Dimethylselenide Demethylation Is an Adaptive Response to Selenium Deprivation in the Archaeon *Methanococcus voltae*

Ulf M. Niess and Albrecht Klein*

Genetics, Department of Biology, Philipps University of Marburg, D-35032 Marburg, Germany

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The archaeon *Methanococcus voltae* needs selenium for optimal growth. A gene group most likely involved in the demethylation of dimethylselenide was discovered, the expression of which is induced upon selenium deprivation. The operon comprises open reading frames for a corrinoid protein and two putative methyltransferases. It is shown that the addition of dimethylselenide to selenium-depleted growth medium relieves the lack of selenium, as indicated by the repression of a promoter of a transcription unit encoding selenium-free hydrogenases which is normally active only upon selenium deprivation. Knockout mutants of the corrinoid protein or one of the two methyltransferase genes did not show repression of the hydrogenase promoter in the presence of dimethylselenide. The mutation of the other methyltransferase gene had no effect. Growth rates of the two effective mutants were reduced compared to wild-type cells in selenium-limited medium in the presence of dimethylselenide.

Selenium is a trace element which is important for many organisms. It is a constituent of proteins, where it is found as selenocysteine or selenomethionine, and of special nucleotides of tRNA bases. High concentrations of selenium are toxic for most organisms. Detoxification can be achieved by volatilization through methylation. Different methylation products have been found. The most abundant one is dimethylselenide, which can probably be generated in different ways from inorganic selenium compounds. Dimethylselenide production is performed by microorganisms and plants and has been monitored in soil and marine environments (9).

Although selenium is widely distributed in the environment, it is not always readily available. While inorganic selenium compounds such as selenite and selenate are soluble, selenides can be very insoluble (12) as is elementary selenium, which can be formed from the oxidized species. Selenium can thus become limiting in anoxic environments. Access to selenium is essential for organisms depending on selenium-containing enzymes in their central metabolism. This is the case for at least two known methanogenic archaea, *Methanocaldococcus jannaschii* and *Methanococcus voltae*. Both organisms convert hydrogen and carbon dioxide to methane, whereby the cells generate their energy. Two selenium-containing hydrogenases, enzymes needed to oxidize hydrogen for the generation of electrons, are involved in the methanogenic pathway (5, 25, 26). Limiting selenium in growth medium for *M. voltae* leads to a reduced growth rate (28), and the knockout of a gene encoding a selenium-containing subunit of a hydrogenase has not been possible (19). While limited growth of *M. voltae* has been observed under selenium depletion, *M. jannaschii* cannot grow without selenium (6, 16).

It was previously shown that *M. voltae* carries genes encoding selenium-free isoenzymes of its selenium-containing hydroge-

nases which are only transcribed upon selenium limitation and most likely supplement the selenium enzymes (3). The cell can thus react to the deprivation of the trace element.

We were interested in learning more about functions of proteins produced only under selenium limitation. The protein patterns in extracts obtained from *M. voltae* cells grown with or without selenium were therefore analyzed. Subsequently, a protein that was induced by selenium deprivation was further characterized. This putative corrinoid protein, together with a methyltransferase, is involved in the liberation of selenium from the organic selenium compound dimethylselenide. The two respective genes are part of a common transcription unit. Their regulation occurs at the level of transcription or by regulation of transcript stability. This inducible demethylation of dimethylselenide constitutes a novel, alternative adaptation strategy of *M. voltae* to selenium limitation.

MATERIALS AND METHODS

Strains and plasmids. Strains and plasmids used in this study are listed in Table 1.

Cultivation and transformation of *M. voltae*. *M. voltae* was grown anaerobically under an H₂-CO₂ atmosphere as described previously (28), with the addition of all amino acids except cysteine and tryptophan to a final concentration of 100 µg ml⁻¹. Tryptophan was used at a final concentration of 25 µg ml⁻¹. Transformation was done in an anaerobic chamber containing a H₂/CO₂/N₂ 5/20/75 (vol/vol/vol) mixture on the basis of the method described by Metcalf et al. (15), with modifications. One milliliter of exponentially growing cells (optical density at 600 nm [OD₆₀₀] of 0.3 to 0.6) was harvested by centrifugation at 13,000 × g for 5 min and resuspended in 0.68 M sucrose at 10⁹ cells ml⁻¹. A total of 5 pmol of linear DNA in 225 µl of 20 mM HEPES buffer, pH 7.0, was mixed with 25 µl of Escort transfection reagent (Sigma-Aldrich, Taufkirchen, Germany) and incubated for 10 min at 25°C. The mixture was added to 0.5 ml of the cell suspension and kept for 2 h at room temperature. The cells were then transferred to growth medium and incubated for 3 h at 37°C followed by the addition of puromycin to a final concentration of 10 µg ml⁻¹. The culture was grown to an OD₆₀₀ of 1. The cells were then plated on agar plates containing 10 µg of puromycin ml⁻¹.

Primers. DNA oligonucleotide primers used in this study are listed in Table 2.

Molecular cloning and hybridization techniques. DNA and RNA preparation, DNA cloning, PCR amplification, sequencing, and Southern and Northern hybridizations were performed employing standard protocols (1, 10, 11, 22, 23). A λZAP library of *M. voltae* with approximately 5 kb of random genomic fragments was kindly provided by Izabela Noll.

* Corresponding author. Mailing address: Genetics, Department of Biology, Philipps University of Marburg, Karl von Frisch St. 8, D-35032 Marburg, Germany. Phone: 49-6421-2823014. Fax: 49-6421-2828971. E-mail: uniess@web.de.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
<i>E. coli</i> XL1 Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacI^qZΔM15 Tn10</i> (Tet ^r)]	Stratagene
<i>E. coli</i> Top 10	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL</i> (Str ^r), <i>endA1 nupG</i>	Invitrogen
<i>M. voltae</i> V1 ^a	<i>uidA</i> ⁺ <i>his</i> ⁺ Pur ^s	20
pNPAC ^b	Integration vector for <i>M. voltae</i> based on pSL1180 Pur ^r Amp ^r	Junsong Sun, our laboratory
pNPAC-sdmA	pNPAC containing flanking sequences of <i>sdmA</i>	This study
pNPAC-sdmB	pNPAC containing flanking sequences of <i>sdmB</i>	This study
pNPAC-sdmC	pNPAC containing flanking sequences of <i>sdmC</i>	This study

^a Strain V1 carries the *uidA* gene under the control of the *vhc* promoter, which is active only under selenium deprivation.

^b Compare with Fig. 1.

Mutagenesis by gene replacement. Knockout of genes was performed by transfection of *M. voltae* cells with DNA comprising a cassette containing the selective marker *pacN* (puromycin acetyltransferase). The *pacN* gene was under the control of the promoter of the S-layer-encoding gene and the terminator of the methyl reductase operon of *M. voltae* and flanked by sequences adjacent to the target gene. Plasmids pNPAC-sdmA, -sdmB, and -sdmC (Table 1 and Fig. 1) were used for the construction. The cassettes comprising the *pacN* resistance cassette flanked by the upstream and downstream regions of the *sdm* gene to be replaced were excised from the plasmids and used for the transfection. The upstream and downstream regions of the *sdmA*, *sdmB*, or *sdmC* genes for the construction of the respective plasmids (described in the legend to Fig. 1) were obtained by PCR amplification with primer pairs corsevorfw, corsevorrv and corsevnachfw, corsevnachrv for *sdmA*; metIvorfw, metIvorrv and metInachfw, met-

Inachrv for *sdmB*; and metIIvorfw, metIIvorrv and metIIinachfw, metIIinachrv for *sdmC*, respectively.

Primer extension. The 5' end of the *sdm* messenger was determined by primer extension (1). Twenty micrograms of total RNA from exponentially growing cells was mixed with 3 pmol of IRD-800-labeled primer excorI (MWG Biotech AG, Ebersberg, Germany) and 0.5 μl each of 2.5 mM deoxynucleoside triphosphate in 12 μl of H₂O heated to 70°C for 10 min and subsequently slowly cooled to 42°C. The reaction was carried out in a total volume of 20 μl of reaction buffer (Invitrogen Life Technologies, Karlsruhe, Germany) containing 10 mM dithiothreitol (DTT) and 200 U of Superscript II reverse transcriptase, according to the supplier's instructions. Forty units of RNase Block (Stratagene, Amsterdam, The Netherlands) was included in the reaction. After 30 min at 42°C, the reaction was stopped by the addition of 180 μl of 10 mM Tris-HCl and 1 mM EDTA, pH

TABLE 2. Synthetic oligonucleotides used in this study

Name	Sequence ^b	Usage
pep2ufw (<i>sdm</i>)	GGW TAY AAY GCW TTY GA ^a	Identification of <i>sdmA</i>
pep1urv (<i>sdm</i>)	GCG TAW CCG TCW GC ^a	Identification of <i>sdmA</i>
p2probe3'	GGA ATG AAC AGA GCA GGT GTT ATG	Probe for <i>sdmA</i>
p2probe5'	AGA TAG GAC CGC CAC CAA CC	Probe for <i>sdmA</i>
m13fw(−20)Primer	IRD-800-GTA AAA CGA CGG CCA GT	Sequencing
m13rvPrimer	IRD-800-CAG GAA ACA GCT ATG ACC	Sequencing
ird800corri2 5'	IRD-800-GTC AGA AGC TAT GGG CGC	Sequencing
ird800P2walker5'B	IRD-800-CTG CCA ATC CTT TTG AAA TTC C	Sequencing
corrimet16RV	IRD-800-CTG CTT CAG TTG TAG GTC C	Sequencing
corrimet16FW	IRD-800-CCA CAG GTA AAC TAC ATC C	Sequencing
corsevorfw	ACT AGT CTT GAC TAT TTC ACT GAC AAT TAA G	Knockout of <i>sdmA</i>
corsevorrv	TCG CGA GTT TTA GAT TAT AGC ACC CCA TAC	Knockout of <i>sdmA</i>
corsevnachfw	GGT ACC GCT TCA AAA TCT GCA AGA ATT GC	Knockout of <i>sdmA</i>
corsevnachrv	GCT AGC CCT TAA ATG TAT GGG TAT TCT ACC	Knockout of <i>sdmA</i>
metIvorfw	ACT AGT GGT GTT ATG TAC GAA GAA GAA G	Knockout of <i>sdmB</i> , probe for <i>sdmA</i> downstream region
metIvorrv	TCG CGA GTA TCG CTA ATC ATC TAT ATC AC	Knockout of <i>sdmB</i> , probe for <i>sdmA</i> down stream region
metIachfw	GGT ACC GGC TAT TAC AAA ATA CGA G	Knockout of <i>sdmB</i>
metInachrv	GCT AGC CAA ATC TTC TGT AGG TTG C	Knockout of <i>sdmB</i>
metIIvorfw	ACT AGT GGT AAC GTT GAC CCT TCC G	Knockout of <i>sdmC</i>
metIIvorrv	TCG CGA CTT TCA TAG TTT GCC TCA TGC TAG	Knockout of <i>sdmC</i>
metIIinachfw	GGT ACC CAC AAA TTA TGT GAA ATA TCC CTA G	Knockout of <i>sdmC</i> , probe for <i>sdmC</i> downstream region
metIIinachrv	GCT AGC CAT CAG TTA CTG GCC ATC C	Knockout of <i>sdmC</i> , probe for <i>sdmC</i> downstream region
excorI	IRD-800-TTG AAA TTC CTT CGA ATG C	Primer extension
RTmetIIfw	GCA ACC TAC AGA AGA TTT GGT G	RT-PCR
RTmetIIB	GAT TGA AAC ACC ATT GAG	RT-PCR
RTmetIIrv	CAA TGC TTA TAC CCA CAC CAG	RT-PCR
RTmetIrvB	C ACC AAA TCT TCT GTA GGT TGC	RT-PCR
RTpac	GT GTA TGA CAA GAA AAC CTG GTG	RT-PCR
pacprobefw	GTT TGT AAA GTG GTA GAA CAA TTT CG	Probe for <i>pacN</i>
pacproberv	CCAGGTTTCTTGTCATACACC	Probe for <i>pacN</i>

^a W, A + T; Y, C + T; R, A + G; K, G + T.

^b IRD-800 is a 5'-terminal fluorescent label (MWG Biotech).

FIG. 1. Construction of the vectors from which the gene replacement cassette was obtained. The *sdm* upstream and downstream regions were obtained by PCR amplification using *M. voltae* DNA as a template, as described in the text. The upstream regions were inserted in between the *Spe*I and *Nru*I sites, and the downstream regions were inserted in between the *Kpn*I and *Nhe*I sites, replacing the *PhmvA* promoter in plasmid pNPAC. The resistance cassette to be used for the gene replacement mutagenesis was then excised by using *Spe*I and *Nhe*I.

8.0. The product was precipitated after phenol extraction and dissolved in 3 μ l of H_2O and 2 μ l of formamide loading buffer (DYEnamic direct cycle sequencing kit; Amersham Biosciences, Freiburg, Germany) and separated on a polyacrylamide sequencing gel next to a sequencing reaction performed with the same primer in an automatic LI-COR II 4000S sequencing apparatus.

Two-dimensional (2D) gel electrophoresis and protein sequencing. For the analysis of protein expression under different growth conditions, *M. voltae* was grown without or with the addition of 10 μ M selenite at 37°C. The generation times of the cultures were 4 or 2 h, respectively. Heat treatment was done by growing the cells at 42°C for at least four generations. In this case, the generation time was 2.5 h. Two hundred-microliter cultures were harvested at an OD_{600} of 0.8 to 1.0. They were harvested by centrifugation at 15°C and lysed by the addition of 8 ml of H_2O . The lysate was centrifuged at 40,000 rpm (Rotor Ti 80; Beckman Coulter GmbH, Krefeld, Germany) for 2 h at 4°C. Two volumes of ethanol was added to precipitate the proteins. The suspension was centrifuged for 30 min at 4,000 $\times$ g at 4°C. The proteins were dissolved in 7 M urea, 2 M thiourea, 100 mM DTT, 2% (wt/vol) CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate), and 0.8% (vol/vol) Pharmalyte 3-10 (Amersham Biosciences). The separation of the proteins was done as previously described (13), with the following modifications. One hundred eighty-millimeter Immobiline dry strips (linear pH range of 4 to 7; Amersham Biosciences) were used for the first dimension. Fifty micrograms or 1 mg of protein for analytical or preparative gels, respectively, was mixed into 7 M urea, 100 mM DTT, 2% (wt/vol) CHAPS, and 0.8% (vol/vol) Pharmalyte 3-10 and soaked into the strips overnight. Isoelectric focusing was performed at 3,500 V for 15 or 20 h for analytical or preparative gels, respectively, in a Multiphor II apparatus (Amersham Biosciences). The proteins were then separated in a sodium dodecyl sulfate–12.5% polyacrylamide gel. Analytical gels were silver stained (4). Proteins to be sequenced were excised from gels stained with PhastGel Blue R (Amersham Biosciences). Trypsin digestion and N-terminal sequencing were performed by Toplab (München-Martinsried).

Protein determination and β -glucuronidase assay. Protein concentrations were determined by using the Roti-Nanoquant (Roth, Karlsruhe, Germany) reagent according to the supplier's instructions. β -Glucuronidase assays were performed with crude cell extracts. One milliliter of exponentially growing cells (OD_{600} of 0.4 to 0.6) was harvested by centrifugation and resuspended in 20 mM potassium phosphate (pH 7.5), 1 mM EDTA, and 100 mM β -mercaptoethanol. The lysed cells were centrifuged in a microcentrifuge at 13,000 $\times$ g at 4°C for 15 min. A total of 15 to 30 μ l of the supernatant was used in a total volume of 330 μ l of lysis buffer including 1.25 mM *p*-nitrophenyl β -glucuronide. The reaction was performed at 28°C. Nitrophenol production was followed by measuring the OD_{405} in a microplate reader.

Nucleotide sequence accession number. The sequences of the *sdmA*, *sdmB*, and *sdmC* genes can be found in GenBank (accession number AJ 575802).

RESULTS

A corrinoid protein is synthesized in *M. voltae* upon selenium deprivation. When cell extracts of *M. voltae* cells grown

either in the presence of selenium or under selenium limitation were analyzed by 2D gel electrophoresis, a protein spot was identified which was only present in selenium-limited extracts (Fig. 2). In contrast to other observed cases, the protein was not visible in extracts of heat-treated cells and probably does not represent a general stress-induced protein.

Primers pep2ufw and pep1urv (Table 2) were derived from the partial peptide sequences, and a PCR product of 0.6 kb in length was amplified. The amplicon was cloned and sequenced, and its identity was confirmed by comparison with the internal peptide sequences (Fig. 3).

A BLAST search identified the protein (SdmA) as a very close relative of corrinoid proteins involved in the demethylation of methylamines in *Methanosarcina* species (14, 27). They

FIG. 2. Sector of a silver-stained 2D gel showing a differentially expressed protein in extracts of *M. voltae* grown with selenium at 37°C (+Se), without selenium at 37°C (–Se), or with selenium at 42°C (+Se 42°C). The differentially expressed protein that was further analyzed is shown by an arrow.

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T E S Q E N M I K Q L
M T E S Q E N M I K Q L S D A V L D M D E
atgacagagtccacagaaaatgataaagcaacttccagatgctgttttagatatggatgaa

G Y N A F E *
D L T E E L S D T Y I K N G Y N A F E G I
gatttaacagaagaattatctgatacatatattaaaaatggatacaacgcattcgaaggaatt

G L A D G M N R
S K G L A D G M N R A G V M Y E E E E Y F
tcaaaaggattggcagatggaatgaacagagcaggtgttatgtacgaagaagaatacttc

I P E L L V C S D A M Y K G L D I L K P H
atactgaattattagtagtctccgatgcaatgaaatccggttggtatattttaaacctcat

L Q Y S E N D E K M K A V V G V V E G D T
ttacaattatccagaaaatgacgaaaaatgaaagcagtagttggtgtcgtagaaggggatact

H D I G K N L F K I M L E T Q G F E V Y D
cagcatttgcaaaaaacttgtttaataatggttagaacaacaaaggtttcgaggtttatgat

L G R D V P P I E F V E K A K E V N A D V
ttaggtagagatgtaccacattgaattcgtagagaagctaaggaagtaaatgcggatgta

I G L S T L M T T T M D N M K V V I D I L
attgattatcaacactatgacaactacaatggataacatgaaagtgttatcgatatctta

K E Q N M K D N T I V M V G G G P I S Q S
aaagaacaaaatgaaagacaataccatcgtaattggtgtggtggtcctatctcacaaagc

I G A D G Y A P E A S K *
F A D K I G A D G Y A P E A S K S A R I A
tttctgtataaaattggcgagatgggttatgctcgtgaagcttcaaaatctgcaagaattgca

K E L V S K L K D S K L N -
aaagaattagtttcaaaattaaaagattcaaaattgaattaa

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FIG. 3. Amino acid sequence of the differentially synthesized gene product identified by comparison with the peptide sequences (boldface type) obtained by microsequencing fragments of the polypeptide eluted from 2D sodium dodecyl sulfate-polyacrylamide gels. The peptides used to design the PCR primers are marked with asterisks.

contain a conserved corrinoid binding motif. The amino acid identities ranged from 35 to 45%. These proteins form complexes with methyltransferases that accept the methyl groups from corrinoid proteins (7, 8). A probe was derived from the identified partial gene sequence, and a λ ZAP genomic library of *M. voltae* was screened. Several clones were obtained which served to extend the known sequence in the 5' and 3' directions. Two genes encoding proteins (SdmB and SdmC) highly related to methyltransferases of *Methanosarcina* were identified. The amino acid sequences of the derived gene products share a high degree of identity (22 to 24%) with respect to the *Methanosarcina* proteins. In addition, the conserved zinc binding motif was detected in both gene products.

These findings might have been an indication that *M. voltae*, like *Methanosarcina*, could use methylamines or other methyl donors as alternative substrates for methanogenesis under selenium limitation. However, unlike in *Methanosarcina*, a gene encoding a protein that could serve as a substrate-specific component of a demethylation-methyltransferase complex was not detected in the neighborhood of the corrinoid protein and methyltransferase genes. Attempts to adapt selenium-limited *M. voltae* to growth on methylamines under selenium limitation also failed.

Regulation of the selenium-dependent expression occurs on the transcript level. In order to investigate whether the regulation of the expression depending on the selenium supply was true for all three identified genes and whether the regulation occurred on the transcript level, Northern hybridization was carried out with probes derived from the *sdmA* gene (Fig. 4). Indeed, the transcripts were detected only at concentrations below 1 μ M selenite in the medium. The apparent higher

FIG. 4. Arrangement of the genes contained in the *sdm* transcription unit and their transcription under different growth conditions. The name for the operon, *sdm*, was chosen on the basis of the dimethylselenide demethylation function. The upper panel shows a schematic of the three genes encoding a corrinoid protein (*sdmA*) and two methyltransferases (*sdmB* and *sdmC*). The gene sizes are given in base pairs. The bar indicates the position of the probe used for the following Northern hybridization. The lower panel shows a Northern analysis of *sdm* transcripts obtained from cultures grown with different conditions or various concentrations of selenite in the medium. (A) Stained filter after the transfer of RNA obtained from cultures grown under the indicated conditions; (B) autoradiograph of the same filter after Northern hybridization, with the gene fragment shown as a bar in the upper panel as a radioactive probe; (C) autoradiograph of a filter carrying equal amounts of RNA from cultures grown with or without selenium at the indicated concentrations. The position of the size marker (in nucleotides) is shown on the left.

amount of transcript at 100 nM Se was due to a higher amount of RNA applied to the filter as seen after methylene blue staining (data not shown). The largest detected transcript (approximately 4 kb) could cover the gene encoding the corrinoid protein and the two adjacent methyltransferase genes. This transcript occurred in different amounts in various RNA preparations. It was also detected in Northern hybridization experiments employing *sdmB* or *sdmC* probes. An open reading frame downstream of the *sdmC* gene was identified in the database due to its overlapping 5' noncoding region. A filter showing the Northern hybridization of the *sdmA* transcript similar to the one shown in Fig. 4 did not cross-hybridize to a probe derived from the open reading frame downstream of *sdmC* (data not shown). This open reading frame is therefore apparently not part of the same transcription unit.

The genes encoding the mentioned selenium-free hydroge-nases in *M. voltae* are positively and negatively regulated with

FIG. 5. Promoter region (left) and primer extension for the determination of the transcription start site (right) of the *sdm* operon. The promoter elements, TATA region, and initiator are shown in boldface type. The start site is underlined. The arrow indicates the primer extension product.

their regulatory *cis* elements located in the intergenic region connecting the two hydrogenase gene groups (2, 18). In order to look for similar elements possibly governing the transcription of the newly found transcription unit, the promoter region was analyzed. For this purpose, the transcription start site was first mapped by primer extension as shown in Fig. 5. Inspection of the intergenic region upstream of the start site yielded TATA box promoter elements. However, neither the positive nor the negative regulatory sequences known from the hydrogenase regulon were detected. It is thus unlikely that the hydrogenase genes and the ones described here belong to a common regulon.

The activities of the *sdmA* and *sdmC* genes are most likely involved in the release of selenium from dimethylselenide. Since it could not be shown that the induction of the putative corrinoid protein and methyltransferase genes permits growth of the cells on methylamines, tests were done to determine whether the genes are involved in opening up an alternative selenium source to *M. voltae* when inorganic selenium salts are unavailable. It has been known that selenium is toxic to bacterial and eukaryotic cells in higher concentrations. The detoxification involves the volatilization by methylation, generating dimethylselenide as a natural organic selenium compound. It was conceivable that *M. voltae* would exploit dimethylselenide as a selenium source under conditions under which the ele-

ment was limiting. This could indeed be shown. It was demonstrated by the repression promoter of a selenium-free hydrogenase, which is turned off in the presence of a sufficiently high selenium concentration in the cell. A reporter gene construct was used to show this effect. *M. voltae* strain V1 carrying the *Escherichia coli* β -glucuronidase gene *uidA* under the control of the regulated *vhc* hydrogenase promoter (20) was grown under selenium limitation and shown to produce β -glucuronidase. Upon addition of dimethylselenide, the hydrogenase promoter was shut down, indicating that the internal selenium concentration necessary to affect the repression had been exceeded (Table 3).

In order to demonstrate the involvement of the corrinoid protein and/or methyltransferase genes in this effect, knockout mutants were constructed as shown in Fig. 6 and 7. Knockout of the *sdmA* gene encoding the corrinoid protein abolished the repression after the addition of dimethylselenide. As a test of whether or not the function of the corrinoid protein was the only necessary condition for the repression of *vhc* promoter activity in the presence of dimethylselenide as the only selenium source, a second gene replacement was constructed. It affected the neighboring methyltransferase gene (*sdmB*) while leaving the corrinoid protein gene intact. In this case, repression was observed in the presence of dimethylselenide. Thus, this methyltransferase has apparently no role in the demeth-

TABLE 3. Glucuronidase expression under the control of the *vhc* promoter^a

Selenium compound (μM)		Glucuronidase activity in extracts from strain			
Selenite ^b	Dimethylselenide	V1	V1 Δ <i>sdmA</i>	V1 Δ <i>sdmB</i>	V1 Δ <i>sdmC</i>
1		<0.3 ^c	<0.3	<0.3	<0.3
0.01		4	3	8	7
	100	<0.3	<0.3	<0.3	<0.3
	10	<0.3	12	<0.3	10
	1	<0.3	4	<0.3	15
	0.01	13	6	10	8

^a The specific glucuronidase activities in the presence of different amounts of selenium compounds in medium are given in mU (nmol nitrophenol/min)/mg of protein. Each value is the average of data from four separate assays.

^b The cultures grown with selenite served as controls to demonstrate the previously described selenium-dependent regulation of the *vhc* promoter (18). The differences among the different genotypes are highlighted in boldface. The fluctuation of the positive results is normal with the very slowly growing cultures in dimethylselenide or very low selenite concentrations. Qualitative measurements with three more independent cultures each of the different genotypes under the different conditions were fully consistent with the results shown here.

^c The residual activities in the presence of selenium (<0.3 mU/mg of protein) are due to basal promoter activity under selenium-dependent repression (18).

ylation of dimethylselenide. In contrast, knockout mutants of the second (downstream) methyltransferase gene (*sdmC*) again did not show repression. This indicates the role of *sdmC* in dimethylselenide demethylation.

The *sdmC* gene is also transcribed from a separate start site. Since the integration of the *pac* cassette, which includes the *mcr* terminator (17), was expected to have a polar effect on genes located downstream in the same transcription unit, it was surprising that the *sdmB* mutant but not the *sdmC* mutant could apparently liberate selenium from dimethylselenide. It was considered that either the terminator of the inserted cassette might be leaky or there might be an independent transcription start site in the intergenic region in front of the *sdmC* gene. Reverse transcription (RT)-PCR was performed with primers located within the *sdmC* gene or bracketing the intergenic region and part of the *sdmC* gene.

The results are shown in Fig. 8. No product from a putative read-through transcript was obtained, ruling out that the terminator of the resistance cassette was leaky. In contrast, the primers amplifying the expected *sdmC* transcript did result in RT-PCR amplification. This result shows that the gene is also transcribed into a shorter messenger out of the intergenic region in front of it, in addition to being part of the polycistronic mRNA carrying all three *sdm* genes.

Dimethylselenide demethylation can open up an alternative selenium source for growth of *M. voltae*. *M. voltae* grows poorly under selenium limitation (<1 μM selenite in the medium). Testing was done to determine whether this growth limitation might be relieved by the addition of dimethylselenide.

M. voltae V1 was grown in the presence of 10 μM or 10 nM selenite. Figure 9A shows that the growth rate was reduced at the lower concentration. The addition of 10 μM dimethylselenide indeed abolished the growth limitation. As expected, the effect of the different mutants in the *sdm* operon on the selenium-controlled *vhc* promoter was reflected in their growth behavior. The *sdmA* and *sdmC* mutants showed reduced growth in the presence of dimethylselenide like that in the total

FIG. 6. Characterization of a gene replacement mutant of the *sdmA* gene. The positions of the replacement (indicated by the boxed regions) of the hybridization probes and the restriction sites used are shown in the upper panel. An autoradiogram of the Southern hybridization is depicted in the lower panel. The replacement was done with linear DNA. It comprised the *pacN* gene under the control of the S-layer promoter and *mcr* terminator. This resistance cassette was flanked by the upstream and downstream regions of the *sdmA* gene. wt, wild type.

absence of added selenium (Fig. 9B and D), while the *sdmB* mutant did not differ from the *sdm* wild type (Fig. 9C). As expected, an *sdmB sdmC* double mutant exhibited the same growth behavior as that of the *sdmC* mutant (data not shown). In all cases, the cultures growing without selenium exhibited a short intermittent lag phase around 20 h after inoculation. This effect might reflect an adaptation to the special growth condition which is not understood.

DISCUSSION

M. voltae requires selenium for optimal growth since seleni-um-containing enzymes are involved in the methanogenic

FIG. 7. Characterization of gene replacement mutants of the *sdmB* and *sdmC* genes. The positions of the replacements (indicated by the boxed regions) of the hybridization probes and the restriction sites used are shown in the upper panel, left side. The autoradiograms of the Southern hybridizations are depicted in the lower panel. The replacement was done with linear DNA. It comprised the *pacN* gene under the control of the S-layer promoter and *mcr* terminator. This resistance cassette was flanked by the upstream and downstream regions of the *sdmB* gene in the case of the *sdmB* deletion or the *sdmC* upstream region and the remaining 3' part of the *sdmC* gene in the case of the *sdmC* deletion.

pathway by which the organism generates its energy. Different possible strategies to evade selenium deprivation occurring in the habitat of the archaeon can be envisioned. The organism may provide genetic information for isoenzymes lacking selenium, which may replace or supplement essential selenoproteins upon selenium deprivation. Alternatively, the organism might attain the ability to obtain selenium from sources not amenable as long as the element is freely available in sufficient concentrations.

It was previously shown that upon selenium limitation, *M. voltae* induces the formation of selenium-free hydrogenases, which are isoenzymes for homologous enzymes containing se-

FIG. 8. RT-PCR performed to show a separate transcription start of *sdmC*. (A) The reaction was performed with primers RTmetIIrw and RTmetIrv bracketing the *sdmC* gene (see schematic). Lanes: 1, size marker; 2 and 4, reactions with 1 and 5 μ g of total RNA, respectively; 3 and 5, control reactions with 1 and 5 μ g of total RNA, respectively, without the addition of reverse transcriptase; 6, PCR control with 20 ng of chromosomal DNA as a template; 7, control reaction with primer RTmetIIrw only; 8, control reaction with primer RTpac and RTmetIrvB bracketing the intergenic region between the *pacN* and *sdmC* genes (see schematic). Lanes: 1, size marker; 2 and 4, reactions with 1 and 5 μ g of total RNA, respectively; 3 and 5, control reactions with 1 and 5 μ g of total RNA, respectively, without the addition of reverse transcriptase; 6, PCR control with 20 ng of chromosomal DNA as a template; 9, size marker. The lengths of the size marker fragments are given in nucleotides.

lenium in their reactive sites (24). The up-regulation of the expression of selenium-free isoenzymes of selenoenzymes involved in methanogenesis has also been reported for *Methanococcus maripaludis*, in spite of its ability to grow at appreciable rates even in the absence of selenium (21).

Here, we describe an alternative strategy to overcome selenium limitation used by *M. voltae*. The organism can up-regulate the transcript level of genes most likely encoding a putative corrinoid protein and a putative methyltransferase and use the gene products to liberate selenium from dimethylselenide.

FIG. 9. Growth of *M. voltae* V1 and different *sdm* mutants in the absence of selenium or with the addition of 10 μ M selenite or dimethylselenide to the growth medium. The 50-ml cultures were grown at 37°C. Samples for OD₆₀₀ measurements were taken at the given time points. (A) V1 *sdm*⁺; (B) *sdmA*; (C) *sdmB*; (D) *sdmC*.

This leads to the repression of the *vhc* hydrogenase promoter which is active only under selenium deprivation.

Although the identity of the two involved proteins has not yet been shown biochemically, the obvious similarity of their genes to the homologous genes in *Methanosarcina*, together with their described involvement in the transformation of dimethylselenide, makes it very likely that they have the assumed functions. We do not know in which form the liberated selenium is used to achieve the observed repression. Inspection of the upstream region (Fig. 5) of the *sdm* operon has not yielded sequences known to be involved in the regulation of the operons coding for the selenium-free hydrogenases. This finding strongly suggests that the operons encoding the hydrogenases and the *sdm* operon do not belong to a common regulon.

The growth behavior of the wild type compared to knockout mutants of the *sdmA* and *sdmC* genes is in good agreement with the observations made with reporter gene constructs

probing into the activity of the *vhc* promoter that drives the transcription of the *uidA* reporter gene. It strongly indicates that dimethylselenide is a natural substrate of the putative SdmA corrinoid protein and the SdmC methyltransferase. It is actually possible that two different methyltransferases are involved in the conceivable stepwise demethylation of dimethylselenide. Whether this is indeed the case could be the subject of further investigations.

As mentioned above, dimethylselenide is a selenium detoxification product found in various environments, including marine habitats. It is therefore available to *M. voltae* in nature. Consequently, the ability of the archaeon to gain selenium from dimethylselenide probably constitutes a major strategy for obtaining the required trace element.

The fate of the liberated methyl groups is not clear since the small amounts of dimethylselenide which can be added to the culture without causing a toxic effect do not allow the forma-

tion of amounts of methane that can be detectable by gas chromatography. Therefore, we also consider dimethylselenide to be at most a minor source for methyl groups for methanogenesis even in slowly growing *M. voltae* cells in their natural habitats.

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