Medium-reductant directed expression of methyl coenzyme M reductase isoenzymes in *Methanobacterium thermoautotrophicum* (strain ΔH)

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Abstract ***Methanobacterium thermoautotrophicum*** **was grown in a chemostat under various controlled conditions in the presence of either sodium sulfide or sodium thiosulfate. After establishment of the steady state, cells were taken and examined for expression of the mRNA transcripts coding for the different forms of methyl coenzyme M reductase (MCR) and methylene tetrahydomethanopterin dehydrogenase (MDH). MCR isoenzyme II expression varied most markedly. Expression was found not only to depend on known parameters temperature, pH and gassing rate, but also on the medium composition, especially the reductant present.**

Key words: Methanogenesis; Chemostat; Transcriptional regulation; Methyl coenzyme M reductase; Methylene tetrahydomethanopterin dehydrogenase; (*Methanobacterium thermoautotrophicum*)

1. Introduction

Methanobacterium thermoautotrophicum grows on molecular hydrogen and CO_2 as its sole energy and carbon source. For several key reactions involved in the reduction of CO_2 to methane, *M. thermoautotrophicum* contains two or more isoenzymes or functionally equivalent enzymes. The different forms of each enzyme appear to be genetically distinctly regulated [1–5].

The final, methane-forming step in methanogenesis is catalyzed by methyl-coenzyme M reductase (MCR). Of this enzyme, two differentially expressed isoenzymes (MCR I and MCR II) have been found [1–5]. Expression of MCR II predominates during the exponential growth phase in a fed-batch culture. MCR I, on the other hand, is preferentially expressed in the later stages of growth [2]. Differential expression of these isoenzymes is regulated at the transcriptional level [3,5]. Similar patterns have been found for the two enzymes that are involved in the reduction of N^5,N^{10}-methenyltetrahydromethanopterin to N^5,N^{10}-methylenetetrahydromethanopterin. One of these types, F_{420}-dependent methylenetetrahydromethanopterin dehydrogenase (F_{420}-MDH) behaves similar to MCR I. Expression of its counterpart hydrogen-using MDH (H_2-MDH) is comparable to, although independent from, that of MCR II [4,5].

Previously, is has been found that MCR II expression was not only favored by conditions characterized by excess substrate/energy supply (high gassing rates; extensive stirring), but also by low temperature (55°C) and alkaline pH (pH 7.5). Preferential expression of MCR I occurred under opposite conditions (i.e., low gassing and fermenter impeller speeds; high temperature (70°C); acidic pH (pH 6.5)). In this study, we will look at a fourth factor — next to gas supply, pH, and temperature — namely medium reduction.

2. Material and methods

2.1. Materials

Hydrogen and carbon dioxyde gasses were supplied by Hoek-Loos (Schiedam, The Netherlands). Synthetic oligonucleotide primers were from Eurogentec (Seraing, Belgium). Molecular biological reagents were from Boehringer-Mannheim (Mannheim, Germany) or Eurogentec. All other chemicals used were obtained from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO, USA), and were of highest grade available.

2.2. Culture methods

M. thermoautotrophicum strain ΔH was grown in a 0.5-l chemostat at 55°C with a culture volume of 300 ml, a dilution rate of 0.067 h^{-1}, and magnetic stirring at 400 rpm. The culture medium contained the following constituents (g/l): KH_2PO_4, 6.8; Na_2CO_3, 3.3; Tris, 6.0; cysteine·HCl·H_2O, 0.6; and 0.1% (v/v) of a trace elements stock solution [6]. Resazurin (0.5 μg/l) was added as a redox indicator. Under the experimental conditions, the pH in the medium was 7.8. In addition to this, the following variations were applied: the medium contained 0.21 or 0.42 g/l NH_4Cl; either Na_2S·$2H_2O$ or $Na_2S_2O_3$ (0.6 g/l) was added as a reductant; and cells were gassed at 3.75 or 12 l/h with H_2/CO_2 (80%/20%, v/v). Cell densities were determined by measuring the optical density at 600 nm. Sulfide concentrations in the chemostat were determined as described by Trüper and Schlegel [7].

2.3. Molecular biological methods

After establishment of steady-state conditions, cells were anoxically sampled, immediately cooled to 0°C, collected by centrifugation, and stored at −80°C. Total RNA was extracted according to Ref. [8]. *M. thermoautotrophicum* DNA was isolated according to Ref. [9].

DIG-labeled oligonucleotide probes were made by PCR amplification of DNA using the Boehringer PCR DIG labeling mix. Primers for amplifying the 16S rRNA gene were the MB1174 and the inverse complement of ARC915 as described by Raskin et al. [10]. Other oligonucleotide primer sequences were based on DNA sequences of the corresponding genes [3,4] and are listed in Table 1.

For Northern blot analyses, RNA preparations and DIG-labeled DNA molecular mass marker III (Boehringer) were subject to glyoxal-dimethyl sulfoxide denaturation and separated on a 1% agarose gel [11] in the absence of sodium iodoacetate. After electroforesis, the nucleic acids were vacuum blotted to a Hybond-N+ membrane (Amersham). For dot-blot analyses, serial dilutions of formaldehyde-denatured RNA samples were spotted on this type of membrane [11]. Membrane-bound RNA was hybridized with DIG-labeled oligonucleotide probes according to Ref. [12]. After autoradiography, relative amounts of the various types of mRNA were compared. As a control, the hybridization signal obtained with the 16S rRNA probe was used.

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Abbreviations: DIG, digoxigenin; MCR, methyl coenzyme M reductase; MDH, methylenetetrahydromethanopterin dehydrogenase

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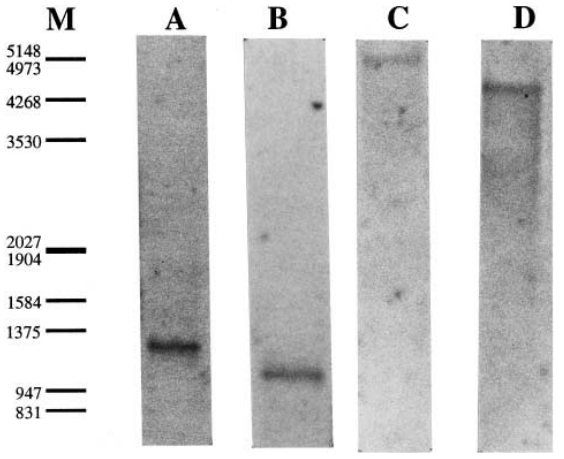

Fig. 1. Specificity of hybridization signals. Autoradiography signals of Northern blot analyses are shown. The following hybridizations were carried out. (M) Marker; (A): 10 μg total RNA with a F_{420}-MDH probe; (B): 10 μg with a H_2-MDH probe; (C): 20 μg with a MCR I probe; (D): 20 μg, with a MCR II probe.

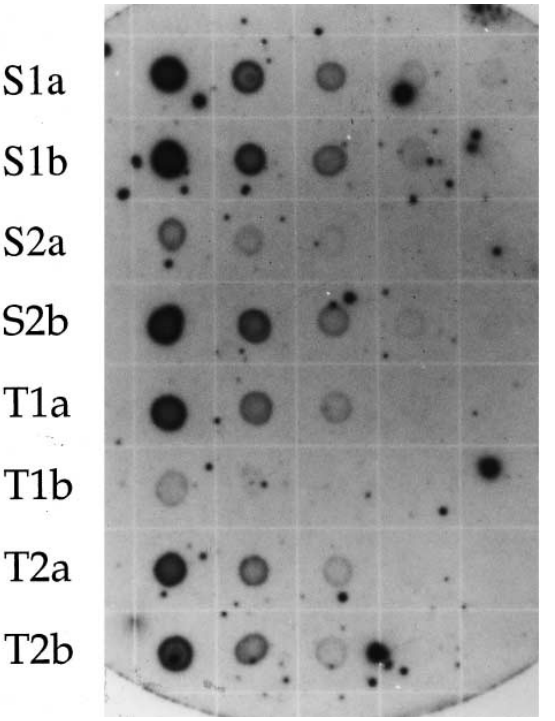

Fig. 2. Dot-blot analysis of MCR II-mRNA levels. From left to right the following amounts of total RNA were spotted: 1.5, 0.5, 0.15, 0.05, and 0.015 μg. The analysis was performed as described in Section 2. Abbreviations used for the various culture conditions are those used in Table 2.

3. Results and discussion

Previously, Bonacker et al. [2] reported that MCR II in *M. thermoautotrophicum* strain Marburg is preferentially expressed in batch cultures when grown under high H_2/CO_2 gassing rates at 55°C and pH 7.5. In repeating their experiments using the fed-batch fermenter operated under comparable conditions with respect to growth temperature, pH, gassing rates and fermenter impeller speeds resulting in comparable growth rates, we could not substantiate the preferential expression of MCR II, but we rather observed large variations in the expression level of the isoenzyme. This might be due to the fact that we employed the ΔH strain of the organism (the Marburg and ΔH strains of *M. thermoautotrophicum* are, in fact, only distantly related). However, a closer comparison of the culture conditions also pointed to a distinct difference in the way sulfide was added as the medium reducing agent. While Bonacker et al. kept the sulfide concentration constant by gassing with a mixture of $H_2/CO_2/H_2S$ (80:20:0.1, v/v), in our case the reductant was added as Na_2S to the culture medium. This led us to examine the influence of the reductant on isoenzyme expression. For this purpose, *M. thermoautotrophicum* was cultured in a chemostat, since this offers the possibility of growing the organism under steady-state conditions, as opposed to the continuously changing conditions in a batch fermenter.

The use of a poorly soluble gaseous energy source like hydrogen introduces a complication. In classical chemostat operation growth is determined by a limiting substrate in the medium. In contrast, growth by hydrogen consuming organisms is readily governed by the gas supply [13]. In order to create a situation in which hydrogen was non-limiting in a number of cultures the ammonium concentration was reduced (0.21 g/l NH_4Cl). This resulted in somewhat lower steady-state cell densities, indicating that ammonium rather than hydrogen had become growth-limiting. A second complication was that the gassing rate affected the sulfide concentrations. Although the culture medium used contained 6 mM sulfide, actual sulfide concentrations in the chemostat were lower due to removal of volatile hydrogen sulfide by the gas flow. Gassing at 12 or 3.75 l/h gave actual sulfide concentrations of 0.29 and 0.68 mM, respectively.

After establishment of a steady state, cells were analyzed for the relative contents of the F_{420}-MDH, H_2-MDH, MCR I and MCR II mRNAs. The specificity of the method was

Table 1
Sequences of oligonucleotide primers used, names of the corresponding genes, GenBank accession numbers and positions corresponding with the oligonucleotide sequences

Enzyme	Gene (accession number)	Primer sequences (corresponding positions)
F_{420}-MDH	mtd (U19362)	AGATGCGGTAACATCGGGACCTC (1481–1503)
		TTACCGTGTGGTGTCCTGAGGAC (2238–2206)
H_2-MDH	mth (U19363)	CTTGCAATACTAGGTGCAGGATG (5151–5173)
		CCGAAGTTCATGGAGTCAGCTGT (4225–4203)
MCR I	mcr (U10036)	TGCACTCACGTTGTTGACTGCAG (2349–2372)
		TTTTCCTGGGGACAGGTTTCTCC (2885–2863)
MCR II	mrt (U0990)	GGAGTCATGATGTCAGAAACAGG (1794–1816)
		GTGTCCTTCATCCTTGAACTGGG (2265–2243)

Table 2
Growth conditions applied in the chemostat, the related steady-state cell densities, and the relative mRNA levels

Culture	Culture conditions			Cell density	mRNA levels			
	Reductant	NH_4Cl (g/l)	Gassing (l/h)	(OD_{600})	F_{420}-MDH	H_2-MDH	MCR I	MCR II
S1a	sulfide	0.21	12	1.38	1 (1)	1 (1)	1 (8)	1 (1)
S1b	sulfide	0.21	3.75	0.98	3	2	2	1
S2a	sulfide	0.42	12	1.49	1	0.5	0.5	0.1
S2b	sulfide	0.42	3.75	1.44	1	2	0.6	0.6
T1a	thiosulfate	0.21	12	1.16	1	1	1	0.4
T1b	thiosulfate	0.21	3.75	1.02	2	1	2	0.06
T2a	thiosulfate	0.42	12	1.52	1	1	1	0.4
T2b	thiosulfate	0.42	3.75	1.47	1	1	1	0.3

The amounts of mRNA were estimated by dot-blot analyses, taking those of condition S1a as a unity. Culturing and other methods were performed as described in the text.
Values between brackets refer to the ratio between mRNA levels of both forms of MDH and MCR, respectively.

tested by performing Northern blot analyses (Fig. 1). As these yielded a specific hybridization signal (Fig. 1), further quantification was done by routine using dot-blots (Fig. 2 and Table 2).

Culture S1a, whose mRNA levels were taken as a reference for the other cultures, contained 8-fold more MCR I than MCR II mRNA (Table 2). This is remarkable, since one would expect MCR II to be preferentially expressed under these conditions (high gassing rate, 55°C, alkaline pH) [2]. The mRNAs of F_{420}-MDH and H_2-MDH were present in equal amounts.

While the F_{420}-MDH, H_2-MDH and MCR I mRNA levels did not vary much under the conditions tested, large changes were found with respect to MCR II. In general, MCR II mRNA levels were lower, when thiosulfate was used as the reducing agent. This would conform the notion that the compound is a poorer reductant than sulfide. In the thiosulfate-reduced cultures higher gassing rates resulted in higher MCR II expression. The opposite, however, was true with the sulfide-reduced media (see S2a and S2b). Here, a 6-fold lower MCR II mRNA level was found at the higher gassing rate (12 l/h). This corroberates the finding mentioned above, that the sulfide concentration was reduced due to the removal of the volatile H_2S. In S1a and S1b two opposing factors, gassing rate and sulfide concentration, apparently counterbalanced each other in a way that the MCR II mRNA levels became equal.

In conclusion, evidence is presented that the three factors described before [2], notably high gassing rate, relative low growth temperature (55°C) and alkaline pH, are not sufficient to direct the specific expression of MCR II. The nature and concentration of the medium reductant plays an important role as well.

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References

[1] Rospert, S., Linder, D., Ellermann, J. and Thauer, R.K. (1990) Eur. J. Biochem. 194, 871–877.
[2] Bonacker, L.G., Baudner, S. and Thauer, R.K. (1992) Eur. J. Biochem. 206, 87–92.
[3] Pihl, T.D., Sharma, S. and Reeve, J.N. (1994) J. Bact. 176, 6384–6391.
[4] Nölling, J., Pihl, T.D., Vriesema, A. and Reeve, J.N. (1995) J. Bacteriol. 177, 2460–2468.
[5] Morgan, R.M., Pihl, T.D., Nölling, J. and Reeve, J.N. (1997) J. Bacteriol. 179, 889–898.
[6] Schönheit, P., Moll, J. and Thauer, R.K. (1979) Arch. Microbiol. 123, 105–107.
[7] Trüper, H.G. and Schlegel, H.G. (1964) Antonie van Leeuwenhoek 30, 225–238.
[8] Chmomczynski, P. and Sachi, N. (1987) Anal. Biochem. 162, 156–159.
[9] Jarrell, K.F., Faguy, D., Hebert, A.M. and Kalmokoff, M.L. (1992) Can. J. Microbiol. 38, 65–68.
[10] Raskin, L., Stromley, J.M., Rittmann, B.E. and Stahl, D.A. (1994) Appl. Env. Microbiol. 60, 1232–1240.
[11] J.E. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: a Laboratory Manual. 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989.
[12] Engler-Blum, G., Meier, M., Frank, J. and Müller, G.A. (1993) Anal. Biochem. 210, 235–244.
[13] Schill, N., van Gulik, W.M., Voisard, D. and von Stockar, U. (1996) Biotechnol. Bioeng. 51, 645–658.