Arch. Microbiol. 95, 29–37 (1974)

The Cytochromes of Some Hydrogen Bacteria

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Received October 1, 1973

Summary. Eight strains of hydrogen bacteria belonging to the genera *Hydrogenomonas, Pseudomonas, Nocardia,* and to coryneform bacteria have been analyzed with respect to the cytochrome patterns. All strains contained *cytochromes a, b, c,* and *o.* Quantitative and qualitative differences reflect the taxonomic heterogeneity of the group. Only in strain *11/x* the growth conditions (auto- or heterotrophic) resulted in a qualitative difference of cytochrome content.

Hydrogen bacteria are characterized by their ability to grow chemolithoautotrophically with hydrogen as a source of energy and reducing power and carbon dioxide as carbon source. All strains known so far are facultative autotrophs and able to use a great variety of organic substrates. The majority of strains are Gram-negative (Davis *et al.*, 1970). Recently, strains have been isolated which belong to the Gram-positive and Gram-variable genus *Nocardia* and to coryneform bacteria (Eberhardt, 1969; Aggag and Schlegel, 1973; Probst and Schlegel, 1973; Tunail, 1973; Tunail and Schlegel, 1972; Schneider *et al.*, 1973).

The hydrogen chemolithoautotrophs, are, therefore, comprized of a multitude of bacteria belonging to different taxonomic groups. With respect to the taxonomic heterogeneity of the group a comparative analysis of physiological properties appears necessary. Those components which are specifically related to the type of energy generation, viz. the oxidation of hydrogen, deserve special attention. The enzymic systems involved in the oxy-hydrogen reaction are bound to the cytoplasmic membranes. Flavoproteins, *cytochromes b, c,* and two CO-binding pigments have been found in *Pseudomonas ruhlandii* (Packer, 1958). Similar data were reported for *Pseudomonas saccharophila* (Bone, 1963), *Hydrogenomonas H 20* (Bongers, 1967), *H. eutropha* (Ishaque and Aleem, 1970), and *H. eutropha H 16* (Pfitzner, 1969). *Micrococcus denitrificans* if grown anaerobically contains *cytochromes d* ($= a_2$) in addition (Sapshead and Wimpenny, 1972).

The present study was undertaken to compare the cytochrome contents of the cytoplasmic membranes from eight strains of hydrogen bacteria belonging to the genera *Hydrogenomonas* (= *Alcaligenes*), *Pseudomonas, Nocardia* and coryneform bacteria.

Materials and Methods

Organisms. 1. *Hydrogenomonas eutropha* (= *Alcaligenes eutrophus*): ATCC 17697. 2. *Hydrogenomonas eutropha H 16* (= *Alcaligenes eutrophus H 16*): ATCC 17699. 3. Strain *N9A* (Schuster, 1967). 4. Strain *B19*: not yet described, probably an *Alcaligenes*-type bacterium. 5. *Pseudomonas facilis* (= *Hydrogenomonas facilis*): ATCC 17695. 6. *Nocardia opaca* strain *1 b* (Aggag and Schlegel, 1973). 7. Strain *14 g*: Coryneform bacterium (Rudolph, 1968; Schneider *et al.*, 1973). 8. Strain *11/x*: Coryneform bacterium (Eberhardt, 1965, 1969). All strains were obtained from the culture collection of the institute.

Culture of Organisms. All strains were grown in the basal medium described by Schlegel *et al.* (1961). For heterotrophic growth the medium was supplemented either with 0.5% fructose (*Hydrogenomonas eutropha, H. eutropha H 16, Hydrogenomonas N9A, Nocardia opaca 1 b*, strain *B19*, strain *11/x*) or 0.5% glucose (*P. facilis*) or 0.5% succinate (strain *14 g*). For autotrophic growth 0.05% $NaHCO_3$ was added to the basal medium, the gas atmosphere consisted of 80% H_2, 10% O_2 and 10% CO_2.

1 l liquid cultures were grown in 2 l fluted Erlenmeyer flasks at 30°C and aerated as described by Schlegel *et al.* (1961).

Growth was followed by turbidimetric determinations at 436 nm in a Zeiss PM 4 spectrophotometer. Cells were harvested at the end of the logarithmic phase (17—24 h) by centrifugation at 5000×g for 20 min at +4°C.

Preparation of Membrane Fractions. Harvested cells were washed with 50 mM potassium phosphate buffer (pH 6.8), resuspended in the same buffer to a turbidity of 150—200 and stored at −20°C. The cells were disrupted by ultrasonic treatment at +4° to +10°C. A sonicator of Schoeller u. Co., Frankfurt a.M. (20 kH, 600 W), was used in combination with a cooled vessel of 20 ml capacity. The sonication time was 30 sec/ml suspension (strain *11/x* and *Nocardia opaca 1 b*: 2 min/ml). The sonicated extracts were centrifuged twice at 4000×g for 20 min at 4°C in order to remove intact cells and cell debris. The cell-free crude extracts were centrifuged at 120000×g for 60 min at +4°C and thus separated into a clear supernatant, "soluble" fraction, and a particulate fraction containing membranes and respiratory carriers.

The particulate fractions were resuspended in 50 mM phosphate buffer (pH 6.8) using a Teflon plunger; the final membrane suspension contained 20—30 mg protein/ml.

Protein Measurement. Protein was determined by the biuret method of Schmidt *et al.* (1963)

Spectrophotometry. The reduced minus oxidized difference spectra were recorded at room temperature in a Zeiss DMR 21 split-beam spectrophotometer, using 10 mm light path cuvettes.

Particulate suspensions were diluted in 50 mM phosphate buffer (pH 6.8) to a protein content of 1—2 mg/ml and solid dithionite was added to reduce the pigments, the reference cuvette was oxidized by air.

To record difference spectra at −196°C, a split-beam spectrophotometer with low-temperature equipment of the Max-Planck-Institut für Ernährungs-

physiologie, Dortmund, was used (Chance, 1957). The cuvette assembly (ca. 2 mm light path) was cooled by liquid nitrogen contained in an oval glass Dewar flask. To measure carbon monoxide difference spectra a dithionite reduced sample was bubbled with carbon monoxide for 30—45 sec and compared with a reduced sample.

Determination of Cytochromes. Cytochromes were identified from α-peaks in their difference spectra (Kamen and Horio, 1970). For quantitative estimation the extinction coefficients were used as proposed by Chance and Williams (1956) and applied to bacterial systems by Asano and Brodie (1964).

α-Maximum (nm)	*Cytochrome*	ΔE_{mM} (mM^{-1} cm^{-1})	Wave length pair (nm)
600—602	*a*-type	16	Max, 630[a]
560—562	*b*-type	20	Max, 575
550—551	*c*-type	19	Max, 540

[a] The absorption difference (ΔE) was measured between the absorption at the α-peak and at the wave length indicated.

Maxima between 620 and 630 nm were presumed to be *cytochrome d* ($= a_2$) and maxima at 415—418 nm of the carbon monoxide spectra showed the presence of *cytochrome o* (Kamen and Horio, 1970).

Results

The cytochromes of eight strains of hydrogen-bacteria were determined qualitatively as well as quantitatively by measuring difference spectra. Both the membrane and the soluble fraction of cell-free extracts obtained by sonication were tested, first at room temperature then in liquid nitrogen.

Characterization of Cytochromes at Room Temperature

The cytoplasmic membranes of all strains tested contained the *cytochromes a, b,* and *c*. Examples for the difference spectra are shown for membranes of autotrophically grown cells of *Pseudomonas facilis*, strain *14 g*, and strain *11/x* (Fig. 1). Strain *11/x* differed from the other strains; the spectrum exhibited an additional maximum at 628 nm indicative for *cytochrome d* ($= a_2$). The qualitative data obtained by the identification of the α-peaks of these spectra are summarized in Table 1. A further characterization making possible more particulars than assignment to *cytochromes a, b,* or *c* was not possible by difference spectrophotometry at room temperature.

Carbon monoxide difference spectra of all membrane preparations exhibited maxima between 415 and 418 nm characteristic for *cytochrome o*. A shoulder at 430 nm in these spectra indicative for a CO-binding *cytochrome* of the *a*-type became visible only in a few cases

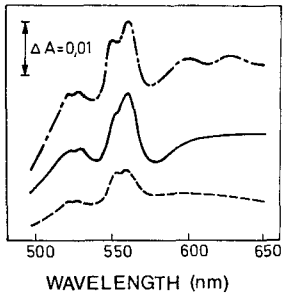

Fig. 1

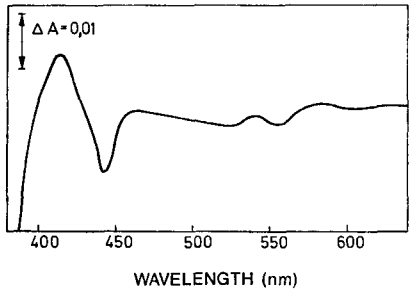

Fig. 2

Fig. 1. Reduced minus oxidized difference spectrum at room temperature. Particulate suspensions (1—3 mg protein/ml) were reduced by solid sodium dithionite. (——) strain *14 g*, (– – – –) *Pseudomonas facilis*, (—·—·—) strain *11/x*

Fig. 2. Carbon monoxide difference spectrum of dithionite reduced particles from *Hydrogenomonas N9A*. Protein concentration: 1.1 mg/ml

Table 1. α-Maxima of room temperature difference spectra from dithionite reduced particulate suspensions

Organism	Culture condition	Wave length (nm) of α-peaks			
		Cyt a-type	*Cyt b*	*Cyt c*	*Cyt d*
Hydrogenomonas eutropha	autotrophic	600	560	553	—
	heterotrophic	600	562	553	—
Hydrogenomonas eutropha H 16	autotrophic	600	560	553	—
	heterotrophic	600	560	553	—
Hydrogenomonas N9A	autotrophic	600	559	551	—
	heterotrophic	600	559	552	—
Strain *B 19*	autotrophic	600	560	551	—
	heterotrophic	600	560	551	—
Pseudomonas facilis	autotrophic	602	560	552	[a]
	heterotrophic	600	560	552	[a]
Nocardia opaca 1 b	autotrophic	600	562	551	—
	heterotrophic	600	562	551	—
Strain *14 g*	autotrophic	602	560	552	—
	heterotrophic	602	560	552	—
Strain *11/x*	autotrophic	600	562	551	628
	heterotrophic	600	562	551	—

[a] Present in soluble fraction.

(Fig. 2). However, the "hidden" maxima of the CO-complex could be recognized by a shift of the adjacent minimum to 440—445 nm.

Low concentrations of *cytochrome b* and *c* were also found in the soluble fractions of all strains. They have presumably been solubilized

Table 2. Quantitative determination of the cytochrome content of particulate fractions reduced with dithionite

Organism	Culture condition	μmole cytochrome/g particle protein		
		Cyt a-type	*Cyt b*	*Cyt c*
Hydrogenomonas eutropha	autotrophic	0.06	0.37	0.56
	heterotrophic	0.02	0.30	0.36
Hydrogenomonas eutropha H 16	autotrophic	0.04	0.67	1.03
	heterotrophic	0.06	[a]	0.61
Hydrogenomonas N9A	autotrophic	0.03	0.53	0.74
	heterotrophic	0.02	0.23	0.32
Strain *B 19*	autotrophic	0.12	0.46	0.75
	heterotrophic	0.08	0.36	0.71
Pseudomonas facilis	autotrophic	0.02	0.37	0.43
	heterotrophic	[a]	0.20	0.28
Nocardia opaca 1 b	autotrophic	0.035	0.072	0.09
	heterotrophic	0.036	0.093	0.1
Strain *14 g*	autotrophic	0.03	0.52	0.36
	heterotrophic	0.03	0.40	0.37
Strain *11/x*	autotrophic	0.04	0.103	0.07
	heterotrophic	0.12	0.37	0.24

[a] Indicates the presence of the component, no quantitative determination possible.

from the membranes. In *P. facilis cytochrome d* (α-peak at 630 nm) has only been detected in the soluble fraction.

Quantitative data derived from these room temperature difference spectra are summarized in Table 2. The order of magnitude of these data corresponds to those reported for *Hydrogenomonas eutropha H 16* (Pfitzner, 1969) and *Micrococcus denitrificans* (Imai *et al.*, 1967). From the differences obtained for cells grown under different growth conditions (autotrophically and heterotrophically) no far reaching conclusions could be drawn, since quantitative measurements of different batches of cells grown under presumably identical conditions resulted always in deviations.

The determination of *cytochromes b* and *c* presented at least some information. The ratio of the specific concentrations of both these cytochromes was relatively constant within the *Hydrogenomonas-Pseudomonas* group; in all instances the concentration of *cytochrome c* was higher. In strain *14 g* (especially after autotrophic growth) and strain *11/x* the ratio was inverted: the concentration of *cytochrome b* was significantly higher than that of *cytochrome c*. Furthermore, cells of strain *11/x* when grown under autotrophic conditions contained *cytochrome d*; however, the total cytochrome concentration was less

($^1/_3$) than after heterotrophic growth. This is contrary to the *Hydrogenomonas* strains and *P. facilis* which had a higher cytochrome content (10—40%) after autotrophic than after heterotrophic growth.

The cytochrome content of *Nocardia opaca* strain *1 b* was remarkably low and independent of the growth substrate.

Difference Spectra at Low Temperature

Difference spectra at the temperature of liquid nitrogen (—196°C) were recorded for the strains *Pseudomonas facilis*, *Nocardia opaca 1 b* (Fig. 3), *Hydrogenomonas eutropha H 16* and strain *14 g*. These spectra exhibited a more detailed resolution of the absorption peaks. In all preparations the absorption maxima were slightly shifted to shorter wave length. Therefore, difference spectra at —196°C of *H. eutropha H 16* and strain *14 g* showed the same characteristic peaks as have been recorded at room temperature, but the maxima were shifted for 2—3 nm to the blue (*H 16*: c_{550}, b_{557}, *14 g*: c_{551}, b_{559}). Additional cytochromes were not detected.

In contrast to these two organisms, in *Nocardia opaca* strain *16* and *P. facilis* low temperature spectra revealed the presence of two *cytochromes b* (*1 b*: c_{549}, b_{558}, b_{562}; *P. facilis*: c_{551}, b_{558}, b_{564}).

In *H. eutropha H 16*, *P. facilis* and strain *14 g* low temperature shifted the α-maximum of the *a*-type *cytochrome* from 600—602 nm (room

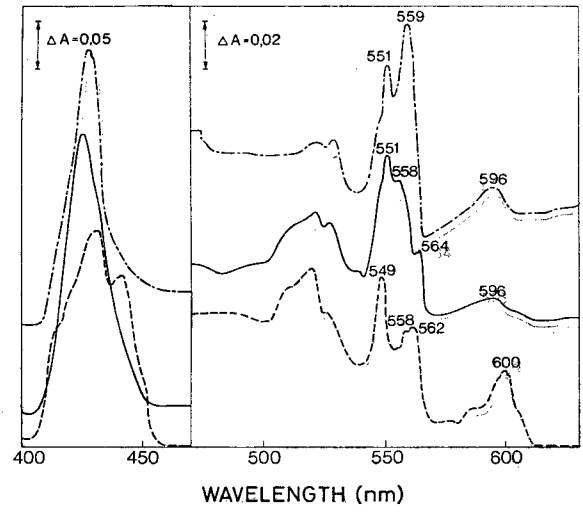

Fig. 3. Reduced minus oxidized difference spectra at the temperature of liquid nitrogen. Particulate suspensions were reduced by solid dithionite. (——) *Pseudomonas facilis*, 3.1 mg protein/ml; (– – – –) *Nocardia opaca 1 b*, 1.5 mg protein/ml; (—·—·—) strain *14 g*, 4.0 mg protein/ml

temperature) to 596 nm. The *a*-type *cytochrome* of *Nocardia 1 b* had an absorption maximum at 600 nm at −196°C and resembled the mitochondrial *cytochrome* a/a_3.

Discussion

With respect to the ability to fix carbon dioxide via the reductive pentose phosphate cycle and to obtain energy and reducing power by the oxidation of hydrogen the hydrogen bacteria appear to be a homogenous group. However, with respect to morphological characteristics, to certain physiological properties and even to the properties and localization of the hydrogenases the group is taxonomically heterogeneous. The physiological heterogeneity of the group is extended by the data on the qualitative and quantitative distribution of cytochromes reported in this paper.

According to the papers quoted in the introduction, the cytochrome patterns, within the *Hydrogenomonas-Alcaligenes* group and the *Pseudomonas*-group of the hydrogen bacteria do not differ significantly. All data presented so far agree with the hypothesis that hydrogen bacteria contain the *cytochromes a*, *b*, *c*, and *o*. The present investigation confirms these results for all eight strains investigated, however, it provides supplementary and modifying data.

a) Four strains of the genus *Hydrogenomonas* contain *cytochromes a*, *b*, *c*, and *o*. The quantitative values vary by a factor of 2. Quantitative differences are not regarded to be significant.

b) *Pseudomonas facilis* contains membrane bound cytochromes which are qualitatively and quantitatively identical with those of the *Hydrogenomonas*-group. However, the soluble fraction contains a *cytochrome d*; this has not been found before either in the membrane fraction of *P. facilis* or in the hydrogenomonads.

c) *Nocardia opaca 1 b* contains the identical set of cytochromes as does the *Hydrogenomonas*-group. However, the concentrations of *cytochromes b* and *c* are significantly less than in all other strains. This difference is, therefore, regarded to be a differentiating character.

d) The cytochrome pattern of two coryneform hydrogen-bacteria, strain *14 g* and strain *11/x* deviates from that of all other strains; the concentration of *cytochrome b* is higher than that of *cytochrome c*. Furthermore, strain *11/x* contains a membrane-bound *cytochrome d*, when grown autotrophically. This is the only case of qualitative difference between autotrophically and heterotrophically grown cells discovered in this investigation.

Our observations indicate that the taxonomic heterogeneity of the eight strains of hydrogen bacteria tested correlates with the differences of their cytochrome patterns. These differences deserve to be emphasized

since the cytochromes are apparently in all instances involved in hydrogen oxidation. Therefore, the cytochrome pattern represents an example of heterogeneity with respect to a property which is a group character of the hydrogen bacteria.

Spectra recorded at −196°C for intensifying and sharpening the absorption bands, support the conclusions drawn from room temperature spectra. In *P. facilis* and *N. opaca 1 b* they reveal the presence of two *cytochromes b* by distinct α-peaks. However, the number of cytochromes in all strains remains uncertain, since the resolution of the α-bands is insufficient even at low temperatures. In a number of different bacteria a further resolution and identification has been accomplished by the use of fourth-order finite difference analysis of low temperature spectra (Shipp, 1972).

Acknowledgements. The authors are indebted to Dr. D. Kuschmitz, Max-Planck-Institut für Ernährungsphysiologie, Dortmund, for measurement of the low temperature spectra.

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