Purification, Characterization, and Molecular Cloning of S-Adenosyl-L-Methionine:Uroporphyrinogen III Methyltransferase from Methanobacterium ivanovii

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Received 11 March 1991/Accepted 24 May 1991

An S-adenosyl-L-methionine:uroporphyrinogen III methyltransferase (SUMT) activity has been identified in Methanobacterium ivanovii and was purified 4,500-fold to homogeneity with a 38% yield. The enzyme had an apparent molecular weight of 58,200 by gel filtration and consisted of two identical subunits of M, 29,000, as estimated by gel electrophoresis under denaturing conditions. The Kₘ value for uroporphyrinogen III was 52 nM. The enzyme catalyzed the two C-2 and C-7 methylation reactions converting uroporphyrinogen III into precorrin-2. Unlike Pseudomonas denitrificans SUMT, the only SUMT characterized to date (F. Blanche, L. Debussche, D. Thibaut, J. Crouzet and B. Cameron, J. Bacteriol. 171:4222–4231, 1989), M. ivanovii SUMT did not show substrate inhibition at uroporphyrinogen III concentrations of up to 20 μM. Oligonucleotide probes from limited peptide sequence information were used to clone the corresponding gene. The encoded polypeptide showed more than 40% strict homology with P. denitrificans SUMT. The M. ivanovii SUMT structural gene is likely to be, as is P. denitrificans cobA, involved in corrinoid synthesis.

Methanogenic bacteria synthesize particular types of corrinoids, mainly 5-hydroxybenzimidazolycobamide (vitamin B₁₂ factor III) (24, 33, 46, 42, and 46) and Coo-3-[α-(7-adenyl)ljcobamide (pseudovitamin B₁₂) (24, 43). Occurrence of cobamidine (factor B) has also been reported in Methanosarcina barkeri (29).

Methanogenic bacteria are known to synthesize large amounts of corrinoids. Values ranging from 0.1 to 1.4 μmol g (dry weight)⁻¹ have been reported (25, 43). The latter value corresponds to a high level of corrinoid compared with that in eubacteria such as Streptomyces, Bacillus, Rhizobium, Agrobacterium, or Pseudomonas spp., in which levels significantly lower (the higher values are 0.1 μmol g [dry weight]⁻¹) have been observed (31). Only propionic bacteria have been shown to synthesize the same amount of corrinoids (31). Therefore, enzymes involved in corrinoid synthesis in methanogenic bacteria might show improved catalytic properties compared with those from organisms producing lower amount of corrinoids.

We described the purification of Pseudomonas denitrificans S-adenosyl-L-methionine:uroporphyrinogen III methyltransferase (SUMT) (6); the cloning of its structural gene, named cobA (8); and its nucleotide sequence (13). P. denitrificans SUMT exhibits two properties which have been suggested to play a regulatory role in cobalamin biosynthesis (6). The enzyme has a low catalytic efficiency (kₗₑₜ = 38 h⁻¹) and shows substrate inhibition at urogen III concentrations above 2 μM. In methanogenic bacteria, like Methanobacterium ivanovii, the regulatory features of the cobamide pathway are expected to be very different from those of P. denitrificans. The present study was undertaken to purify

SUMT from a methanogenic bacterium, to study its basic kinetic properties, and to clone its structural gene. M. ivanovii was chosen to carry this work out since it is easily cultivable (22) at a scale allowing enough cells to be obtained for the purification of an enzyme that should be present in low amounts.

MATERIALS AND METHODS

General methods. Standard procedures for DNA isolation, manipulation, analysis, amplification, Southern blots, and colony hybridization have been used (39). The procedures to mobilize plasmid DNA from Escherichia coli to P. denitrificans were already described (8). M. ivanovii genomic DNA was purified as previously described (40). Nucleotide sequence was performed with Sequenase version 2.0 (United States Biochemical Corporation) with α-35S-dATP (Amersham) on plasmid DNA as previously described (10) or on single-strand DNA according to the manufacturer's specifications and protocols.

Media, bacteriological techniques, and chemicals. P. denitrificans and E. coli were grown in Luria broth (39) for routine culturing. M9 medium (30) was supplemented when required with 20 mg of L-cysteine liter⁻¹. The growth temperature was either 37°C for E. coli or 30°C for P. denitrificans. M. ivanovii was cultivated as previously reported (40) at 37°C in the complex medium described by Whitman et al. (45). E. coli strains were grown anaerobically in jars using the BBL GasPak anaerobic system (Becton Dickinson and Co., Cockeysville, Md.).

A 1-liter culture of E. coli B5548 with plasmid pXL1832 or pUC13 was grown in LB medium supplemented with ampicillin up to an optical density of 1 (610 nm), and then the cells were pelleted by centrifugation. Strain SC510 Rif' harboring plasmid pKT230 or pXL1841 was cultured with 30 ml of PS4 medium in 250-ml Erlenmeyer flasks as already described (8).

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TABLE 1. Bacterial strains and plasmids used

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant properties</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B5548</td>
<td>F(^-) ΔlacU169 rpsL thi cystG44 relA</td>
<td>From P. Cossart and B. Gicquel, obtained by PJ transduction of the cystG44 mutation from NF1400 into MC4100 (11)</td>
</tr>
<tr>
<td>DH5(\alpha)</td>
<td>F(^-) endA1 hsdR17 supE44 thi-1 λ(^-) recA1 gyrA96 relA, 8089lacZΔM15</td>
<td>Clontech Laboratory, Palo Alto, Calif.</td>
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<tr>
<td>MC1060</td>
<td>ΔlacIOPZYA×74 galU galK strA2 hsdR</td>
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<td><em>Pseudomonas denitrificans</em></td>
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<td></td>
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<tr>
<td>SC510 Rif(\beta)</td>
<td>Rif(\beta) isolate (100 μg ml(^{-1})) of SC510</td>
<td>5</td>
</tr>
<tr>
<td>SBL25</td>
<td>Cobalamin-producing strain from which SC510 derives through mutageneses</td>
<td>5</td>
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<tr>
<td><em>Methanobacterium ivanovii</em></td>
<td></td>
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<tr>
<td>DSM261</td>
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<td>22</td>
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<td><strong>Plasmids and phages</strong></td>
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<td>pBSK(+)</td>
<td>Amp(\beta) ColE1, multicloning site</td>
<td>Stratagene, La Jolla, Calif.</td>
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<tr>
<td>pRK2013</td>
<td>Km(\beta) ColE1, carries the tra genes of RK2</td>
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<td>pXL694</td>
<td>Amp(\beta) ColE1, Prp promoter vector followed by cDNA human angioinogen</td>
<td>15</td>
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<td>pG10</td>
<td>M13 derived, part of <em>M. ivanovii</em> corA gene</td>
<td>This study</td>
</tr>
<tr>
<td>pXL1809</td>
<td>Amp(\beta) ColE1, corA under the control of Prp and ribosome binding site rII</td>
<td>This study</td>
</tr>
<tr>
<td>pXL1841</td>
<td>Km(\beta) RSF1010, corA under the control of Prp</td>
<td>This study</td>
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<tr>
<td>M13mp19</td>
<td>M13 derived, multicloning site</td>
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</tr>
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</table>

*Bacterial strains and plasmids and plasmid constructions.*

Bacterial strains and plasmids used in this study are described in Table 1.

For pXL1809 construction, 40 μg of *M. ivanovii* genomic DNA was digested with EcoRI and BglII; the fragments were then separated by agarose gel electrophoresis, and fragments in the range size of 3 to 3.5 kb were purified by electrophoresis and cloned into EcoRI-BamHI-digested pBSK\(+\). To create pXL1832, a PCR of the pXL1809 sequence with the primers 1277 (5′ GCCGCAATTCACTATTGG TAGGTATTTTA 3′) and 1278 (5′ GCCGCAAGCTCTATTAC ATAAATT 3′) was carried out in the same conditions as for the enzymatic amplification of an internal fragment of *M. ivanovii* SUMT structural gene, except that only 20 cycles were performed. The PCR products were digested by *NdeI* and *SstI* (primers 1277 and 1278 have on their 5′ ends, respectively, *NdeI* and *SstI* restriction sites as underlined) and then ligated with the EcoRI-NdeI (120 bp) and the EcoRI-*SstI* (3.1 kb) purified fragments from pXL694 (15). The 1.5-kb EcoRI-BamHI-BamHI fragment from pXL1832, obtained by partial digestion with BamHI of EcoRI-linearized pXL1832, was then cloned into pKT230 digested with the same restriction enzymes to give pXL1841.

*Enzyme assay and determination of SUMT properties.*

SUMT activity was assayed in cell extracts as previously described (6), except that incubations were carried out at 100 μM S-adenosyl-L-methionine (SAM) and 1 μM urocan III. One unit of SUMT was defined as the amount of enzyme necessary to transfer 1 nmol of methyl group per h under these conditions. Kinetic parameters (K\(_m\) for urocan III and V\(_{max}\) values) were obtained from the primary plot of initial velocity against urocan III concentration (from 0.05 to 2.0 μM) at 100 μM SAM. The curve was fitted to weighted experimental data with Enzfitter, a nonlinear regression data analysis program (Elsevier Science Publishers BV, Amsterdam).
dam, The Netherlands). Determinations of protein concentrations, native molecular weight estimation, and sodium dodecyl sulfate (SDS)-PAGE of purified SUMT were carried out as previously described (14).

**Reaction catalyzed by SUMT from M. ivanovii.** Purified SUMT (41 μg; 63 U) was incubated anaerobically in the dark at 30°C for 4 h in 0.1 M Tris hydrochloride, pH 7.7, containing 5 mM dithiothreitol (DTT), 1 mM EDTA, 140 μM [methyl-3H]SAM (6.8 μCi μmol⁻¹), and 0.86 μM urogen in a total volume of 350 ml. After incubation, tetrpyrrolic acids were trapped on a small DEAE-Sephadex A 25 column (5 ml of swollen gel) and were oxidatively esterified with 5% (vol/vol) sulfuric acid in methanol (18 h, 20°C). The methyl ester derivatives were extracted with dichloromethane, purified by thin-layer chromatography using standard methods (6), and identified as previously described (6).

**Purification of SUMT from M. ivanovii.** The pH of Tris hydrochloride buffers was adjusted to 7.6 at 20°C. All high-performance liquid chromatography separations were performed at 20°C with a Gilson 305 gradient system (Gilson, Villiers LeBel, France).

(i) Preparation of a cell extract. Bacteria (12 g [wet weight] of cells of M. ivanovii) were resuspended in 80 ml of 0.1 M Tris hydrochloride–5 mM DTT–1 mM EDTA (buffer A) and were sonicated at 4°C with 30-20-s bursts from a Branson B 30 ultrasonic disintegrator. Cell debris was separated by centrifugation at 50,000 × g for 1 h, and the clear supernatant was passed through a column of DEAE-Sephadex A 25 (1 ml of swollen gel) which had been equilibrated with buffer A.

(ii) Ammonium sulfate fractionation. The protein fraction precipitating between 55 and 75% saturation with ammonium sulfate was collected by centrifugation (10,000 × g for 30 min) and resuspended in 6.0 ml of 0.1 M Tris hydrochloride–0.5 mM DTT–1.7 M ammonium sulfate (buffer B).

(iii) Hydrophobic interaction chromatography. The enzyme solution was applied at a flow rate of 1.0 ml min⁻¹ onto a Phenyl-Superose HR 10/10 column (Pharmacia) previously equilibrated with buffer B, and proteins were eluted at 2.0 ml min⁻¹ with a 70-ml linear decreasing gradient of 1.7 to 0 M ammonium sulfate in buffer B and immediately adjusted to 25% (vol/vol) glycerol. Active fractions were pooled (SUMT was eluted with approximately 0.6 M ammonium sulfate) and passed through a column of Sephadex G-25 which had been equilibrated with 0.1 M Tris hydrochloride–0.5 mM DTT–25% glycerol (buffer C).

(iv) First anion-exchange chromatography. The protein eluate was loaded on a Mono Q HR 5/5 column (Pharmacia) equilibrated with buffer C. Proteins were eluted at 1.0 ml min⁻¹ with a 15-ml linear gradient of 0 to 0.4 M potassium chloride in buffer C.

(v) Second anion-exchange chromatography. Fractions containing SUMT activity (eluted with 0.2 M potassium chloride) were pooled and mixed with 5 ml of buffer C. The sum was applied to a Mono Q HR 5/5 column eluted at 1.0 ml min⁻¹ with a 25-ml 0 to 0.3 M linear gradient of potassium chloride in buffer C. The fraction containing SUMT activity was concentrated to 200 μl with a Centricon 10 microconcentrator (Amicon).

(vi) Gel permeation chromatography. The protein solution was injected on a Bio-Sil SEC 250 column (Bio-Rad) eluted at 0.5 ml min⁻¹ with 50 mM sodium sulfate–20 mM sodium dihydrogen phosphate (pH 6.8)–0.5 mM DTT–25% glycerol. SUMT was eluted as a symmetrical peak.

**Protein sequencing.** About 500 pmol of SUMT was subjected to microsequencing by using the sequential automated Edman degradation method with an Applied Biosystems model 477 apparatus. The sequence NH₂-VVVLYGAPGD PELITLKAVNVLKXADVVL . . . was determined. In order to obtain an additional sequence, the same quantity of protein was submitted to complete trypsin digestion. The fragments were then separated on a Brownlee RP C8 reverse-phase high-performance liquid chromatography column (2.1 by 10 mm; flow, 0.2 ml min⁻¹), eluting with an 80-min gradient of 0 to 50% (vol/vol) acetonitrile in 0.07% aqueous trifluoroacetic acid. A peptide eluted in a well-separated peak at 31% acetonitrile was sequenced, giving the sequence NH₂-IITGTLHENAGK.

**Nucleotide sequence accession number.** The nucleotide sequence shown in Fig. 5 has been submitted to GenBank under accession number M62874.

**RESULTS**

Characterization of SUMT from M. ivanovii. The purification of M. ivanovii SUMT is summarized in Table 2. The enzyme was purified about 4,500-fold with a 38% yield.

(i) Identification of products of SUMT activity. These studies were performed with a purified SUMT preparation which was homogeneous, as judged by SDS-PAGE, and showed a single symmetrical peak when analyzed by gel filtration high-performance liquid chromatography and one clean sequence when subjected to NH₂-terminal amino acid sequencing. The products of SUMT activity were identified as factor I octamethyl ester and sirohydrochlorin octamethyl ester after oxidative esterification, thus indicating that SUMT from M. ivanovii catalyzes both methylation at C-2 and C-7 of urogen III leading to precorrin-1 and precorrin-2 (see structures in Fig. 1). No trimethylated pigment in a detectable quantity was obtained. The precorrin-1-to-precorrin-2 ratio of intermediates produced in incubation conditions described in Materials and Methods was approximately 65:35, a value not very different from that (1:1) obtained in experiments with SUMT from P. denitrificans (6).

(ii) M₅ determination. The enzyme, when subjected to SDS-PAGE, migrated as a single protein band with a molecular weight of 29,000 ± 1,000 (Fig. 2). The M₅ determination of the native enzyme by gel filtration gave a value of 58,200 ± 2,000, indicating, as for P. denitrificans SUMT, a homodimeric structure.

(iii) Kinetic parameters. The Kₘ for urogen III was found to be 52 ± 8 nM, a value about 20-fold lower than that obtained for the enzyme from P. denitrificans. The Vₘₐₓ value was 1,537 U mg⁻¹. Unlike SUMT from P. denitrifi-
cancs, the enzyme from M. ivanovii did not show substrate inhibition at concentrations of up to 20 µM urogen III.

(iv) Absorption spectrum. In 0.1 M Tris hydrochloride–0.5 mM DTT–25% glycerol, purified SUMT showed a typical protein spectrum with a single absorption maximum at 265 nm, and no absorbance in the visible region. Assuming an $M_r$ of 58,200, the molar extinction coefficient at 265 nm was 2.0 $\times 10^5$ M$^{-1}$ cm$^{-1}$.

Cloning of the M. ivanovii SUMT gene. From the NH$_2$-terminal peptide sequence (30 residues) and the sequence of an internal fragment (12 residues) of the purified enzyme, three different oligonucleotides probes were designed. Primers A1 and A2 (sense primers) are, respectively, 256- and 48-fold degenerate 27-mers derived from the N-terminal sequence; primer B (antisense primer) is a 96-fold degenerate 25-mer derived from the sequence of the internal fragment. Primers A2 and B take into account the known codon usage in M. ivanovii (41). Primers A1 or A2 could be used with primer B for a PCR with M. ivanovii genomic DNA for generating a probe specific for the SUMT structural gene as already described for the cloning of a full-length porcine urate oxidase cDNA (26). Two PCRs with M. ivanovii genomic DNA were performed with primers A2 and B (reaction 1) or primers A1 and B (reaction 2). Those PCR products, when analyzed by PAGE, give fragments of 615 and 240 bp for reaction 1 and 630 and 170 bp for reaction 2. The specific product of reaction 2 should have a size of 21 bp.

FIG. 1. Structures. Compound 1, urogen III; compound 2, precorrin-1; compound 3, precorrin-2; compound 4, factor I octamethyl ester; compound 5, sirohydrochlorin octamethyl ester.

FIG. 2. SDS-PAGE of purified SUMT. Lane A, pure SUMT (100 ng); lane B, molecular weight markers (in thousands) (10 to 20 ng per band). Gels were stained with silver.
in excess of the one of reaction 1, since primers A1 and A2 correspond to N-terminal peptide sequences that are seven residues apart. The size difference is in the range of what was observed between the fragments of 615 and 630 bp. The 615-bp fragment was cloned into the replicative form of M13mp19 phage. In the clones having the expected insert, the obtained sequence should be, just after the EcoRI site, a sequence coding for the last 21 residues of the SUMT-determined NH₂-terminal sequence, since in the sense primer A2, the sequence corresponds to residues 9 to 14 of the sequence. For two of six clones, the forecasted sequence was observed. This also allowed the identification of the undetermined residue corresponding to a lysine codon (AAA) in the DNA sequence. One replicative form was kept for this study and named pg10.

Southern hybridization, using 32p-labelled pg10 as a probe against several digestions of M. ivanovii genomic DNA, revealed that a 3.2-kb EcoRI-BglII fragment hybridizes with the probe (Fig. 3). It was also found that M. ivanovii BglII-HindIII, BglII-KpnI, and BglII-PstI fragments of, respectively, 2.0, 5.8, and 1.9 kb also hybridized with the probe (Fig. 3). The EcoRI-BglII-digested chromosomal fragments of 3.0 to 3.5 kb were purified by agarose gel electrophoresis, ligated with EcoRI-BamHI-digested pBSK+, and introduced into the E. coli strain DH5α. Among 800 recombinant colonies, one showed a strong positive hybridization signal with pg10 insert as a probe. Restriction analysis of the corresponding plasmid DNA (named pX1800) indicated that a 3.2-kb fragment had been inserted (Fig. 4). Supercil DNA sequencing with primer A1 allowed reading of a sequence that overlapped the one obtained from the sequence of pg10 with the M13 −20 universal primer, indicating that at least part of the SUMT structural gene was cloned.

Nucleotide sequence and sequence analysis of the gene encoding SUMT activity. The strategy for sequencing was to extend the sequence from the part encoding the NH₂-terminal of the purified SUMT by supercoil DNA sequencing with oligonucleotides specific for the sequence on both strands. The sequence of a 955-bp fragment, presented in Fig. 5, was obtained. It contains an open reading frame (ORF) coding for a 231-amino-acid protein (M₉, 24,900) from base 34 (ATG) to 729 (TGA). The NH₂-terminal sequence of the encoded polypeptide is strictly identical to the amino acid sequences determined from the purified SUMT, except that the amino-terminal methionine has been removed. Removal of the amino-terminal methionine has been proposed to occur when the penultimate amino acid is valine (4, 20). The SUMT internal amino acid sequence, obtained after trypsin digestion, corresponds in Fig. 5 to bases 634 to 669; as expected, a basic amino acid (arginine) is found before the trypsin cleaving site. No other ORF was found in the sequence. The deduced polypeptide sequence was analyzed with the Hopf and Woods program (21) and presented the characteristics of a soluble protein, as expected from biochemical data.

The sequence 5' GGTGA 3', complementary to the 3' end of Methanobacterium 16S rRNA (2), is found 5 bp upstream from the initiation codon. Only one gene from M. ivanovii has been sequenced (41). This gene codes for a protein highly homologous to NiFH (niorgenase Fe protein) from the nitrogen-fixing archaeabacteria. The A+T content of the ORF/nifH is very close (63%) to that (62.8%) of the SUMT gene. The noncoding region has a higher A+T content (67.4%), and the average A+T content is 64%, which is consistent with that (63%) of the M. ivanovii genome (3). The codon usage of the sequenced gene is very similar to that of ORF/nifH (41), with a preference for codons ending in A and U and little use for codons containing GC or CG (data not shown). This gene was named corA since it encodes an activity that has been shown to be involved in corrinoid synthesis in P. denitrificans (6).

Sequence homology with other proteins having SUMT activity. M. ivanovii CorA and P. denitrificans CobA protein sequences were compared by using the Kanehisa program (23). A total of 40.4% strict identity between the two proteins was found on most of their length (data not shown). CorA was also shown to have more than 47% strict identity with Bacillus megaterium SUMT (36) and 41.7% strict identity with the carbox-terminal domain of the E. coli CysG protein (32), which has been shown to have SUMT activity (32, 44). Sequences homologies between these four proteins are reported in Fig. 6. The recently determined sequence of the Salmonella typhimurium CysG protein (47) is not presented in this figure, since it is very homologous to the E. coli enzyme (94% strict identity). Highly conserved domains among these SUMT proteins are observed; they are likely to
VOL. 173, ATG ccataattettttataatttaaacggtgaacac
CCA GGA
most
gions reflect conserved regions by
AAT (V/I)FGRGGEE, homologies were (GXGPG and VXXXXXGDP) sequences cans SAM (35), which methyltransferase sequences, (11) for the enzyme defect might be
AAT TGG GAC
tmT ACA
and 24,900, sequences of such SUMT sequences, (12) for CobA, CobI, CobL, and CobM P. denitrificans SAM methyltransferases of the cobalamin pathway (12), indicating that they must be of great relevance for
V TGG TTA
in
AAA CAA
of
AAA CCT
Val, which suggests that the
AAA GAT
SUMT activity
AAA CAT
for the enzyme
AAA CAT
three regions of homologies among the known (CobA and CobI) and the proposed (CobF, CobJ, CobL, and CobM) P. denitrificans SAM methyltransferases of the cobalamin pathway (12), indicating that they must be of great relevance for methyltransferase activity (Fig. 6). However, four significant homologues were found between SUMT consensus sequences (GXGPG and VXXXXXGDP) and the conserved sequences of 5-methylcytosine-forming methyltransferases (35), which are also SAM-dependent C-methyltransferases, suggesting that there is no conserved domain in the primary sequences of such proteins concerning either the active site or a SAM-binding site.

In addition to the homologies between their primary sequences, SUMT from B. megaterium, M. ivanovii, and P. denitrificans have very similar molecular weights (25,800, 24,900, and 29,200, respectively), which confirms that E. coli CysG protein (M_r, 49,928) carries another domain.

Expression of Methanobacterium SUMT activity in E. coli and P. denitrificans. Plasmid pXL1809 was introduced into strain B5548, an E. coli cysG mutant carrying the cysG44 mutation (11) and was found not to complement this mutant on M9 medium without cysteine either aerobically or anaerobically. This result suggests that either M. ivanovii SUMT is not sufficiently expressed in E. coli or, as previously reported for the P. denitrificans CobA protein (13), this enzyme cannot complement the cysG mutation since this defect might involve, in addition to a SUMT activity deficiency, a lack in precorrin-2 oxidation and iron chelation. The last two reactions might not be catalyzed by M. ivanovii SUMT and are necessary for siroheme synthesis (27).

A construction of pXL1832 was carried out, as shown in Fig. 4, in which M. ivanovii SUMT structural gene was linked to both the strong E. coli Ptp promoter and the efficient ribosome binding site from the lambda cII gene (15), which was 9 bp upstream of the corA initiation codon. pXL1832-dependent SUMT expression was studied in strain B5548, which should allow the detection of a weak SUMT activity in cell extracts. The strain harboring plasmid pXL1832 showed a 52-fold-higer SUMT activity than the control strain (with plasmid pUC13) (310 versus 5.9 pmol h^{-1} mg of protein^{-1}, whereas SUMT activity of a cysG+ E.
coli is between 60 and 160 pmol h$^{-1}$ mg of protein$^{-1}$), indicating that M. ivanovii SUMT is expressed from the E. coli transcription and translation signals. However, the expression level was not sufficient to visualize the corresponding band when the total cellular proteins were analyzed by SDS-PAGE (data not shown). Plasmid pXL1832, like pXL1809, was not able to complement the E. coli cysG mutant B548, indicating that SUMT expression is not sufficient for complementation of the cysG mutation.

The expression cassette was subcloned on a wide-host-range 
<ref>incQ-derived</ref> vector (Fig. 4), giving plasmid pXL1841, which was introduced by conjugative transfer into P. denitrificans SC510 Rif$. SUMT activity was studied further in one transconjugant cultivated in PS4 medium, and a 17-fold increase compared with that in the control strain SC510 Rif$^+$ (KT230) was found (1,700 versus 100 pmol h$^{-1}$ mg of protein$^{-1}$). This result established that plasmid pXL1841 carries the expression signals allowing expression of the M. ivanovii SUMT structural gene in P. denitrificans. Whether or not this expression is dependent upon E. coli 7pTrp and the $\lambda$ cI1 ribosome binding site remains to be demonstrated; however, this result confirmed that the ORF found on the nucleotide sequence encodes a protein showing SUMT activity.

**DISCUSSION**

We report in this study the purification of a SUMT activity from M. ivanovii. This enzyme catalyzes the same reactions as the previously purified P. denitrificans SUMT enzyme involved in corrinoid synthesis (6). However, this newly described enzyme did not show inhibition by its substrate, urogen III, a property which has been suggested to play a regulatory role in cobalamin synthesis in P. denitrificans. M. ivanovii SUMT had a $k_{cat}/K_m$ of about 4.7 $\times$ 10$^4$ s$^{-1}$, whereas the value of this parameter for the P. denitrificans enzyme was 1.1 $\times$ 10$^4$ s$^{-1}$ (6). This 45-fold-higher value for the specificity constant is mainly due to the very low Michaelis constant of M. ivanovii SUMT for urogen III (52 nM). It might reflect the adaptation of the enzyme to the low intracellular level of urogen III in cultured cells (concentrations of <50 nM were currently measured) (17).

The property of substrate inhibition reported for P. denitrificans SUMT was shown not to be restricted to the highly mutagenized cobalamin overproducing strain SC510. Purified SUMT from SBL25, which derives from the wild-type P. denitrificans (28) by only two mutageneses, was shown to present a pattern of substrate inhibition quite similar to that of SC510 Rif$^+$ (5). Moreover, purified SUMT from B. megaterium, whose primary sequence has more than 40% strict identity to both the M. ivanovii and the P. denitrificans SUMT, has also been shown to be negatively regulated by urogen III (36). This suggests that SUMT substrate inhibition is a general feature in aerobic eubacteria.

The interesting property of the M. ivanovii enzyme, compared with P. denitrificans SUMT, is the absence of substrate inhibition. The differences between the two enzymes is interpreted in view of the amount of corrinoids produced by these two organisms. The P. denitrificans strain, from which the CobA protein has been purified, derives from a strain that has been isolated from poultry litter (28) and produces 100 nmol of cobalamin liter$^{-1}$ per g of dried cell. P. denitrificans is a gram-negative aerobie rod that should require cobalamin at very low levels, as does E. coli, for which it is admitted that 25 molecules of cobalamin per cell are sufficient to support growth, in a metE background (7).

One way to limit cobalamin synthesis would be to negatively regulate the enzyme activity in the first step by urogen III, when this substrate accumulates above a certain concentration. On the contrary, methanogenic bacteria synthesize more corrinoids than wild-type P. denitrificans and other eubacteria (see Introduction) for unknown reasons. Therefore, it is consistent that the activity of the key enzyme for their corrinoid pathways does not exhibit a regulation.

Furthermore, $F_{a30}$ must be synthesized in high amounts, in methanogenic bacteria, since it is the prosthetic group of a protein constituting about 10% of the cell protein content (37). $F_{d30}$, the nickel-containing uroporphyrinoid prosthetic group of methyl-CoM methylreductase component C (19), combines structural elements of both the corrin and the porphyrins and contains two methyl groups at positions C-2 and C-7 (18); it has been proposed to be synthesized from precorrin-2 (18). In this instance, corrinoids and $F_{a30}$ would share the same biosynthetic pathway up to precorrin-2, and SUMT would operate on both pathways. The properties of M. ivanovii SUMT reflect this requirement for high levels of corrinoid and $F_{a30}$ synthesis.

**ACKNOWLEDGMENTS**

We express our gratitude to J. Lunel and J.-F. Mayaux for their support during this work. We thank the CITI2 (Centre de Traitement Interuniversitaire d’Informatique & Orientation Biomédicale, Paris, France) for nucleic and protein sequences analysis programs. We thank F. Cuine for the NH$_4$-terminal sequencing of M. ivanovii SUMT and T. Ciora for oligonucleotide synthesis. We acknowledge L. Sibold, J.-P. Aubert, and M. Henriet from the Pasteur Institute, without whom this work would not have been possible, and we are particularly grateful to L. Sibold, who has since died. We thank R. Longin and his team for the cultivation of M. ivanovii.

This work was supported by both Rhône-Poulenc Rorer S.A. and Rhône-Poulenc S.A.

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