The method of Schmidt *et al.* (1963) was employed. Standard curves were performed using bovine serum albumin. Cell suspensions were

sonicated (4 min/ml in the sonic disintegrator "Braun-Sonic 300", Quigley-Rochester, inc. N. Y.) and the disintegrated cell suspensions were heated with 40% KOH for 15 min in a boiling water bath. Cell-free extracts were heated only for 5 min with 40% KOH.

Manometric Techniques. For the manometric measurements, a round Warburg apparatus (B. Braun, Melsungen, frequency 150 min, amplitude 4 cm) was used. The temperature of the water bath was 30° C. a) Measurement of hydrogen oxidation by intact cells: the cells were harvested by centrifugation, washed twice and resuspended in 0.067 M phosphate buffer at pH 7.0. Vessels in which the consumption of H_2 and O_2 was to be determined, received 2.0 ml cell suspension in the main compartment and 0.2 ml of 20% KOH in the center well. The flasks were flushed with hydrogen for 5 min and then with a gas mixture of 90% H_2 and 10% O_2 (3 l/flask). Flasks, in which the total hydrogen, oxygen, and carbon dioxide uptake was to be measured, contained 2.0 ml suspension and 0.2 ml of sodium bicarbonate solution (11 mg/ml). The bicarbonate was added to enhance the equilibrium of the carbon dioxide between the gas and fluid phases. The flasks were flushed with hydrogen for 5 min and then with a gas mixture of 10% O_2 + 10% CO_2 + 80% H_2 (3 l/flask). b) Measurement of oxygen uptake by heterotrophically grown cells: The flasks contained 1.8 ml of cell suspension in 0.067 M phosphate buffer, pH 7.0, in the main compartment, 0.2 ml of 20% KOH in the center well and 0.2 ml of the organic substrate solution in the side arm. Flasks for measuring endogenous respiration contained 0.2 ml water instead of the organic substrate in the side arms and air as the gas phase. c) Hydrogenase of intact cells: the hydrogenase activity was measured using methylene blue as H-acceptor. In the main compartment was placed 2.0 ml cell suspension in 0.067 M phosphate buffer, pH 7.0, and in side arm 0.2 ml methylene blue (10 μmoles). The flasks were flushed with hydrogen for 10 min. About 5 min after flushing, the methylene blue was tipped into the flasks from the side arms. d) Hydrogenase of cell-free extracts: the hydrogenase of cell-free extracts was measured using different H-acceptors (5 mM methylene blue, 2.5 mM NAD, 2.5 mM dichlorophenolindophenol, 2.5 mM benzyl- and 2.5 mM methylviologen). The flasks contained 130 μmoles phosphate buffer (pH 7.0) and the cell-free extract in the main compartment, and 0.2 ml solution of the H-acceptors in the side arms.

Preparation of Cell-Free Extracts. Cell-free extracts were prepared by sonic disintegration (Disintegrator Braun-Sonic 300) or by using a French-Pressure cell. Suspensions in 0.067 M phosphate buffer, pH 7.0, were sonicated for 3 min/ml. The temperature of the suspension was kept always below 10° C with the help of salt-ice bath. For preparing extracts with the French-press, 30 ml cell suspension (1 g wet weight/4 ml phosphate buffer) were mixed with about 10 μg DNase before being broken in a French pressure cell at approximately 60 kg/cm². Intact cells were removed by two-fold centrifugation for 15 min at 4000 g and the resultant supernatant served as the crude extract. The latter was centrifuged at 10000 g for 30 min at 2° C into a sediment S_1 and a supernatant U_1. The supernate U_1 was further centrifuged at 140000 g for 1 h at 2° C into a sediment S_2 and a supernatant U_2. The latter supernatant was then centrifuged at 140000 g for 3 h to give the sediment S_3 and the supernatant U_3. The sediment-fractions were resuspended in 0.067 M phosphate buffer, pH 7.0. All fractions were kept under hydrogen atmosphere in the ice-bath and were generally used within 1 or 2 days.

Determination of Malate Dehydrogenase and NADH-Oxidase in Cell-Free Extracts. The activity of the two enzymes was determined in a Zeiss-photometer (PL 4) at a wave length of 356 nm. One enzyme unit is that which transfers 1 μmole substrate/min. The specific activity is given as mU/mg protein. a) Assay of malate dehydrogenase and NADH-oxidase: assay mixture: 100 μmoles Tris-HCl-buffer,

pH 9.2; 0.2 ml $NADH_2$ 4.5 mM; 0.2 ml K-oxalacetate 0.023 M; 0.1 ml extract in a total volume of 3.00 ml. The test was started by the addition of extract. b) Assay of NADH-oxidase: The assay mixture was the same as above but with the addition of 0.2 ml H_2O instead of the K-oxalacetate. The malate dehydrogenase activity was the difference between the activity of both enzymes (a) and the activity of NADH-oxidase (b).

Determination of Sugars. The sugar contents of the cells were assayed according to the anthrone method of Trevelyan and Harrison (1952).

Chemicals. Methylene blue and 2.6-dichlorophenol-indophenol were obtained from Merck, Darmstadt; methyl- and benzylviologen were purchased from Serva, Heidelberg; NAD, $NADH_2$ and oxalacetic acid were obtained from Boehringer, Mannheim. All chemicals used were of analytical grade.

Results

The organism was isolated from a soil sample collected from the surroundings of a defective town gas pipe. The first aim of the enrichment was the selection of propane oxidizing bacteria; among the propane oxidizing strains a screening was made for those strains which were able to grow autotrophically as hydrogen bacteria. Strain *1 b* originally attracted attention by its unusual colony morphology; on mineral agar the colonies were characterized by cone-shaped outgrowths which were not formed under heterotrophic conditions on complete medium (Siebert, 1969). This property was lost during successive cultivation on nutrient agar. Since the bacterium was recognized as a *Nocardia* and since the growth rate was rather high, it was more closely investigated.

Morphological Characterization

On solid agar media at 30° C colonies are formed within one to three days. The colonies are round, raised, rough, and opaque and become slightly lobed and wrinkled with elevated margin and depressed center after several days. At the periphery of the colony the mycelial type of growth becomes visible (Fig. 1 a). The cells grow as branching hyphae which project intensively into the agar and frequently interlace with the hyphae of the surrounding colonies. An aerial mycelium is not formed. The hyphae appear unfragmented during the first day of growth (Fig. 1 b), however, with prolonged incubation discrete cytoplasmic condensations develop which are separated by clear areas. The colonies have a soft consistency, can be easily spread and suspended in water by means of the platinum loop. The colour of the colonies varies according to the substrate: cream on nutrient-agar; cream with more pink tinge on glucose- or fructose-agar, grey when grown under autotrophic conditions or with propane as a substrate, and grey to cream under alkane (C_{11}—C_{18}) vapors.

In liquid culture during the exponential phase of growth on fructose and under autotrophic conditions, the cells grow homogeneously as long

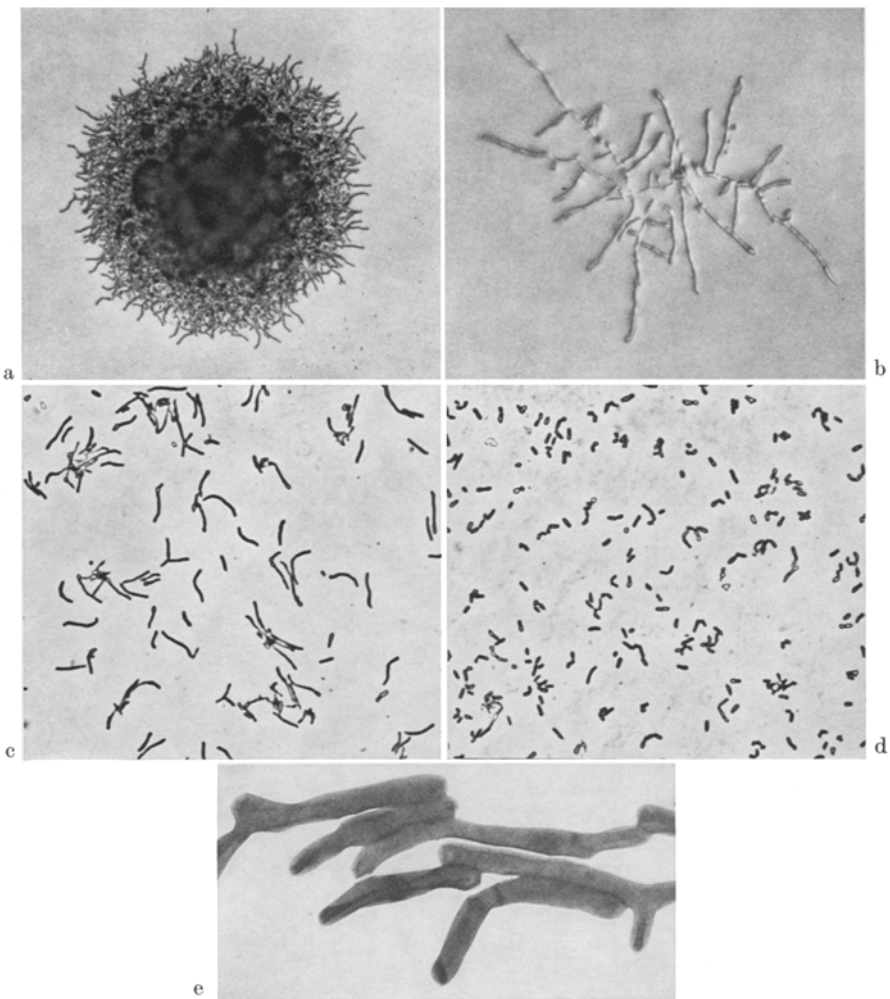

Fig. 1 a—e. The hydrogen bacterium *Nocardia opaca* strain *1 b*. a Colony grown on nutrient agar for 2 days; b colony grown on nutrient agar for 1 day; c fructose grown cells in the logarithmic phase; d fructose grown cells in the stationary phase; e electron micrographs of fructose grown cells in the logarithmic phase. Magnification: a 25fold; b 312.5fold; c and d 625fold (light field; after staining with methylene blue; agar method); e 4625fold, electron microscopy after phosphotungstic acid staining

irregular rods and filaments with a diameter of about 0.8—1.0 μm. The filaments are up to 60 μm long, branching and not septate during the early stages of the logarithmic growth phase (Fig. 1 c and e). At the end of the log phase the filaments soon form transverse walls, and the whole

mycelium breaks up into short cylindrical rods (Fig. 1 d). On fresh culture media these short rods grow again into long rods and filaments. When alkanes ($C_{11}-C_{18}$) are used as substrates, the cell-growth is restricted to the oil/water interface; this kind of growth renders the direct observation of the bacteria impossible in a phase contrast microscope, unless an organic solvent is added to the bacterial film. In unagitated culture, the cells remain firmly attached to the oily phase on the surface leaving the aqueous medium clear.

Physiological Properties

The cells (fructose-grown) are Gram-positive and not acid-fast (decolorization is achieved by 3% HCl in ethanol). Nitrate reduction: +;

Table 1. Utilization of

	Nocardia strain *1 b*[a]	*Nocardia autotrophica*	*Arthrobacter* strain *7 C*	*Mycobacterium rhodochrous* H 768	*Mycobacterium rhodochrous* W 3408	*Nocardia petroleophila* 1002
Agar[b]						
Fructose	+	+	+	+	+	+
Glucose	+	+	−	+	+	+
Mannose	+	+	+	+	+	−
Maltose	+	+	−	−	−	+
Xylose	−	+	−	−	−	+
Lactose	+	−	−	−	−	+
Cellobiose	+	+	+	−	−	+
Sucrose	+	+	−	+	−	+
Gluconate	+	+	+	+	−	+
Benzoate	+	−	−	−	+	−
Succinate	+	+	+	+	+	+
Phenylalanine	−	+	−	+	−	−
Nutrient broth	+	+	+	+	+	+
Quinic acid	+	−	−	+	−	−
Shikimic acid	+	−	−	+	−	+
Carbon dioxide[c]	+	+	+	−	−	−
Propane	+	+	−	−	−	−
Butane	−	+	−	−	−	−
Alkanes (C_6-C_{10})	−	+	−	−	−	−
n-undecane	+	+	−	+	−	−
n-tetradecane	+	+	−	+	+	−
n-hexadecane	+	+	−	+	+	−

[a] All substrates except benzoate and alkanes were used at a concentration of 0.5% in mineral salts medium solidified by 2% agar. Benzoate was used at a concentration of 0.2%. Alkanes were used as described in text.

[b] All strains tested except *Nocardia petroleophila* 1002 and *H 16* showed slight growth

catalase: +; oxidase: −; citrate utilization: +; starch utilization: −; gelatine hydrolysis: −; casein hydrolysis: −; cellulose hydrolysis: −; Voges-Proskauer-reaction: −; indol formation: −; purple milk: unchanged; sensitivity to lysozyme: insensitive.

Production of acid (but no gas) from sorbitol, inositol, mannitol, maltose, fructose, glucose, and lactose: +; production of acid from mannose and arabinose: −; utilization of trimethylenediamine as C- and N-source: −; utilization of thymine, β-alanine, cytosine, urea, nitrite, nitrate, uric acid, ammonium salts as N-sources: excellent growth; utilization of barbituric acid as N-source: moderate growth; neither uracil nor orotic acid nor nitrogen gas could be utilized as N-sources.

various carbon sources

Mycobacterium rhodochrous 560	*Mycobacterium rhodochrous* ATCC 13556	*Mycobacterium rhodochrous* W 3406	*Mycobacterium rhodochrous* 765	*Nocardia opaca T 16*	*Nocardia opaca* ATCC 17039	*Nocardia opaca* ATCC 4276	*Hydrogenomonas eutropha H 16*
+	−	+	+	+	+	+	+
+	−	+	+	+	+	+	−
+	−	+	+	+	+	+	−
−	−	−	−	−	+	−	−
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+	+	+	+	+	+	+	−

on Difco agar (2% in doubledestilled water). The growth response for the other C-sources was considered positive (+) or negative (−) to that on agar alone. The plates were incubated for one week at 30° C.

[c] The plates were incubated under a gas atmosphere of 80% H_2 + 10% O_2 + 10% CO_2.

The ability of the strain *1 b* to utilize different substrates as carbon sources was tested on solid agar media and compared with that of other hydrogen bacteria and nocardia-like bacteria (Table 1). All the plates were inoculated with a cell suspension in bidestilled water prepared from a 48 h culture on nutrient agar slants. All the substrates except benzoate (0.2%) and alkanes were added in 0.5% concentration in mineral salts agar medium. In case of propane and butane the inoculated plates were incubated under atmospheres of 60% air + 40% propane and butane, respectively.

The C_5-C_{17}-alkanes were added in 0.5 ml quantities to sterile filter paper discs placed in the lid of the petri dishes. The plates were then sealed with adhesive tape and incubated in inverted position at 30° C.

Nocardia strain *1 b* unlike *Hydrogenomonas eutropha* utilizes many sugars; some of these are listed in Table 1 for comparative purposes. The described strain *Nocardia 1 b* proved to be an extremely versatile bacterium. It is able to grow autotrophically with hydrogen and CO_2 and organotrophically with hydrocarbons as well as with a multitude of organic compounds (71 out of 138 tested substrates). Since it grows, although modestly, on a mineral agar (Difco agar), which does not support any growth of *E. coli* or *H. eutropha*, *Nocardia 1 b* is even able to use impurities present in normal Difco agar.

Tentative Identification

Comparing the morphological, cultural, and physiological characteristics of strain *1 b* with the descriptions given in Bergey's manual (1957) or by Waksman (1961) or Tsukamura (1970) clearly indicates that the strain belongs to the genus *Nocardia* or according to the classification of Jensen (1953) to the genus *Proactinomyces*. Strain *1 b* is especially similar to *Nocardia opaca* as reported by Den Dooren de Jong (1926); the similarity pertains to colonial and cell morphology as well as to the physiological properties. Among the strains held in culture collections under the name *N. opaca*, strain *1 b* is most similar to *N. opaca* ATCC 17039. The similarity is most pronounced with regard to microscopical appearance, colonial morphology and the substrate spectrum. The strains only differ with respect to the utilization of butane, which is used by the ATCC strain but not by *1 b*, and to growth on hydrogen and carbon dioxide. The ATCC strain even after prolonged incubation under autotrophic conditions and various attempts for adaptation did not grow as a hydrogen autotroph.

The strain *1 b* shares its ability to grow as a hydrogen autotroph with *Nocardia autotrophica* as described by Takamiya and Tubaki (1956) and renamed by Hirsch (1960). Strain *1 b* is different from *N. autotrophica*,

however, with respect to several characters. The colonies of *N. autotrophica* are "snow-white in appearance, because of the abundance of aerial hyphae . . ." (Takamiya and Tubaki, 1956); spores are formed. *N. autotrophica* grows on xylose, phenylalanine, however, does not use quinic acid, shikimic acid and lactose. Furthermore, autotrophic growth is slow and extremely poor. The statement that *N. autotrophica* does not grow in agitated submerged culture (Hirsch, 1961) has been confirmed.

On the basis of these comparative studies we are inclined to refer to our strain as *Nocardia opaca 1 b*.

Growth Rates in Liquid Media

For a small number of substrates the doubling time has been measured, i.e. the time interval needed for duplication of the optical density of the suspension. 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 (Fig. 2). The doubling times (in hours) during exponential growth were the following: gluconate 2.3; fructose 2.35; sucrose 2.55; glucose 2.65; maltose 2.95; lactate 3.7; nutrient broth (Difco) 4.7; lactose 5.25; carbon dioxide + hydrogen 7.5.

For cells growing exponentially in a fructose medium the relationship between turbidity (optical density), protein contents and cell dry weight has been determined (Fig. 3). The three values increase nearly in a parallel manner. Throughout the interval of eight hours the protein contents of the cells was about 54% indicating that no reserve materials are formed. The optical density, which was measured in the range of extinction below 0.4 at 436 nm was firmly related to the dry weight: 1 ml suspension of an extinction of 1.0 contains 0.17 mg cells (dry weight).

For a few substrates the oxidation rates (Q_{O_2}) were determined. 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 the oxygen uptake was measured manometrically (Fig. 4). The specific oxidation rates were (in μl O_2/mg cell protein · h) 123 for gluconate, 118 for fructose, 109 for sucrose, and 80 for glucose. The endogeneous respiratory rates for the cells grown on the four different substrates were approximately equal around 35.

The optimal growth temperature (Fig. 5 A) and the pH-optimum for growth (Fig. 5 B) were estimated in cultures growing in the fructose medium. The highest growth rates were found between 28 and 30° C; above 40° C the organism failed completely to grow. The pH-curve exhibits a rather broad optimal area between 6 and 8 and has its peak at pH 7.3.

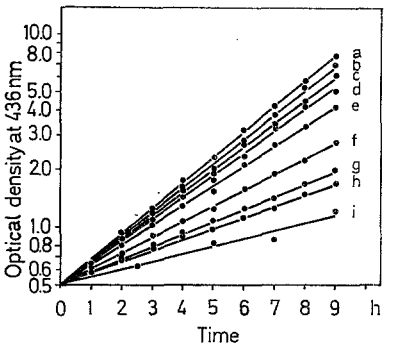

Fig. 2

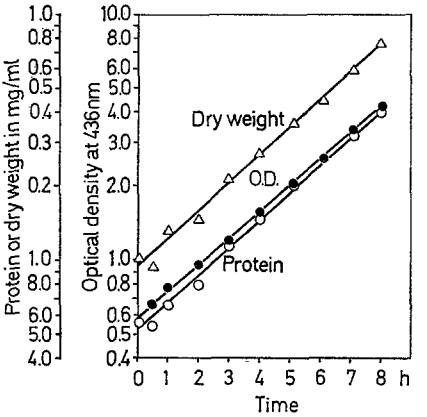

Fig. 3

Fig. 2. Growth of *Nocardia opaca* strain *1 b* on several carbon sources. 200 ml-Erlenmeyer flasks each containing 50 ml medium were shaken in a constant temperature bath at 30° C. The concentration of organic substrates was 0.5%; *a* gluconate, *b* fructose; *c* sucrose; *d* glucose; *e* maltose; *f* lactate; *g* nutrient broth (Difco); *h* lactose; *i* 10% CO_2 + 80% H_2. Samples were taken at intervals during exponential growth and the extinction was measured at 436 nm

Fig. 3. Increase of turbidity, protein, and dry weight of the cells during the logarithmic growth phase. Cells were grown in a 6 l-round bottom flask containing 2 l-mineral salts medium + 0.2% fructose; pH 7.0; temp. 30° C; magnetic stirring at 600 rpm

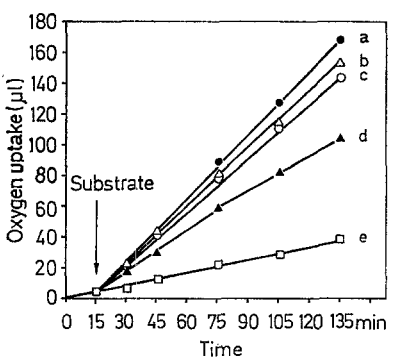

Fig. 4. Substrate oxidation by washed cells, suspended in phosphate buffer (0.067 M, pH 7.0). Each Warburg vessel contained in the main compartment: 1.8 ml cell suspension; side arm: 4.0 mg substrate: *a* gluconate; *b* fructose; *c* sucrose; *d* glucose; center well: 0.2 ml of 20% KOH. Vessel *a* contained 0.52 mg protein; other vessels contained 0.48 mg protein. After 15 min endogenous respiration, the substrates were tipped from side arms. Vessel *e* served as a control to measure endogenous respiration

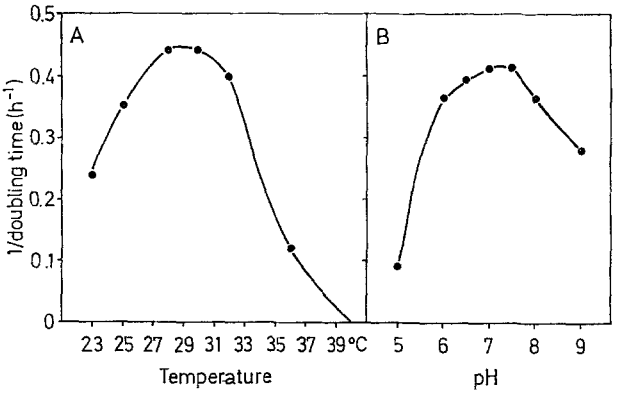

Fig. 5 A and B. Effect of temperature (A) and pH (B) on the growth rate during growth in a fructose medium. Cells were grown in 200 ml-Erlenmeyer flasks containing 50 ml fructose medium stirred magnetically at 30° C. Turbidity at 436 nm was measured at intervals during the logarithmic phase of growth. The pH was checked each hour; HCl or NaOH were added to readjust to the original pH

Table 2. Storage material synthesis by non-proliferating cell suspensions of strain *1 b* during incubation in the presence of fructose

Incubation	Time of incubation (h)	Turbidity (E_{436})	Dry weight (mg/ml)	Protein (mg/ml)	Carbohydrate (µg/ml)	Carbohydrate (% of dry weight)
In the absence of N-source	0	7.2	1.53	0.67	203	13
	5	10.4	1.63	0.67	362	22
	9	15.2	1.72	0.67	397	23
	18	20.0	1.93	0.66	408	21
	23	21.0	2.36	0.66	455	19
In the presence of NH_4Cl (0.1%)	0	0.84	0.16	0.09	20.2	12
	7	2.7	0.56	0.28	53.5	9
	21	9.8	2.08	0.91	207.0	0

Washed cells obtained from an exponentially growing culture were suspended in ammonia-free mineral salts medium containing 1% fructose. 0.1% NH_4Cl was added to one culture vessel. The vessels were incubated at 30° C. Total carbohydrate was measured after the anthrone method.

Synthesis of Carbohydrate Storage Material

Since in preliminary experiments a turbidity increase had been observed even when the cells were incubated in a fructose medium in the absence of a nitrogen-source, the increase of turbidity, dry weight, protein, and carbohydrate has been followed in cell suspensions con-

taining 1% fructose in the absence and presence of a nitrogen-source (Table 2). In the absence of a nitrogen-source turbidity and dry weight increase while the protein content remains constant. The increase in dry weight is only partially due to an increase of the total carbohydrate contents of the cells. The unproportional high increase of the turbidity compared to dry weight appears to indicate that a lipid is accumulated in addition to the carbohydrate. In fact, relatively large amounts of lipid have been obtained by extracting the lyophilized cells with chloroform; the addition of ethyl-ether resulted in the precipitaion of only traces of flocculent material indicating that poly-β-hydroxybutyrate is not the main storage lipid.

Chemolithoautotrophic Growth and Gas Uptake

In contrast to other *Nocardia* strains known for their ability to grow under autotrophic conditions and exhibiting only very poor growth, *Nocardia opaca* strain *1 b* grows very well in a mineral salts medium under a gas atmosphere of 80—85% hydrogen, 10% carbon dioxide and 5—10% oxygen. For optimal growth in either a 1 l-suction flask or a 6 l-round bottom flask, both magnetically stirred, certain requirements have to be fulfilled: a) the inoculum must be in an active growth condition and has to be added to the growth medium to give an initial turbidity (extinction at 436 nm) of at least 0.5—0.6; b) stirring has to be intensive and efficient in order to keep the cells in homogeneous suspension and to avoid aggregation and granular growth; c) the gas mixture has to be supplied continuously or has to be replaced after intervals of about four hours. In culture vessels with magnetic stirring or in Erlenmeyer flasks shaking in a Warburg bath (Fig. 2) the cells grow with a doubling time of 7.5 h and reach a final optical density of $E_{436} = 2.5$ to 3.0. Cell densities as high as $E_{436} = 10.0$ have been observed in 10 l-fermenters (Braun, Melsungen). In these vessels the culture medium is intensively stirred by impellers at 400—600 rpm and continuously gassed by the gas mixture at a rate of 300 ml gas/min. The cells grown under these conditions exhibit higher hydrogenase activities than those grown in the smaller culture vessels.

The effect of oxygen partial pressure in the gas atmosphere on hydrogen bacteria has been studied by several investigators. It seems to be a general phenomenon that high oxygen concentrations inhibit the growth as well as the gas uptake and even exert a bactericidal effect on hydrogen bacteria (Kluyver and Manten, 1942; Schatz and Bovell, 1952; Schlegel *et al.*, 1961; Eberhardt, 1969); the majority of hydrogen bacteria do not tolerate more than 40% oxygen; beyond this concentration no colonies are formed on solid media. *Nocardia opaca* is rather sensitive to oxygen concentrations higher than 20%. If non-proliferating

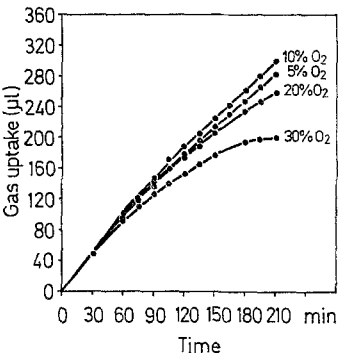

Fig. 6

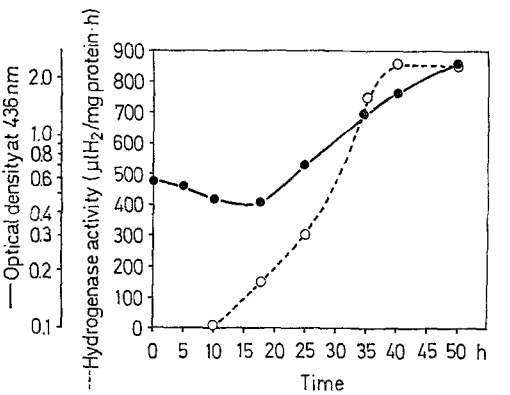

Fig. 7

Fig. 6. Dependence of gas uptake on the oxygen contents of the atmosphere. Cells (0.95 mg protein/vessel) were suspended in 0.067 M phosphate buffer, pH 7.0. Temp. 30° C; atmosphere: 10% CO_2, O_2 as indicated, rest H_2

Fig. 7. Time course of hydrogenase formation and growth in a mineral salts medium under an atmosphere of 80% hydrogen, 10% oxygen and 10% carbon dioxide. Cells used as inoculum had been grown heterotrophically (0.5% fructose; air) and washed twice. Samples were removed at intervals, washed and suspended in 0.067 M phosphate buffer, pH 7.0, and tested for hydrogenase activity with methylene blue as H-acceptor at 30° C. Optical density at 436 nm in full circles, hydrogenase activity in open circles

cell suspensions of low density are exposed to atmospheres of hydrogen, carbon dioxide and varying concentrations of oxygen, the rate of gas uptake becomes depressed by high oxygen concentrations and reached approximately zero after 3.5 h at 30% oxygen (Fig. 6). In another experiment the effect of oxygen on the growth and the hydrogenase activity was investigated. Aliquots of 100 ml mineral salts media were inoculated with autotrophically grown cells (with hydrogenase activity of 700 µl H_2 mg protein · h) and incubated under gas atmospheres containing carbon dioxide, hydrogen and different concentrations of oxygen. The results summarized in Table 3 show clearly that the hydrogenase activity is suppressed by oxygen and the degree of suppression varies depending on the oxygen partial pressure. A high oxygen concentration of 20% of the gas mixture did not only suppress the hydrogenase activity but also inhibited completely the cellular growth.

The rate of hydrogen oxidation by washed non-proliferating cells of *H. eutropha H 16* is influenced by carbon dioxide (Schlegel and Bartha, 1961). In the presence of carbon dioxide it is about 4.5 times as high as in its absence (Eberhardt, 1966). *Nocardia opaca 1 b* tested under similar conditions did not respond to carbon dioxide in such a pronounced

Table 3. The influence of oxygen concentration in the gas phase on the growth and on hydrogenase activity of chemolithoautotrophically growing cells

	Concentration of oxygen in the gas atmosphere (%)			
	2.5	5	10	20
Hydrogenase activity (μl H_2/mg protein · h)	1530	1020	743	157
Doubling time (h)	8.4	8.0	8.5	no growth

Hydrogenase activity was measured in intact cells after 48 h incubation using methylene blue as H-acceptor (see methods). The doubling times were estimated during the phase of logarithmic growth. Incubation gas atmospheres: 10% carbon dioxide, oxygen as indicated, rest H_2.

Table 4. Rate of hydrogen oxidation in the presence and absence of carbon dioxide by non-proliferating cells grown autotrophically in small vessels and in 10 l-fermenters

Grown in	Gas uptake (in μl total gas/mg protein · h)			
	in an atmosphere containing		Uptake ratio $+CO_2$-CO_2	in 100% H_2 with methylene blue
	H_2:O_2:CO_2 = 8:1:1	H_2:O_2 = 9:1		
1 l-Erlenmeyer flask	1000	695	1.44	821
10 l-fermenter	2857	1785	1.6	3651

Washed cells were suspended in 0.067 M phosphate buffer, pH 7.0. The gas uptake was measured manometrically at 30° C.

fashion, however, the ratio was 1.5. With cells grown in a 10 l-fermenter the corresponding values were 2857 and 1785 μl total gas/mg protein · h, respectively (Table 4). In both cases the stimulating effect exerted by carbon dioxide lead to an increase of the gas uptake rate of about 1.5 fold.

The stimulatory effect of CO_2 on the rate of hydrogen oxidation does not manifest itself in all hydrogen bacteria to the same extent. In *Hydrogenomonas eutropha H 16* and *Pseudomonas ruhlandii* there is a 4—5 fold increase while in others such as *Pseudomonas saccharophila*, strain *12x*, and strain *12/60x* there is almost no response (Bartha, 1962; Eberhardt, 1966, 1969).

Hydrogenase and Its Adaptive Behaviour

In heterotrophically grown cells (with fructose as a substrate) no hydrogenase activity can be detected. The induction of hydrogenase

activity has been followed during a growth experiment. Cells, which had been subcultured at least ten times on nutrient agar slants and were then grown in a fructose medium under air, were used as inoculum for 3 l mineral medium contained in a 6 l-round bottom flask and incubated under a gas atmosphere of $80\%\ H_2 + 10\%\ CO_2 + 10\%\ O_2$. Samples were withdrawn at intervals and turbidity and hydrogenase activity were determined (Fig. 7). A 20 h lag-phase passed before exponential growth commenced. In the heterotrophically grown cells as well as during the first 10 h of incubation under autotrophic conditions no hydrogenase activity could be detected at all. Thereafter, hydrogenase was formed at a nearly linear rate until it reached an upper level of about 850 µl H_2/mg protein · h (632 enzyme units/g protein). This is the average enzyme activity reached by cells growing in medium sized culture vessels. The succession of the appearance of hydrogenase activity and of the commencement of growth deserves mentioning; obviously the establishment of a certain level of hydrogenase and other lithoautotrophic systems is prerequisite for autotrophic growth.

Repression of Hydrogenase Formation by Fructose

In order to elucidate the interactions of fructose and hydrogen with respect to enzyme formation a little further the influence of fructose on the synthesis of hydrogenase has been studied. A cell suspension growing autotrophically in the exponential growth phase was equally divided into three flasks. The cell suspension in the first flask (I) was supplemented with fructose (0.5%), while the second flask (II) served as a control. Both suspensions were incubated under a gas mixture $H_2:O_2:CO_2 = 8:1:1$. The third flask (III) received 0.5% fructose and was incubated under air. The results shown in Fig. 8 indicate that after the addition of fructose hydrogenase formation became repressed and the enzyme was diluted out during the growth of the cell suspension. Suspension I continued to grow without a lag phase and grew even faster (doubling time 5.75 h) than the control suspension II (7.5 h doubling time). Suspension III (fructose + air) after a lag phase of 5 h started to grow exponentially with a doubling time of 2.8 h which is typical for fructose-grown cells.

Hydrogenase in Cell-Free Extracts

The crude cell-free extract obtained from autotrophically grown cells by sonic disintegration contains hydrogenase whose specific activity is in the same order of magnitude as that of intact cells (Table 3). If in the manometric assay the hydrogen acceptor methylene blue (MeB) was replaced by dichlorophenolindophenol (DCPIP), benzyl- or methylviologen (BV, MV) or NAD, the rate of hydrogen consumption was less,

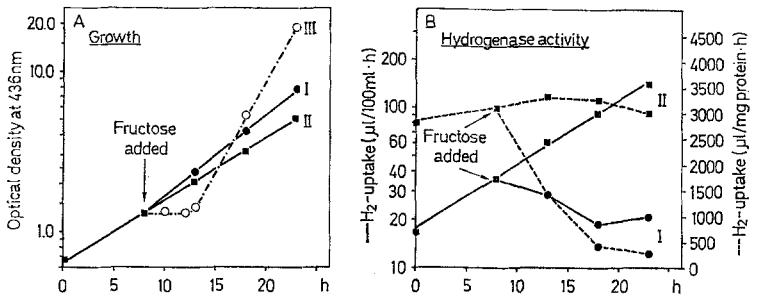

Fig. 8 A and B. Effect of fructose on growth and hydrogenase synthesis of autotrophically grown cells. An autotrophically grown cell suspension was divided into three vessels. Fructose (0.5%) was added to *I* and *III*. *I* and *II* were incubated under a gas mixture of hydrogen:oxygen:carbon dioxide = 8:1:1; III was kept under air. Samples were taken at intervals; growth was followed by optical density measurements; hydrogenase activity was determined with methylene blue as H-acceptor manometrically with intact cells. The specific activity is based on protein determinations

however, still remarkably high. It is astonishing that two-electron acceptors (methylene blue, DCPIP, NAD) as well as a one-electron acceptor were reduced. With oxygen as H-acceptor the rate of gas uptake was the lowest, indicating that the oxidase system is much more sensitive to disintegration than the hydrogenase(s).

In order to localize the hydrogenase activity in the cell-free system, the crude extract (about 10 mg protein/ml) was separated into different fractions by fractional centrifugation. The sediment S_1 obtained after centrifugation of the crude extract for 30 min at 10000 g exhibited very little hydrogenase activity with all the H-acceptors used. This sediment fraction is white-grey in color and seems to consist of the fragmented cell walls, storage materials and cell debris. Almost the whole hydrogenase activity is retained in the supernatant (U_1). This supernating fraction was centrifuged for 1 h at 140000 g and gave a supernate U_2 and a sediment S_2. A repetitive centrifugation of U_2 for 3 h at 140000 g gave U_3 and S_3. The efficiency of the fractionation procedure was controlled by determining NADH-oxidase as an indicator of a particle bound enzyme and malate dehydrogenase as a soluble enzyme. As can be seen in the last two lines of Table 5, the distribution pattern of these two enzymes in supernatant and sediment fractions agrees with expectations and indicates that the fractionation procedure was effective.

The distribution in the supernating and the sedimenting fractions indicates the presence of more than one hydrogenase enzyme in this *Nocardia*. All fractions were able to reduce all H-acceptors. NAD is even

Table 5. Hydrogenase activity of the crude cell-free extract of *Nocardia opaca 1 b* and enzyme fractions with several hydrogen-acceptors

H-Acceptor	Hydrogenase activity (µl H_2/mg protein · h) at 30° C						
	Crude extract	U_1	S_1	U_2	S_2	U_3	S_3
MeB	2754	3423	49	3674	1686	1515	2370
NAD	577	706	36	720	811	368	495
DCPIP	845	1011	62	1055	869	716	1149
BV	1187	1500	92	1487	1105	1282	1259
MV	169	300	43	337	160	219	314
O_2	62	64	0	10	4	4	0
	Specific active (mU/mg protein) at room temperature						
NADHLOx	71	66	77	32	199	10	82
Malate-DH	53	76	29	77	24	95	13

reduced by the sediment fractions which mainly consist of small particles. This result is in contrast to the experiences made with other hydrogen bacteria and deserves further investigation. The same distribution pattern was given by cell-free extracts prepared by the French pressure cell.

The oxy-hydrogen reaction was only catalyzed by the crude extract and by the supernatant U_1. When this fraction was centrifuged for 1 h at 140000 g the activity was recovered neither in the supernatant nor in the sediment. However, on mixing both fractions (1:1, v/v) the activity has been reconstituted. This observation may in part denote the presence of two or more components necessary for the oxy-hydrogen reaction one of which is soluble and is retained in the supernatant. Preliminary experiments showed that the one component is bound to particles and is heat labile being inactivated by heating for 15 min at 70° C, and the soluble component is not completely destroyed by heating for 15 min at 70° C.

These preliminary experiments show that the localization of hydrogenases in *Nocardia opaca 1 b* is different from that in other hydrogen-bacteria, e.g. *H. eutropha H 16*. Since the enzyme appears to be rather stable, further studies seem profitable.

Discussion

The Gram-positive hydrogen bacteria have not received much attention. They are only seldom isolated by employing the standard procedure for the enrichment of hydrogen autotrophs and grow slowly

compared to the Gram-negative hydrogen autotrophs. The strain *1 b* isolated as a propane oxidizing bacterium and tentatively assigned to *Nocardia opaca* has been easily recognized to belong to the filamentous coryneform bacteria since it forms hyphae on agar media as well as during exponential growth, especially on hydrocarbons. Further studies on the placement of this strain in the genus *Nocardia* are, however, necessary. Reviews and experimental studies stress the difficulties of delineating genera like *Arthrobacter*, *Brevibacterium*, *Mycobacterium*, and *Nocardia* and suggest appropriate routes for further research (Veldkamp, 1970; Bousfield, 1972; Kanetsuna and Bartoli, 1972). Strain *1 b* should be included in future taxonomical work.

The cells of strain *1 b* grow rather fast with a generation time of 7.5 h under autotrophic conditions, and reach a concentration of 1.5 to 2.5 g dry weight per liter. The strain lends itself for further biochemical and physiological investigations.

The hydrogenase activity of the cells is variable and depends on the oxygen concentration of the gas mixture. Cells grown in small vessels developed only 20% of the specific activity of cells grown in a 10 l-fermenter. The hydrogen uptake rate of these cells amounts to 3651 µl H_2/mg · h (corresponding to an enzyme activity of intact cells of 2730 enzyme units/g protein); this figure is about as high as the hydrogenase activity of *Hydrogenomonas eutropha H 16* (Eberhardt, 1966). In contrast to *H. eutropha*, the hydrogenase is an inducible enzyme and is completely absent from fructose grown cells. The formation of hydrogenase is repressed by fructose, when the sugar is added to cells growing autotrophically under a hydrogen-oxygen-carbon dioxide atmosphere. With regard to the regulation of hydrogenase formation strain *1 b* resembles, therefore, e.g. *Micrococcus denitrificans* rather than *H. eutropha*.

The hydrogenase activity and its distribution in "soluble" and "particulate" fractions seems interesting and deserves further attention.

Acknowledgements. The authors gratefully acknowledge the assistance of colleagues who sent us their strains: Drs. P. Hirsch, Kiel; D. H. Webley, Aberdeen; R. Gordon, New Brunswick; E. F. Lessel, ATCC Washington.

Preliminary encouraging experiments on the autotrophic growth of strain *1 b* were performed by B. Friedrich, Dr. C. Bovell, and I. Probst. Mrs. Flaskerud and Dr. Mendgen supplied photographs and the electron micrograph.

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Dr. Mansour Aggag
Prof. Dr. H. G. Schlegel
Institut für Mikrobiologie
der Gesellschaft für Strahlen- und
Umweltforschung mbH, München
D-3400 Göttingen, Grisebachstraße 8
Federal Republic of Germany