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Cloning, sequencing and expression of the genes encoding the sodium translocating N^5-methyltetrahydromethanopterin : coenzyme M methyltransferase of the methylotrophic archaeon *Methanosarcina mazei* Gö1

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Abstract The N^5-methyltetrahydromethanopterin:coenzyme M methyltransferase of *Methanosarcina mazei* Gö1 is a membrane-associated, corrinoid-containing protein that uses a transmethylation reaction to drive an energy-conserving sodium ion pump. The eight open reading frames encoding the eight different subunits of the methyltransferase were identified and sequenced. All of these subunits are shown to be heterologously expressed in minicells of the *Escherichia coli* mutant DK6. Sequence comparisons with the methyltransferases of thermophilic and hypothermophilic methanogenic archaea are presented. The participation of the gene product of *mtr*D in sodium ion translocation as well as a consensus sequence of a corrinoid binding motif in MtrA are discussed.

Key words: N^5-Methyltetrahydromethanopterin:coenzyme M methyltransferase; Sodium ion translocation; Corrinoid; Methanogenic archaeon

1. Introduction

The N^5-methyltetrahydromethanopterin:coenzyme M methyltransferase of *Methanosarcina mazei* Gö1 (referred to as methyltransferase in this paper) was shown to function as an energy-conserving sodium ion pump [1,2]. The enzyme was purified from acetate-grown *Ms. mazei* Gö1, reconstituted in ether lipid liposomes of the same organism, and thereby identified as being responsible for the translocation of sodium ions across the membrane [3]. The protein has been reported to be composed of at least six different polypeptides with apparent molecular masses of 34, 28, 20, 13, 12, and 9 kDa. The N-terminal amino acid sequences have been determined [3].

The enzyme of the hydrogenotrophic organism *Methanobacterium thermoautotrophicum* has also been studied very extensively. Thauer and coworkers detected eight different subunits in the purified enzyme by biochemical [4] and genetic analyses [5]. The dependence of the activity on sodium ions was demonstrated [6,7], the energy-conserving nature of this enzyme could not be studied because a method to generate vesicular proteosomes from *Mb. thermoautotrophicum* cells is not yet available. Sequence data for methyltransferases of *Methanococcus jannaschii* and *Methanopyrus kandleri* are also accessible ([8], accession number Y14428) but biochemical data on the corresponding enzymes are still lacking. Several reasons justify cloning and sequencing of the methyltransferase genes of *Ms. mazei* Gö1: (i) this organism is a mesophile, whereas the known methyltransferase sequences are from thermophilic or hypothermophilic organisms, (ii) *Ms. mazei* Gö1 is methylotrophic implying that the Na^+ pump has to operate in a reversed fashion when methyl groups originating from methanol or trimethylamine have to be oxidized via N^5-methyltetrahydromethanopterin, (iii) as already outlined, only for *Ms. mazei* Gö1 do we have a complete picture of the involvement of the methyltransferase in the bioenergetics of methanogenesis.

The cloning and sequencing of the genes encoding this methyltransferase revealed highly conserved structural elements. For the first time expression studies with genes of the methyltransferase were performed successfully in minicells showing that all of the subunits of this archaeal protein can be expressed in *Escherichia coli*.

2. Materials and methods

2.1. Organisms and plasmids

Ms. mazei Gö1 (DSM 3647) was obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany, and grown under strictly anaerobic conditions as already described [9]. *E. coli* DH5α (*supE*44 Δ*lac*U169(Φ 80*lac*ZΔM15) *hsdr*17 *rec*A1 *end*A1 *gyr*A96 *thi*1 *rel*A) [10] was grown on LB at 37°C. *E. coli* DK6 (F^- Str^r *hsd*R *min*A *min*B *pur*E *pdx*C *his ilv met* Δ(*unc*B-*unc*C) [11] was grown at 30°C on LB, for minicell preparation the medium was supplemented with 1% glucose and 0.5 mg/l hydroxocobalamin, methylcobalamin, and cyanocobalamin at a time. The vector pBluescriptII SK (Stratagene, San Diego, CA, USA) was employed for cloning and expression experiments.

2.2. Procedures

Chromosomal DNA of *Ms. mazei* Gö1 was isolated by a modified Marmur preparation [12,13] with one exception – treatment with lysozyme was not necessary. The DNA was restricted, size-fractionated by gradient centrifugation and cloned into pSK^+. All techniques used were standard methods [10]. DNA sequence of both strands was determined by the chain termination method of Sanger using an ALF DNA sequencer from Pharmacia (Freiburg, Germany) and analyzed using the Wisconsin Genetics Computer Group sequence analysis software package, version 8.1 (University of Wisconsin Biotechnology Center, Madison, WI, USA). The preparation of minicells and subsequent techniques of gel drying, fluorography and autoradiography were performed by the method of Homma [14]. The heterologous expression of the *mtr* genes in a minicell-producing mutant of *E. coli* was achieved in two different clones: *E. coli* DK6/pMTR 28-12.5 containing pSK^+ with an insert of *mtr*E to *mtr*B and *E. coli* DK6/pMTR 23-34 containing pSK^+ with an insert of *mtr*A to *mtr*H. The respective inserts were amplified by PCR using pTL2 and pTL3 as template. The expression was started by induction of the plasmid-coded *lac* promoter with IPTG.

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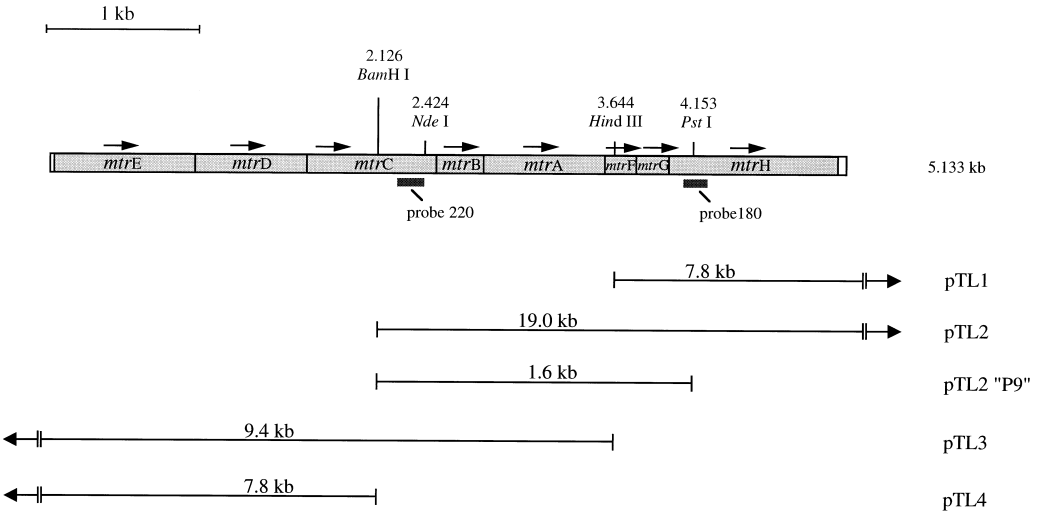

Fig. 1. Schematic map of the cloned chromosomal DNA region from *Ms. mazei* and the organization of the entire *mtr* operon. The DNA fragments of *Ms. mazei* Gö1 were cloned in pBluescript SK$^+$. Genes are indicated by shadowed boxes and orientation of the genes is indicated by arrows.

3. Results and discussion

For cloning the genes encoding the methyltransferase, a homologous probe was generated by the polymerase chain reaction (PCR) using genomic DNA from *Ms. mazei* Gö1 as template and degenerated oligonucleotides derived from the N-terminal amino acid sequence (5′-ATG TT T/C AAG TT T/C GA T/C AAG AAG CA A/G G-3′, sense) and from an internal peptide of the 34 kDa subunit (5′-CCA T/G AG T/G GT T/C TC T/G GC A/C/G/T GC-3′, antisense). The identity of the resulting 180 bp fragment was verified by sequencing and comparison with the experimentally derived N-terminus; after labeling with [α-^{32}-P]dATP it was used further as homologous probe. Southern blot analysis of genomic DNA restricted with various enzymes revealed a 7.8 kbp *Hin*dIII and a 19 kbp *Bam*HI fragment which hybridized with the homologous probe. These fragments were cloned into the appropriate restriction site of pBluescript SK$^+$ yielding pTL1 (containing the 7.8 kbp *Hin*dIII insert) and pTL2 (containing the 19 kbp *Bam*HI insert) (Fig. 1). 1.5 kbp of the *Hin*dIII fragment were sequenced leading to the recognition of *mtr*F, *mtr*G, *mtr*H, and a putative transcriptional termination signal of the methyltransferase. For sequencing the large *Bam*HI insert of pTL2, subcloning by restriction with *Pst*I was necessary. The resulting plasmid which contains a 2.1 kbp insert was designated pTL2 'P9' (Fig. 1). By restriction of pTL2 with *Bam*HI and *Nde*I a second probe was generated which rendered the finding of pTL3 (9.4 kbp insert) by further Southern hybridization. A restriction with *Bam*HI and *Hin*dIII and religation in an appropriately restricted pSK$^+$ vector resulted in plasmid pTL4, which contained an insert of only 7.8 kbp and thus allowed easier sequencing.

The DNA sequence for all the genes encoding the methyltransferase subunits starts with the initiation codon ATG and each of these codons is preceded by a consensus ribosomal binding site (AGGAGGT) [15]. Upstream of the sequence encoding the 28 kDa subunit an (A+T)-rich intergenic region is found with several potential TATA boxes which could be components of a promoter directing transcription initiation [16] (Fig. 2A). About 35 bp downstream of the stop codon of *mtr*H an inverted repeat follows and approximately 60 bp downstream (nucleotides 5250–5264) is a T-rich stretch of DNA (Fig. 2B). The significance of these regions in general is not completely understood; however, T stretches can function as transcriptional termination signal in methanogenic archaea [15]. The identity of seven of the eight genes is con-

Table 1
Comparison of the deduced amino acid sequences of the methyltransferase from the methylotropic organism *Ms. mazei* Gö1 [6] with the corresponding methyltransferases from the hydrogenotrophic methanogens *Mb. thermoautotrophicum* [4,5], *Mc. jannaschii* [6] and *Mp. kandleri* (accession number Y14428).

Gene products[a] of the *mtr* operon from *Ms. mazei* Gö1	*Mb. thermoautotrophicum*		*Mc. jannaschii*		*Mp. kandleri*	
	Similarity (%)	Identity (%)	Similarity (%)	Identity (%)	Similarity (%)	Identity (%)
MtrH	58.8	48.0	60.1	52.4	56.8	47.6
MtrE	67.7	58.1	63.8	52.4	58.9	49.5
MtrA	72.8	50.4	73.9	49.6	55.3	41.6
MtrD	69.4	**60.7**	65.0	**57.4**	68.7	**58.8**
MtrC	54.9	45.9	57.0	45.5	52.8	40.6
MtrF	61.9	36.5	57.8	29.7	–	–
MtrB	59.4	39.6	54.0	32.0	43.9	30.6
MtrG	64.7	43.7	76.8	49.3	63.6	45.5

Values give percentages of similar and identical amino acids with respect to the deduced amino acid sequences from *Ms. mazei* Gö1. The gene product with the highest amino acid identity and the corresponding values are shown in bold.
[a]In taking pattern from the sequence data of the methyltransferase from *Mb. thermoautotrophicum*.

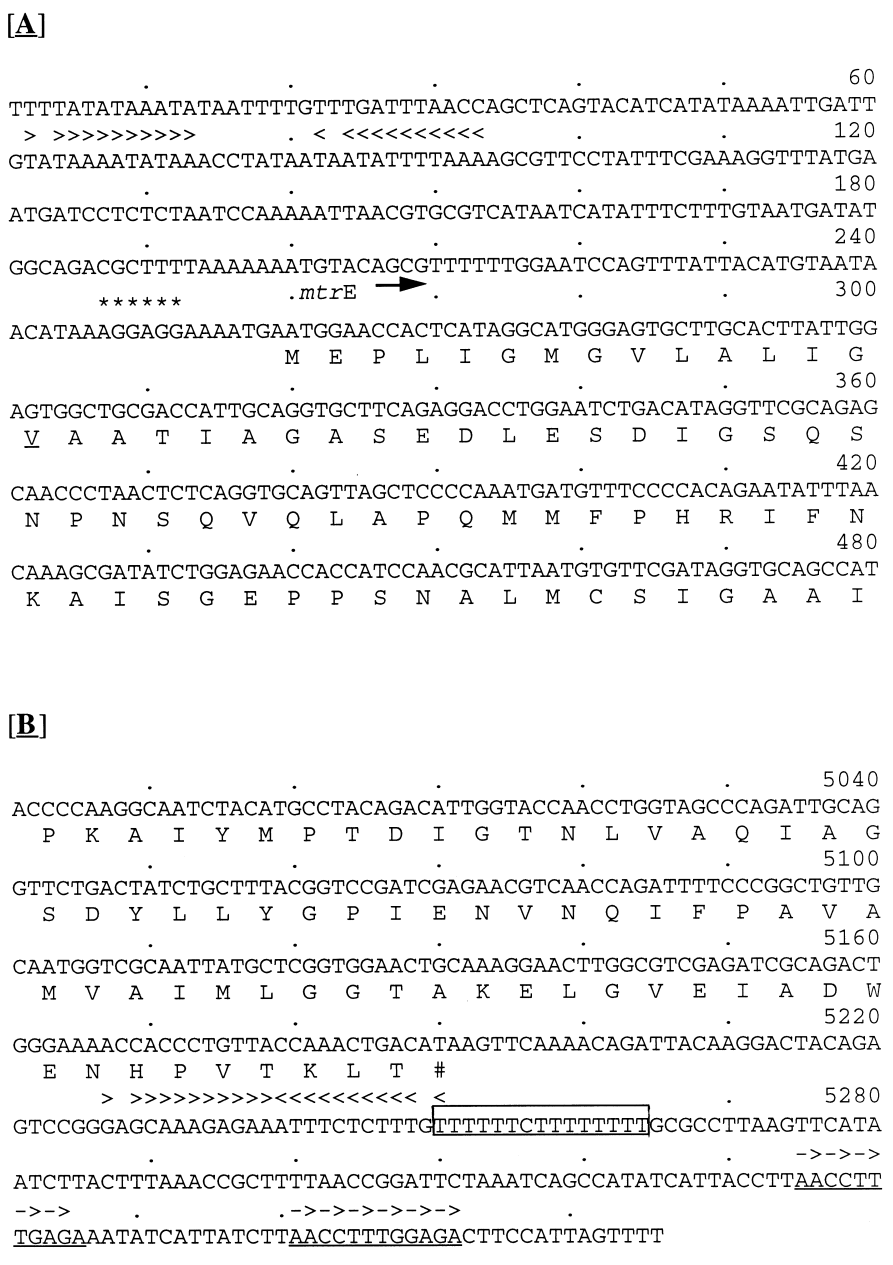

Fig. 2. Nucleotide sequence of the start (A) and end (B) of the *mtr* operon from *Ms. mazei* Gö1. A potential Shine-Dalgarno sequence is marked by asterisks. A T-rich stretch of DNA as possible transcription termination signal is boxed, and inverted repeats as well as stem-loop structures are marked by arrows. The complete DNA sequence of the *mtr* operon has been submitted to GenBank and is available under accession number AF042381.

firmed by a comparison of the deduced with the experimentally derived N-termini. The complete DNA sequence of the *mtr* operon has been submitted to GenBank and is available under accession number AF042381.

The deduced amino acid sequences were compared with those of the methyltransferases from *Mb. thermoautotrophicum, Mc. jannaschii* and *Mp. kandleri*. The comparison revealed similarities to *Mb. thermoautotrophicum* and *Mc. jannaschii* of 54–77%, for *Mp. kandleri* these values were generally lower (Table 1). Amino acid identities reached values of 30–50%. The only remarkable exception is the gene product of *mtr*D (identity 57–61%). These data indicate the presence of highly conserved structural elements in this gene product. From the hydrophilicity profiles [17] of the eight different polypeptides (data not shown) it can be concluded that the gene product of *mtr*D is one of the possibly five integral membrane proteins of the methyltransferase. A participation in sodium ion translocation is therefore imaginable.

The gene product of *mtr*A reached similarity values of the deduced amino acid sequences of 55–74% and identities of 40–50%. MtrA of *Mb. thermoautotrophicum* was shown to harbor the corrinoid prosthetic group [4,7,18] which is involved in the methyl transfer reaction. The corrinoid of the methionine synthase [19], methylmalonyl-CoA mutase [20] and glutamate mutase [21] is bound in the base-off form, and a consensus sequence for cobamide binding was recognized. This se-

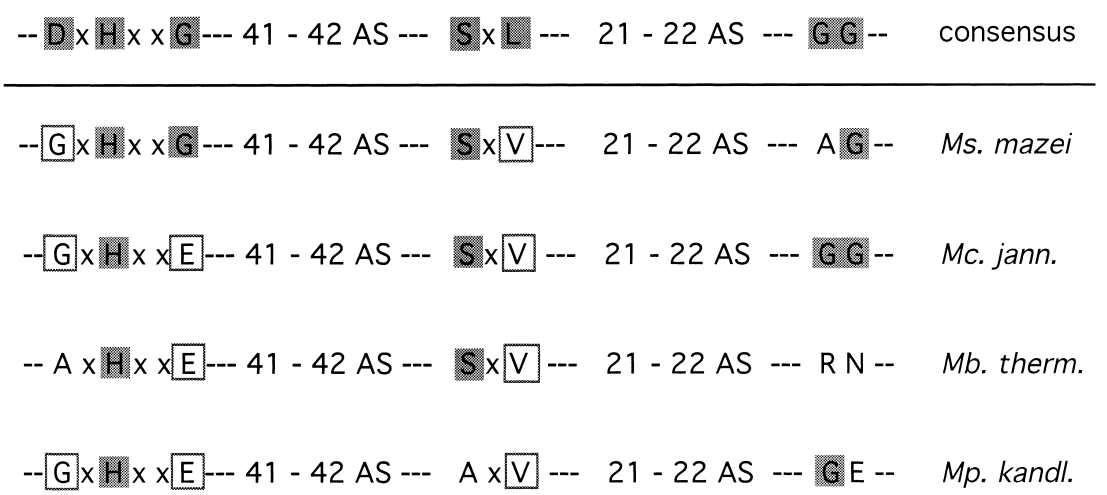

Fig. 3. Comparison of a *mtr*A sequence section from the methyltransferases of *Ms. mazei* Gö1 (amino acids 33–106), *Mc. jannaschii* (amino acids 37–108), *Mb. thermoautotrophicum* (amino acids 33–104) and *Mp. kandleri* (amino acids 33–108) with the consensus binding motif for corrinoids in 'base-off' form. Agreements with the consensus sequence are shadowed in gray, correspondences just within the sequences of the methanogenic organisms are boxed.

quence, in a slightly modified form, is also present in MtrA of *Ms. mazei* (Fig. 3). It can also be seen in MtrA of *Mc. jannaschii*, but is less pronounced in *Mb. thermoautotrophicum* and *Mp. kandleri*. EPR spectra of the *Ms. mazei* methyltransferase yielded signals characteristic for a base-on cobamide [22]. However, it was stressed that the lower ligand could be the benzimidazole base or a protein-derived ligand such as histidine. In light of the sequence homology and the recent results of the role of a histidine residue in MtrA of *Mb. thermoautotrophicum* [18] it can be concluded that cobamide binding in MtrA of *Ms. mazei* proceeds benzimidazole-off but 'His-on'.

The expression of the plasmid-encoded *mtr* genes in minicells was identified by incorporation of [^{35}S]methionine followed by SDS-PAGE analysis and autoradiography (Fig. 4). For the identification of *mtr*E an analysis by SDS-gradient PAGE was necessary, since the pure plasmid pSK$^+$ expressed a protein of similar size (Fig. 4). It is thus demonstrated for the first time that the genes of the methyltransferase can be heterologously expressed in *E. coli*. These results indicate that it might be possible to overexpress single subunits of the enzyme for a detailed study on sodium translocation by this archaeic protein.

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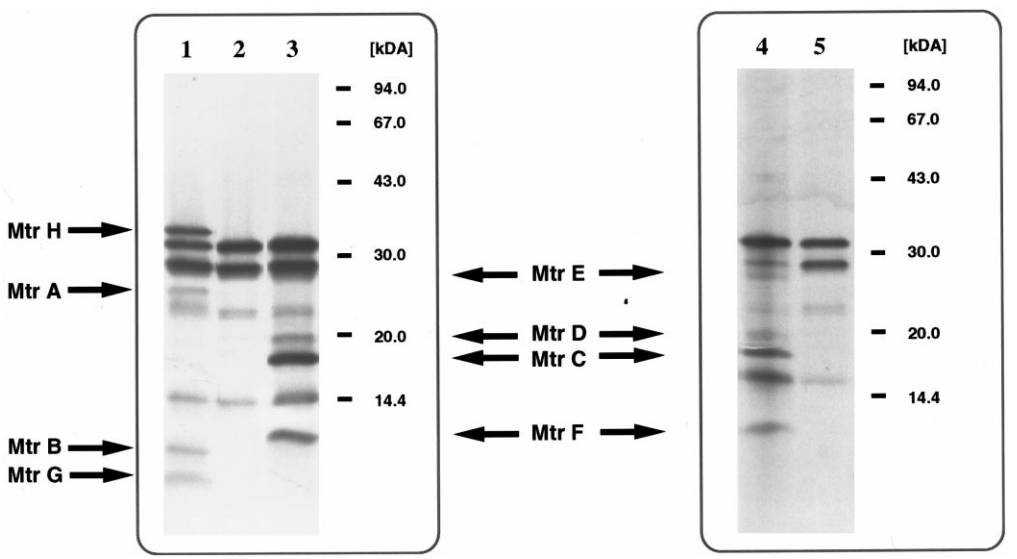

Fig. 4. Expression of the *mtr* genes in minicells of *E. coli* DK6. Autoradiography of the [^{35}S]methionine-labeled proteins. Lanes 1–3 show an analysis after SDS-PAGE by Schägger and von Jagow. Lanes 4 and 5 present an analysis after SDS-gradient PAGE. Lane 1, cell extract of *E. coli* DK 6/pMTR 23-34; lane 2, cell extract of *E. coli* DK 6/pSK$^+$; lane 3, cell extract of *E. coli* DK 6/pMTR 28-12.5; lane 4, cell extract of *E. coli* DK 6/pMTR 28-12.5; lane 5, cell extract of *E. coli* DK 6/pSK$^+$.

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