PRESENCE OF A CYTOCHROME b_{559} IN *METHANOSARCINA BARKERI*

W. KÜHN, K. FIEBIG, R. WALTHER and G. GOTTSCHALK
Institut für Mikrobiologie der Universität Göttingen, Grisebachstraße 8, D-3400 Göttingen, FRG

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1. Introduction

The methanogenic bacteria are considered as a group of organisms that is distinct from the conventional prokaryotes. Their 16 S ribosomal RNAs bear only little resemblance to the corresponding RNAs of other bacteria [1]; the cell walls of methanogenic bacteria lack peptidoglycan [2] and their lipids contain ether-linked alkyl chains [3]. Furthermore, some unusual electron carriers, coenzyme F_{420} and factors F_{430} and F_{342} have been found in these organisms [4,5]. These carriers could not be detected in a variety of other anaerobic or of aerobic bacteria. So far known to the authors cytochromes have never been detected in methanogenic bacteria [6–8].

In this connection it was surprising to find a cytochrome in membrane preparations of *Methanosarcina barkeri*. The evidence for it is presented here.

2. Materials and methods

2.1. *Cultivation of* Methanosarcina barkeri

M. barkeri strain Fusaro (DSM 804) was obtained from the Deutsche Sammlung von Mikroorganismen (DSM), Göttingen. The growth medium used was as in [9]. In one experiment (see table 1, last line) a synthetic medium was used in which yeast extract, casitone and cysteine hydrochloride were omitted. *M. barkeri* was transferred 5 times in the synthetic medium before the culture for cytochrome determinations was inoculated. The final concentration of methanol and the methylamines was 200 mM and 100 mM, respectively. *M. barkeri* was mass-cultured in 10 l carboys at 37°C. At the end of the logarithmic growth phase the cells were harvested by centrifugation, frozen in liquid nitrogen, and stored at −70°C.

2.2. *Purity tests*

In order to ensure the purity of the cultures to be used for cytochrome determinations, the cell suspension was carefully examined in a phase contrast microscope. Furthermore, 0.25 ml aliquots of the cultures to be harvested were withdrawn and inoculated into the following prereduced media: (i) AC medium (Difco); (ii) CMC medium (cooked meat medium of Oxoid supplemented with carbohydrates as recommended [10]); (iii) the *M. barkeri* medium as above, except that the usual substrate was replaced by 0.1% glucose. In order to demonstrate the reliability of the transfer procedures, tubes with *M. barkeri* medium containing the same growth substrate as the main culture were also inoculated.

For the preparation of the test media the Hungate technique as modified [11] was used. The atmosphere contained 80% N_2 and 20% CO_2. All tests were run in duplicate.

2.3. *Preparation of membrane fractions*

Cells were thawed and washed 4 times in 50 mM potassium phosphate buffer (pH 7.0). After addition of a small amount of DNase the cells were disrupted by passing them 3 times through an Aminco French pressure cell (9500–13 500 N/cm^2). Cell debris was removed by centrifugation (2 times for 20 min, 8000 × *g*). The supernatant was centrifuged for 2 h at 100 000 × *g*. The pellet fraction containing the membranes was washed twice with the above buffer. After the last centrifugation the membranes were resuspended in the same buffer and carefully

homogenized. This membrane solution was diluted to give a final concentration of ~5 mg protein/ml. Protein was determined by the biuret method using KCN [12].

2.4. *Measurement of spectra*

All spectra were recorded at room temperature in a Perkin Elmer/Hitachi model 556 spectrophotometer. Reduced minus oxidized difference spectra were obtained by reducing the sample cuvette with a few grains of $Na_2S_2O_4$. The reference cuvette contained the air-oxidized sample. To carry out measurements of reduced plus CO minus reduced difference spectra the 2 cuvettes were reduced with $Na_2S_2O_4$ and CO was bubbled through the sample cuvette in the dark for 1 min. The spectra were recorded after 5 min incubation with CO. The heme of the *b*-type cytochrome was characterized as the pyridine hemochrome derivative after extraction with acetone–HCl by the method in [13]. All spectra were measured in cuvettes of 1 cm pathlength. The slitwidth was 1 nm. The linearity of the baseline was checked before each measurement.

3. Results

Membrane fractions of cells of *M. barkeri* grown on monomethylamine contained a cytochrome as was apparent from the reduced minus oxidized difference spectrum (fig.1). This spectrum showed maxima at 559, 529 and 428 nm, respectively. The heme was extractable with acetone–HCl, and the pyridine hemochrome derivative exhibited a difference spectrum with maxima at 556, 525 and 419 nm (fig.2) indicating that protoheme was the prosthetic group [14,15]. These findings demonstrate that *M. barkeri* grown on monomethylamine contains a cytochrome *b*.

The effect of carbon monoxide on the spectral properties of this cytochrome *b* was investigated. The reduced cytochrome reacted with CO, and the resulting difference spectrum is shown in fig.3. It had peaks at 575, 541 and 418 nm and troughs at 559 and 433 nm.

Since these results were quite unexpected it was necessary to carefully rule out that contaminants were present in the cultures and contributed to the spectra.

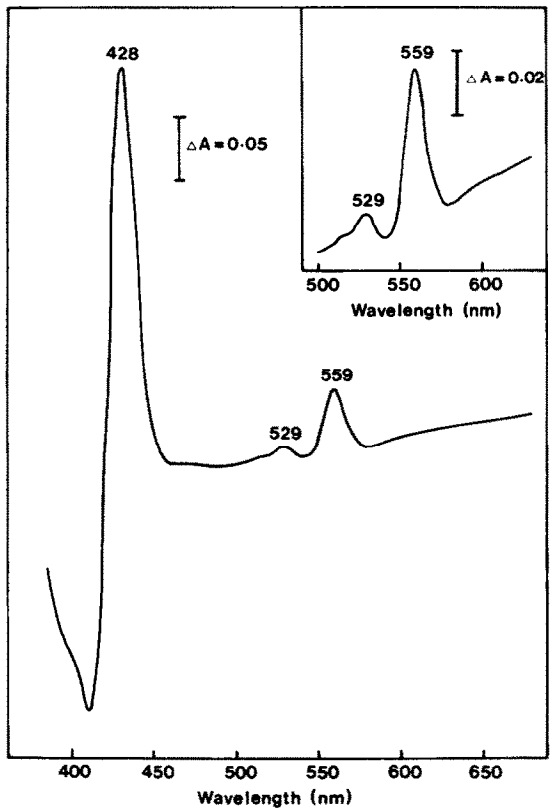

Fig.1. Reduced minus oxidized difference spectrum of a membrane fraction of *M. barkeri* grown on monomethylamine. The membrane fraction was prepared as in section 2.3. The membrane suspension contained 5.2 mg protein/ml.

That the cultures used were free of contaminants was examined microscopically and by inoculating aliquots of the culture to be used for the preparation of membranes into three different media (see section 2.2). The controls were incubated at 37°C for 5 days and checked for contaminants by phase-contrast microscopy. In all cases only sarcina cells, which originated from the inoculum, could be seen. When *Methanosarcina* medium containing monomethylamine was inoculated with an aliquot of the culture growth and methanogenesis occurred.

Since *M. barkeri* shows also good growth with methanol [16] and with dimethyl- and trimethylamine [9] cells were grown on these substrates and analyzed as above. In addition, the cytochrome content of the various fractions was estimated from the

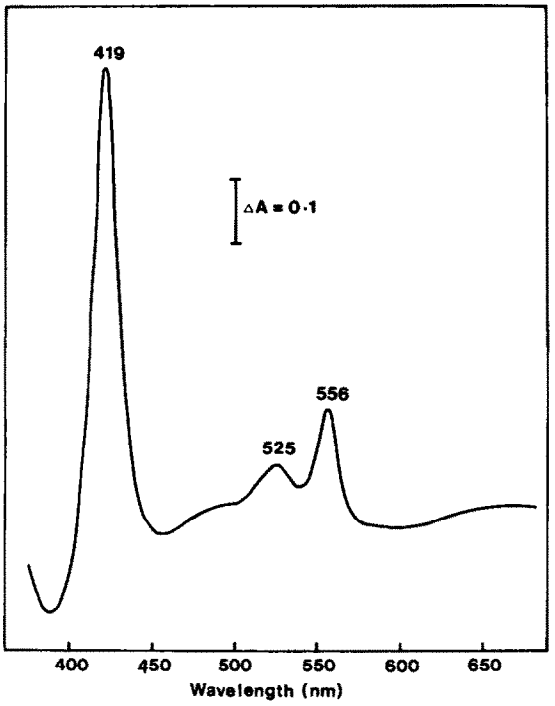

Fig.2. Reduced minus oxidized difference spectrum of the pyridine hemochrome of the pigment extracted from membranes of *M. barkeri*. Membranes containing 66 mg protein were extracted with acetone–HCl as in section 2.4.

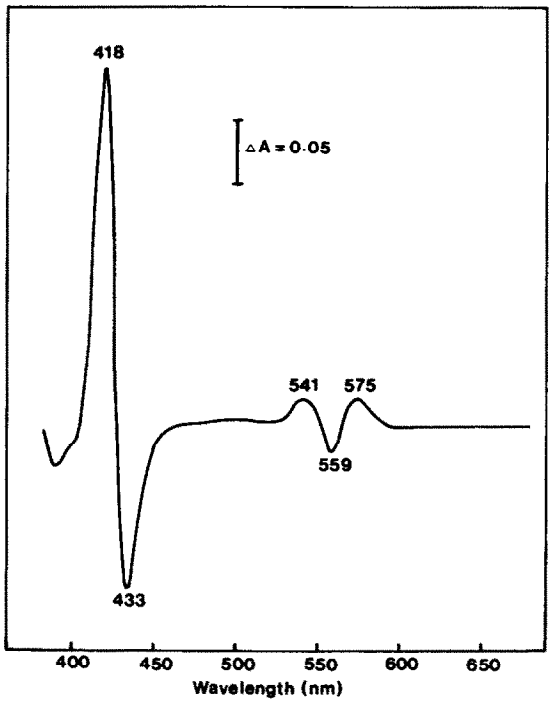

Fig.3. $Na_2S_2O_4$-reduced plus CO minus $Na_2S_2O_4$-reduced difference spectrum of a membrane fraction of *M. barkeri*. The membrane suspension contained 5.2 mg protein/ml.

Table 1
Spectral properties and cytochrome content of membrane fractions of *Methanosarcina barkeri* grown on various substrates

Growth substrate	Absorption maxima in reduced minus oxidized difference spectra (nm)			Cytochrome content (μmol/g particle protein)	Absorption maxima and minima in reduced plus CO minus reduced difference spectra	
	α	β	γ		Maxima	Minima
Methanol	559	529	428	0.30	575, 541, 418	559, 433
Monomethylamine	559	529	428	0.38	575, 541, 418	559, 433
Dimethylamine	559	529	428	0.27	575, 541, 418	559, 432
Trimethylamine	560	530	429	0.38	575, 541, 418	559, 433
Trimethylamine (synthetic medium)	560	530	429	0.26	575, 541, 418	559, 431

The cytochrome contents were estimated from reduced minus oxidized difference spectra. The difference absorption coefficient used was the one of mammalian cytochrome *b* ($\Delta\epsilon$ = 22 $mM^{-1}.cm^{-1}$; [17]). The 559 nm minus 577 nm wavelength pair was used

reduced minus oxidized difference spectra. The data obtained are summarized in table 1. It is apparent that a *b*-type cytochrome with the same characteristics as the one from monomethylamine-grown cells was present in the membrane fractions from all the samples examined and that the cytochrome content did not vary significantly.

M. barkeri is able to grow in a mineral medium supplemented with vitamins, and it was of interest to examine whether this organism is able to carry out a de novo synthesis of this cytochrome. As shown in table 1 for cells grown on trimethylamine, the same type of cytochrome and nearly the same amounts were found in the membranes irrespective of the presence or absence of yeast extract and casitone in the growth medium.

4. Discussion

In the dendrogram of relationships of methanogenic bacteria as proposed [1], *M. barkeri* represents the group IIb which bears only little resemblance with the other groups of methanogenic bacteria. In fact, *M. barkeri* and other possibly existing species of this genus are unique among these organisms in their ability to form methane from compounds containing methyl groups. These compounds are: methanol, acetate and the three methylamines [9,18]. Therefore, the detection of a cytochrome in *M. barkeri* does not allow the assumption that this pigment may also occur in other genera of the methanogenic bacteria; its presence may be connected to the metabolism of the above methyl compounds. On the other hand, it now becomes necessary to analyze other species of the methanogens very carefully for the presence of cytochromes. So far only *Methanobacterium thermoautotrophicum* has been studied thoroughly in this respect, and this species seems to lack cytochromes [6].

M. barkeri is able to grow and to form cytochrome b_{559} in a mineral medium supplemented with vitamins. It does not require hemin as a precursor for cytochrome synthesis as *Bacteroides fragilis* does [19] and it can be concluded that *M. barkeri* possesses the enzyme machinery to synthesize the porphyrin ring.

The cytochrome *b* content of *M. barkeri* membranes is comparable to that of membranes from methylotrophic bacteria [20]. Although cytochrome b_{559} of *M. barkeri* binds carbon monoxide it seems unlikely that its physiological function is that of an oxidase.

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