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Characterization of a Corrinoid Protein Involved in the C1 Metabolism of Strict Anaerobic Bacterium *Moorella thermoacetica*

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ABSTRACT **The strict anaerobic, thermophilic bacterium *Moorella thermoacetica* metabolizes C1 compounds for example CO_2/H_2, CO, formate, and methanol into acetate via the Wood/Ljungdahl pathway. Some of the key steps in this pathway include the metabolism of the C1 compounds into the methyl group of methylenetetrahydrofolate (MTHF) and the transfer of the methyl group from MTHF to the methyl group of acetyl-CoA catalyzed by methyltransferase, corrinoid protein and CO dehydrogenase/acetyl CoA synthase. Recently, we reported the crystallization of a 25 kDa methanol-induced corrinoid protein from *M. thermoacetica* (Zhou et al., Acta Crystallogr F 2005; 61:537–540). In this study we analyzed the crystal structure of the 25 kDa protein and provide genetic and biochemical evidences supporting its role in the methanol metabolism of *M. thermoacetia*. The 25 kDa protein was encoded by *orf1948* of contig 303 in the *M. thermoacetica* genome. It resembles similarity to MtaC the corrinoid protein of the methanol:CoM methyltransferase system of methane producing archaea. The latter enzyme system also contains two additional enzymes MtaA and MtaB. Homologs of MtaA and MtaB were found to be encoded by *orf2632* of contig 303 and *orf1949* of contig 309, respectively, in the *M. thermoacetica* genome. The *orf1948* and *orf1949* were co-transcribed from a single polycistronic operon. Metal analysis and spectroscopic data confirmed the presence of cobalt and the corrinoid in the purified 25 kDa protein. High resolution X-ray crystal structure of the purified 25 kDa protein revealed corrinoid as methylcobalamin with the imidazole of histidine as the α-axial ligand replacing benziimidazole, suggesting base-off configuration for the corrinoid. Methanol significantly activated the expression of the 25 kDa protein. Cyanide and nitrate inhibited methanol metabolism and suppressed the level of the 25 kDa protein. The results suggest a role of the 25 kDa protein in the methanol metabolism of *M. thermoacetica*. Proteins 2007;67:167–176.**

Key words: X-ray crystal structure; corrinoid protein; methanol metabolism

INTRODUCTION

Moorella thermoacetica (formerly *Clostridium thermoaceticum*) is a thermophilic, anaerobic acetogenic bacterium, which using the autotrophic Wood/Ljungdahl acetyl-CoA pathway converts C1 compounds such as CO_2, CO, formate, and methanol to acetate.[1–4] This involves a total synthesis of acetate from two moles of CO_2. One mole of CO_2 is reduced via formate and tetrahydrofolate intermediates to 5-methyltetrahydrofolate, the methyl group of which is transferred onto the cobalt atom of a corrinoid iron sulfur protein (C/Fe-S) forming a Co-methylcorrinoid.[5] In the final step, the methylcorrinoid and coenzyme A (CoA) were condensed with another mole of CO_2 forming acetyl-CoA catalyzed by the bifunctional Ni-Ni enzyme carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS).[6–10]

The first indication of the involvement of corrinoids in the synthesis of acetate by the acetyl-CoA pathway was obtained by Poston et al.,[11] who found that the methyl group of synthetically produced methylcobalamin was incorporated into the methyl group of acetate by cell extracts of *M. thermoacetica*. This led to an investigation of the corrinoid content of *M. thermoacetica*.[12] Out of 15 different corrinoids found, 11 were identified. The most abundant B_{12}-derivatives were 5-methoxybenzimidazolylcobamide (Factor III_m) and cobyric acid, which both were present mostly as their Co-5′-deoxyadenosyl (coenzyme) derivatives, and also as Co-methyl derivatives. When intact cells of *M. thermoacetica* were exposed to $^{14}CO_2$ the Co-methyl groups of the two Co-methyl corri-

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noids got labeled and their ^{14}C-methyl groups were converted to the methyl group of acetate by extracts of *M. thermoacetica* fortified with pyruvate.[13]

It is now well established that derivatives of vitamin B_{12} play important roles in bacterial C1 metabolism.[14,15,7,16] The cobalamin-dependent methionine synthase is the most studied methyltransferase (Mtr).[17,18] Other corrinoid-dependent transferases have been isolated from acetogens,[19] methanogens,[20–24] and methylotrophs[25,26] and the methyl donors in these reactions include methyltetrahydrofolate, methyltetrahydromethanopterin, methanol, acetate, methylamines, methylethers, and halomethanes.

The corrinoid proteins isolated from acetogenic bacteria that participate in the Wood/Ljungdahl pathway of acetate biosynthesis have been reviewed.[7] Three corrinoid proteins have been isolated from *M. thermoacetica.* The best characterized corrinoid protein in the Wood/Ljungdahl pathway is the C/Fe-S, and as discussed above it transfers the methyl group of methyltetrahydrofolate to the CODH/ACS. It is a αβ dimer having two subunits of 33 and 55 kDa. The smaller subunit carries the corrinoid 5-methoxybenzimidazolylcobamide, whereas the larger subunit has the [4Fe-4S] cluster.[5,19] A second corrinoid protein designated MtvC isolated by Naidu and Ragsdale[27] is part of a three component vanillate *O*-demethylase system. This enzyme system may have a broad specificity and be involved in the transfer of methyl groups from a number of methoxylated aromatic compounds functioning as methyl donors.[28] A similar system has been described for the acetogen *Acetobacterium dehalogenans*.[29,30]

Recently we reported the preliminary crystallography study of a 25 kDa corrinoid protein.[31] Based on the N-terminal amino acid sequence of the protein it was identified to be a homolog of MtaC, which is a corrinoid protein and a component of the methanol:CoM Mtr system of methane producing archaea.[20,32] This enzyme system consists of three components MtaA, MtaB, and MtaC. MtaB catalyzes the transfer of the methyl group from methanol to the corrinoid cofactor of MtaC, while MtaA catalyzes the transfer of the methyl group from the corrinoid of MtaC to CoM. Genes encoding homologs of MtaA, MtaB, and MtaC are found to be present in the *M. thermoacetica* genome (http://www.tigr.org). Here we provide physiological, genetic, and structural evidence that the 25 kDa polypeptide is a corrinoid Mtr. The role of this protein in the methanol metabolism and acetate biosynthesis of *M. thermoacetica* has been discussed.

MATERIALS AND METHODS

Bacterial Strain and Growth Conditions

Moorella thermoacetica strain ATCC 39073 was grown on 200 m*M* methanol or 1% (wt/v) glucose as a carbon source at 58°C under 100% CO_2 gas in semi-defined Drake's minimal medium[33] in 125-mL bottles, 4-L flasks, or 100-L fermentors as previously described.[34,35] Cultures were harvested at mid to late log phase ($OD_{600} \sim 1.0$) by centrifugation at 6000*g* and stored at −80°C until used.

Purification of the 25 kDa Protein

The 25 kDa corrinoid protein was purified from cytosolic extracts as described.[31]

Assays and Measurements

UV-visible absorption spectra of fractions containing the corrinoid protein were recorded with a dual wavelength spectrophometer (Shimadzu, model 2051PC). The sample preparations for spectral analysis were according to Ljungdahl et al.[36] Proteins were estimated using the Lowry method as described.[34] SDS-polyacrylamide gel electrophoresis of proteins was according to Laemmli.[37]

DNA and RNA Sources, PCR, and Northern Hybridization

M. thermoacetica genomic DNA was isolated using Puregene DNA purification system (Gentra, Minneapolis, MN). Total RNA was isolated using RNeasy mini kit from Qiagen (Qiagen Valencia, CA). Prior to use RNA was treated with RNase-free DNase I (Roche Applied Sciences, Indianapolis, IN). For Dot-blot hybridization experiments RNA was denatured with formaldehyde (15% v/v in 5 × SSC at 60°C for 1 h) prior to application onto nylon membranes (ICN, Costa Mesa, CA). Hybridization (Northern or Dot-blot) experiments were carried out using the Genius system (Roche Applied Sciences) as described.[38,39] The PCR and the labeling of the PCR products with digoxygenin (DIG) were carried out using the FailSafe PCR System (EPICENTRE, Madison, WI). The DIG-labeled PCR product used as a probe in the hybridization experiments was a 462-bp fragment amplified from *orf1948* of contig 303 of the *M. thermoacetica* genome with 5′-TGACCAGGAGTTTGTTGAGC-3′ (forward primer) and 5′-CCGACGATTACTTTTACCCG-3′ (reverse primer) using *M. thermoacetica* genomic DNA as template.

N-Terminal Protein Sequence

To determine the N-terminal sequence of the 25 kDa protein the purified protein was subjected to SDS-PAGE and then trans-blotted onto PVDF membranes (Bio-Rad). After briefly stained with Coomassie Brilliant Blue the polypeptide was excised from the blot and sequenced at the Integrated Biotechnology Laboratories at the University of Georgia.

Antibodies and Western Blotting Experiment

Polyclonal antibodies against the purified 25 kDa protein were raised in adult New Zealand white rabbit at the animal facility of the University of Georgia. Antibodies against CO dehydrogenase/acetyl-CoA synthase (CODH/ACS), Mtr, and corrinoid iron-sulfur protein (Co/Fe-S) were kindly provided by Steve Ragsdale of the

TABLE I. Data Processing Statistics for the Refinement Data Set

Resolution range (Å)	50.00–1.60 (1.66–1.60)
Wavelength (Å)	1.5798
Space group	$P2_12_12$
Cell dimensions (Å)	$a = 55.69$, $b = 62.74$, $c = 34.54$
Unique reflections	13939 (625)
Completeness (%)	84.1 (38.6)
$I/\sigma(I)$	46.8 (10.2)
R_{sym} (%)[a]	7.7 (21.6)
Redundancy	5.7 (2.9)

[a]$R_{sym} = \Sigma\Sigma_j|I_j - \langle I\rangle|/\Sigma\langle I\rangle$.

University of Nebraska. Western blotting experiments were carried out according to Bio-Rad.

Crystallization of the 25 kDa Protein and Collection of Diffraction Data

Crystallization and collection of a diffraction data to 1.9 Å resolution on a copper rotating anode source have been described previously.[31] The position of a single anomalous scatterer was determined by the program SHELXD in single wavelength anomalous scattering (SAS) mode.[40] Initial phases were calculated with the program SOLVE.[41] Phase improvement and automated model building were performed with RESOLVE.[42] A higher resolution data set was collected at a wavelength of 1.5798 Å at beamline 22ID of the Advanced Photon Source. A continuous sweep of 300 consecutive 1° oscillation images was recorded with a Mar300 CCD detector at a crystal-to-detector distance of 110 mm with an exposure of 4 s per image. Data reduction was carried out with the HKL2000 suite[43] (Table I). Further automated model building was carried out with ARP/wARP[44,45] using structure factor amplitudes[46] derived from the higher resolution data set with the program TRUNCATE of the CCP4 suite.[47] Iterative model validation, rebuilding, and refinement were carried out with MOLPROBITY,[48,49] XFIT[50] and the CCP4 program REFMAC5,[51] respectively. ARP/wARP and CCP4 programs were controlled through the CCP4I interface.[52] Coordinates of the refined model were deposited at the Protein Data Bank (PDB)[53] using the program PDB_EXTRACT[54] with access code 1Y80.

M. thermoacetica Genome Sequence

A draft annotated nucleotide sequence of the *M. thermoacetica* (ATCC39073) genome has been completed at the Joint Genome Institute Department of Energy and can be viewed at http://www.tigr.org.

RESULTS

Purification and Spectral Properties of the 25 kDa Protein

The 25 kda corrinoid protein was purified from cytosolic extracts of methanol grown cells of *M. thermoacetica* following ammonium sulfate precipitation, ion exchange chromatography, and gel filtration as described.[31] By size exclusion chromatography on Superose 12 column, an approximate molar mass of the protein was estimated to be 25 kDa (not shown). Since the predicted molar mass of the protein based on the amino acid sequence is 22,329 Da,[31] the purified protein as isolated is apparently a monomer in its native form. The amount of cobalt was estimated to be 0.9 mol per mol of the protein with negligible interferences from other metals. UV-visible spectra of the purified protein exhibited absorptions at 357 and 542 nm in oxidized form which were shifted to 363 and 551 nm following treatment with cyanide and to 369 and 580 nm after boiling with cyanide. These absorption maxima are typical for corrinoids as previously reported by Ljungdahl et al.[36]

Identification of the 25 kDa Protein From the *M. thermoacetica* Genome Sequence

The *N*-terminal amino acid sequence of the purified 25-kDa protein was determined to be M(P)TYEELS-QAVFEGD. This sequence is identical to the predicted *N*-terminal amino acid sequence of the polypeptide encoded by *orf1948* of contig 303 of the *M. thermoacetica* genome. The *orf1948* is 630 bp long and encodes 210 amino acids (see Fig. 1). Analysis of the deduced amino acid sequence of *orf1948* revealed its similarity to MtaC, a component of the methanol:CoM Mtr system of methanogenic archaea,[16] and also to the C-terminal amino acid sequence of 5-methyltetrahydrofolate S-homocysteine Mtr from several bacteria including *Thermatoga maritima* (accession no. B72397, 35.1% identity), *Mycobacterium tuberculosis* (accession no. G70513, 34.1% identity), and *Bacillus halodurans* (accession no. B72397, 39.5% identity) (see Fig. 2). Both MtaC and the above enzymes shared a common sequence motif Asp-X-His-X-X-Gly-X_{41}-Ser-X-Leu-$X_{26–28}$-Gly-Gly, which has been rationalized as the signature for the corrinoids, and the His residue serves as the α-axial ligand to the corrinoid.[15] The above motif is also common to a subset of other B_{12} enzymes including methyl-malonyl-CoA mutase, glutamate mutase, and methionine synthase.[15] In *M. thermoacetica* the methyl transfer reaction of the Wood/Ljungdahl pathway was catalyzed by methyltetrahydrofolate-dependent Mtr and the corrinoid iron-sulfur protein (C/Fe-S). The primary structure of C/Fe-S (ORF1921 of contig 303) lacks the above signature and has corrinoid with water instead of His as α-axial ligand. In the *M. thermoacetica* genome *orf1948* was surrounded by two additional ORFs, *orf1949* and *orf1947* (see Fig. 1). The *orf1949* is 1401 bp long encoding 476 amino acids, and *orf1947* is 801 bp long encoding 276 amino acids. The three ORFs were organized in the order *orf149*>*orf1948*>*orf1947* (see Fig. 1), which we now referred to as the corrinoid cluster of *M. thermoacetica*. The corrinoid cluster was located 3009 bp upstream of the CODH/ACS cluster (see Fig. 1) that contained the genes encoding CODH/ACS (ORFs 1919 and 1920), C/Fe-S (ORFs 1921 and 1924) and Mtr (ORF

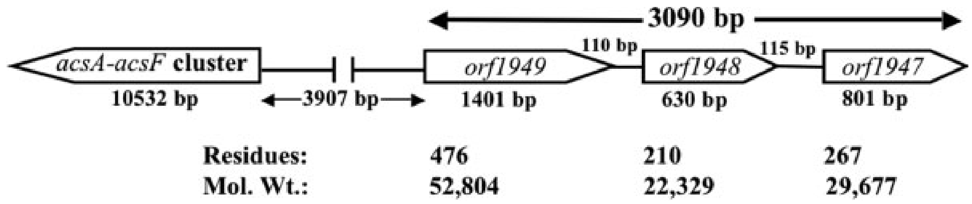

Fig. 1. The organization of the genes of the corrinoid cluster and the relationship between the genes and the corresponding products derived from the annotated *M. thermoacetica* genome. The *acsA-acsF* cluster encoding CODH/ACS, C/Fe-S, and Mtr[19] was located 3907 bp upstream of the corrinoid cluster.

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G1948        1    MPTY.EE... .......... .......... .......... .......... ..LSQAVFEG  14
Mmaze_MtaC   1    MLKSKWRFRN MLDFTEASLK KVLTRYNVAL EKAMTPEDAA EELYPKDELI YPIAKAIFEG  60
Afulg_MtaC   1    MADVRKE... .......... .......... .......... .......... ..FVQALADL  15
Mbark_MtaC   1    .......... MLDFTEASLK KVLTRYNVAL EKALTPEEAA EELYPKDELI YPIAKAIFEG  50
F83853       621  SNLTLE.ER. .......... .......... .......... .......... ..LANYIVEG  636
B72397       561  PRAEVKEEK. .......... .......... .......... .......... ..LVEIILSG  577
G70513       641  G.LPLF.ER. .......... .......... .......... .......... ..LAQRIVDG  655

G1948        15   DEAQVVELTR SLLSGGAEPL EVINKGLIAG MDRVGVLFKN NEMFVPEVLM SANAMNAGVE  74
Mmaze_MtaC   61   EEDDVIEGLE AAIKAGKDPI ALIDDALMVG MGVVTRLYDE GIIFLPNVMM SADAMLDGIE  120
Afulg_MtaC   16   DEAKTVELTK KRVESGEDPF TILEDV.RKA TDIIGKRFEE GRYFVSDLIM AGEILKQVME  74
Mbark_MtaC   51   EEDDVVEGLQ AAIEAGKDPI DLIDDALMVG MGVVIRLYDE GVIFLPNVMM SADAMLEGIE  110
F83853       637  SKDGLTEDLD KALAKYDDPL DIINGPLMNG MDEVGRLFNN NELIVAEVLQ SAEVMKASVA  696
B72397       678  NRSELEKLVE DFLKE.KDPL SVIEEHLRPA MERIGELYDK GKIFLPQLIL AAQTVKPVFD  736
G70513       656  ERNGLDADLD EAMTQ.KPPL QIINEHLLAG MKTVGELFGS GQMQLPFVLQ SAEVMKAAVA  714

G1948        75   VVKQSQQAFD MPS.VGKIVL GTVKGDLHDI GKNLVAMMLE SGGFTVYNLG VDIEPGKFVE  133
Mmaze_MtaC   121  FCKENSETAP VT..KGTVVC HVAEGDVHDI GKNIVTALLR ANGYNVVDLG RDVPVDEVLN  178
Afulg_MtaC   75   ILRPLLGEKK AES.KGKVVI GTVEGDVHDI GKNIVIALLE AEGFEVVDIG VDQPPEAFVE  133
Mbark_MtaC   111  YCKENSGATP KT..KGTVVC HVAEGDVHDI GKNIVTALLR ANGYNVVDLG RDVPAEEVLA  168
F83853       697  HLEPHMEKKA DDHGKGKIIL ATVKGDVHDI GKNLVEIILS NNGFRIVNLG IKVTSNELIE  756
B72397       737  KLTSMLPSDS QGETF...VI ATVKGDVHDI GKNIVASVIR SSGYRVVDLG KDVDTSEIVE  793
G70513       715  YLEPHMERSD DDSGKGRIVL ATVKGDVHDI GKNLVDIILS NNGYEVVNIG IKQPIATILE  774
                                                  *   *

G1948        134  AVKKYQPDIV GMSALLTTTM MNMKSTIDAL IAAGLRDRVK ..VIVGGAPL SQDFADEIGA  191
Mmaze_MtaC   179  AVAENNPILV TGTALMTTTM YAFKEVNDKL LEKGYK..IP ..FACGGGAV NQDFVSQYAL  234
Afulg_MtaC   134  AANQHNPDVV GLSGLLTEAI ESMKRTVEAL RKAGYK..GK ..IIIGGGRT SEEAKEYTGA  189
Mbark_MtaC   169  AVQKEKPIML TGTALMTTTM YAFKEVNDML LENGIK..IP ..FACGGGAV NQDFVSQFAL  224
F83853       757  AVARENPDAI GLSGLLVKSA QQMVLTAQDL KQQQIS..IP ..ILVGGAAL TRKFTNTKIA  812
B72397       794  AVEKERPVAL GLSAMMTTTV GRIKEVVEKL KEKNLK..IP ..VIVGGASL NEKLAKELGA  849
G70513       775  VAEDKSADVV GMSGLLVKST VVMKENLEEM NTRGVA..EK FPVLLGGAAL TRSYVENDLA  832
                                 *  *                                 **

G1948        192  DGYAPDAASA TELCRQLLE. .......... .... 210
Mmaze_MtaC   235  GVYGEEAADA PKIADAIVAG TTDIAALRDK FHKH 268
Afulg_MtaC   190  DDWADDAAVG VRKIKALVGV E......... .... 210
Mbark_MtaC   225  GVYGEEAADA PKIADAIIAG TTDVTELREK FHKH 258
F83853       813  PEYDGLVVYA KDAMNGLELA NKLMKPDE.. .... 840
B72397       850  DYYAKNASEA VKILKSLGR. .......... .... 868
G70513       833  EIYQGEVH.. .......... .......... .... 840
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Fig. 2. Multiple sequence alignment of the 25 kDa polypeptide (*orf1948*) from *M. thermoacetica* with MtaCs from *Methanosarcina mazei* Go 1 (accession no. NP_633672); *Archaeoglobus fulgidus* DSM 4304 (accession no. NP_068847), *Methanosarcina barkeri* (accession no. CAA69619), and 5-methyltetrahydrofolate S-homocysteine methyltransferases from *Bacillus halodurans* (strain C-125) (accession no. F83853); *Thermatoga maritima* (accession no. B72397); and *Mycobacterium tuberculosis* (accession no. G70513). Conserved residues were shown in bold face. Residues marked by asterisk belong to the conserved corrinoid-binding domain of the protein.

1925).[19] The polypeptide encoded by *orf1949* revealed similarity to MtaB (see Fig. 3), the second component of the methanol:CoM Mtr system, from several methanogenic archaea.[20] In methanogenic acrchaea, *mtaC* and *mtaB* were co-transcribed from a single polycistronic operon, while *mtaA* encoding the third component MtaA of the enzyme system was present separately at a distant location.[20] As described below *orf1949* and *orf1948* of *M. thermoacetica* were also co-transcribed from a polycistronic operon. Search for the gene encoding the third component of the Mtr system MtaA revealed its similarity to the polypeptide encoded by ORF2632 of contig 309 in the *M. thermoacetica* genome (see Fig. 4) as the deduced *orf2632* shows similarity to MtaAs from several archaea. Therefore, homologs of all three components of the methanol:CoM Mtr system of methanogenic archaea are present in *M. thermoacetica*. The polypeptide encoded by *orf1947* of the corrinoid cluster revealed similarity (33.6% identical residues) to C/Fe-S of *M. thermoacetica*, and also to the N-terminal amino acid sequence of 5-methyltetrahydrofolate S-homocysteine Mtrs from several bacteria (not shown).

Northern Blot Analysis and Regulatory Sequences

The *orf1949* and *orf1948* of the corrinoid cluster of *M. thermoacetica* were separated by 110 bp, while *orf1948*

A

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G1949        1     MDYKPVKTFS ELEVKSLDDF VYGIAPHPVK AKNGMVIGAG TVYPEINMTL PPMNIEESTM
Mmaze_MtaB   1     MAATR...FT KMAYASADEM TFGVSKYPVK AGLGLEIGAG YTIPEVNYAP RPEAG..ASK
Mbark_MtaB   1     MAAKR...YT SMAYANADEM TFGVSKYPVK AGLDLEIGAG YTIPEINYAP RPEAG..ASK
Mace_MtaB    1     MAAKR...YT SMAYASADEM SFGVSKYPVK AGLGLEIGAG YTIPEVNYAP RPEAG..ASK

G1949        61    PEVRRQYAEM IEGILKRARD LYAPGIIVEL ELLPETTMKP EWGIEINKIL RDKMHEYEDK
Mmaze_MtaB   56    EKLIKEYERI TTDIMARMVQ VGFPAVILET EHVQQMSNNP SWGAEVAHAQ KTIMEEYHDE
Mbark_MtaB   56    EKLIKEYERI TTDVMERMVQ VGFPAIILET EHVQQMSNNP SWGAEVAHAQ KTIMEKYHDE
Mace_MtaB    56    EKLVKEYERI TTDIMGRMVQ VGFPAVILET EHVQQMSNNP SWGAEVAHAQ KTIMEEYHDE

G1949        121   YGLKSLLRCT PNDTREILRP PLMKRGELLE NMFITFEKCA EDGADILSIE STGGKEVHDE
Mmaze_MtaB   116   YGIKCALRHT IGDIRENRDF LQLRGDKYSV FLEA.FEECA KAGADLLSVE SMGGKEVFDY
Mbark_MtaB   116   YGIKCALRHT IGDIRENREF LQLRGDKYSV FLEA.FEQCA ENGADLLSVE SMGGKEVFDY
Mace_MtaB    116   YGIKCALRHT IGDIRENRDF LQLRGDKYSV FLEA.FEECA KSGADLLSVE SMGGKEVFDH

G1949        181   ALVTCNIRKA IFALGVLGVR DMRFLWSNIV RIAERTGAIA GGDTACGFAN TALALAEQGM
Mmaze_MtaB   175   AVLRNDIAGM LYAIGCLGSI DMELIWSDIS AIAKKTGTVS AGDTDCAQAN TAMFIGGGLL
Mbark_MtaB   175   AVLRNDIPGL LYSIGCLGSI DMELIWTDIS KIAKKTGTIS AGDTDCAQAN TAMFIGGGLL
Mace_MtaB    175   AVLRNDVAGM LYAIGCLGSI DMEMIWSDIA AIAQKTGTVA AGDTDCAQAN TAMFIGGGLL

G1949        241   IPRVF...AA VDRVATIPRS LVAFEMGAIG PDKDCGYEGP YMKAIAGVPI SMEGKTAACA
Mmaze_MtaB   235   DKNLAHTLAI LARAISAPRS LVAYECGAVG PGKDCGYENV VIKAITGMPM TQEGKTSTCA
Mbark_MtaB   235   NKNLAHTIAV IARAISAPRS LVAYEAGAVG PGKDCGYENI IVKAITGMPM TMEGKTSTCA
Mace_MtaB    235   DKNLAHTLAI LARAISAPRS LVAYECGAMG PGKDCGYENI IIKAITGKPM TQEGKTSTCA

G1949        298   HLSAIGNIAA CVCDMWSNES VQNVKLLSAP APVVSTEQLI YDCRIMNEAA ADGRSFALKM
Mmaze_MtaB   295   HSDVMGNLIM QCCDCWSNES VEYHGEFGGT TVQCWGESLA YDCAIMNTAL ETKNDKV..L
Mbark_MtaB   295   HSDVMGNLVM QCCDCWSNES VEYHGEFGGT TVQCWSETLA YDCAIMNTAL ETKNDKV..L
Mace_MtaB    295   HSDVLGNLIM QCCDCWSNES VEYHGEFGGT TVQCWSETLA YDCTIMNTAL ETKNEKV..L
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B

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G1949        358   RDWLAASDSR LDPQAYVLRP DIVLEISQEL VKEKDA.FIA TKKAAALAAE VIKRGLARGE
Mmaze_MtaB   353   RDLIMLSDRY RDPQAYVLAY DNAYRIGQAI VKDGDNIYLR AKNAAIACCD IVSEG.AAGK
Mbark_MtaB   353   RDLMMLSDRY RDPQAYMLAY DNAYRVGQSI VKDGDNIYLR AKNAAIECCN IIEEG.AAGK
Mace_MtaB    353   RDLFMLSDRY RDPQGYVLAY DNAYKVGEAI VKDGEDIYLR AKNAAVACCD IVSEG.AAGK

G1949        417   VQVSSREKKW LDIISSQIET IPDDWEEFWY EIQKEL..DL EKFRPEEYDL EVIMARGASA
Mmaze_MtaB   412   LELSRFETKA LADAKASLDS LTDDMDKFMD DCLTKYKSEV KVFLPENYGF ..........
Mbark_MtaB   412   LELSRFETKA LADAKAALEA LPDDMDKFMD DCLTKYKSEV KVFKPENYGF ..........
Mace_MtaB    412   LELSRFETKA LADAKASLDS LTDDMDKFMD DCLTKYKSEV KVFLPENYGF ..........

G1949        475   GN
Mmaze_MtaB         ..
Mbark_MtaB         ..
Mace_MtaB          ..
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Fig. 3. Multiple sequence alignment of the protein encoded by *orf1949* of contig 309 of *M. thermoacetica* genome with MtaBs from *M. mazei* Go 1 (accession no. NP_633671), *M. acetovorans* C2A (accession no. AAK07601) and *M. barkeri* (accession no. CAA69620). Conserved residues were shown in bold face.

and *orf1947* by 115 bp. A putative promoter sequence, 5′-ATGACC-N_{15}–TTAAT-3′, resembling the *E. coli* consensus σ promoter (5′-cCTTGACa–N_{15-21}–TATAaT-3′)[55] was located 19-bp upstream of the *orf1949* start codon. No secondary promoter structure was apparent in the two intergenic regions, suggesting all three genes belong to a single polycistronic operon. To verify this presumption total RNA from *M. thermoacetica* was subjected to Northern hybridization with a 462-bp DIG-labeled DNA fragment amplified from *orf1948* as a probe. The Northern blots exhibit a strong hybridization signal at 3200 bp which is close to the combined size of *orf1947, orf1948*, and *orf1949* [Fig. 5(A)] including the intergenic regions 3009 bp, suggesting a polycistronic message for the corrinoid cluster. The hybridization signal was much stronger with RNA from methanol grown cells than with RNA from glucose-grown cells. Hybridization signals were virtually undetectable with RNA from glucose plus nitrate grown cells grown which is expected since nitrate was reported to inhibit the C1 metabolism and the Wood/Ljundahl pathway in *M. thermoacetica*.[33] RNA from cyanide-grown cells also failed to yield any hybridization (not shown) which could be due to inhibition of CO dehydrogenase/acetyl-CoA synthase,[56] the most crucial enzyme of the Wood/Ljundahl pathway. The Northern blots show smear immediately following the hybridization signal at 3200 bp, indicating degradation of the transcripts, which is not unusual for polycistronic transcripts as reported earlier.[35] To verify the level of transcripts under different growth conditions, total RNA was subjected to dot-blot hybridization with the same probe used in Northern hybridization experiments. Results [Fig. 5(B)] show strong hybridization signals for RNA from methanol-grown cells than from glucose-grown or

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G2632        9     TPKRRFLSAL FGGRVDRTPV ANPTSLVTVE IMERTGAYFP DAHLDAEKMA RLAATSYEVL
Mmaze_MtaA   8     TLKTRLLAAL KGEPVDKVPV CSVTQTGIVE IMDVVGAPWP EAHTNPEIMA KLALANHELS
Mace_MtaA    8     TLKTRLLAAV KGEPVDKVPV CSVTQTGIVE IMDEVGAPWP EAHTNPEIMA KLALANYELS
Mbark_MtaA   5     TPKERLYRAL RKQQVDRMPA VCFTQTATVE QMEACGAYWP EAHSDAEKMA TLAEAAHTVV

G2632        69    GFDTIMPVFS AHTESAALGV PVDWGDKMSW PVNTSHPITD PEQIVIPDSF LEEPSMRT-V
Mmaze_MtaA   68    GLEAVRLPYC LTVLVEAMGC EINMGTKNRQ PSVTGHPYPK DLEGAAVPAD LLQRGRIPVV
Mace_MtaA    68    GLEAVRVPYC LTVLVEAMGC EINMGTKNRQ PSVTGHPFPK ALDGAAVPAD LLQKGRIPAV
Mbark_MtaA   65    GFEAVRVPFD ITAEAEFFGC GIKAGDLKQQ PSVIKPSVKN LEDLEKLKNY NLKEGRF---

G2632        128   LDAIKILRSQ YGDRVAIIGK TYGPWSLAYH LVGTENFLME TILNPDKARR YLEVLLEASI
Mmaze_MtaA   128   LEAIKIIREK VGPDVPIVGG MEGPVTVASD LVSVKSFMKW SIKKTDLLEQ ALDIATEASI
Mace_MtaA    128   LEAIKIIREK VGPDVPIIGG MEGPITVASD LVSVKSFMKW SIKKPDLFEQ SLDLATAASI
Mbark_MtaA   122   ---------- ---------- ---------- ---------- ---------- --------NV

G2632        188   LSAKAQIKAG ADAILWGDH- ATGDLVSAEY YRDFLMKVHQ YVTR---EVG APIILHICGN
Mmaze_MtaA   188   IYANAMVEAG ADVIAIADPV ASPDLMSPDS FRQFLKSRLQ KFAS---SVN SVTVLHICGN
Mace_MtaA    188   AYANAMVEAG ADIIAIADPV ASPDLMSPDS FKQYLQPRLQ KFSS---SVS SVTVLHVCGN
Mbark_MtaA   124   AYAKAMVENG ADTIAIIDPT ASYELIGGEF YEKYALPYQK KIVDAMKELD VATVLHICGN

G2632        244   TTKFIPYIVE AGFDAFHFDS KVD-AKLAKE LAGNKMSLIG NINNPVTLLA GTPEDVKKET
Mmaze_MtaA   244   VNPILSDMAD CGFEGLSVEE KIGSAKKGKE VIGTRARLVG NVSSPFTLLP GPVDKIKAEA
Mace_MtaA    244   VNPILSYMAD CGFEGLSVEE KIGSVKKAKE VIGTRARLIG NISSPFTLLP GPVDKIKAES
Mbark_MtaA   184   TTNGLGIMDK TGVNGISVDQ KVD-IKTATG NVK-NALIVG NLDPVAVLWN GTPEEIAEVS

G2632        303   LYAIEAGVEI VGPECAIPLT TPLENILAIT ETAKEYQIHK KLGGETQ
Mmaze_MtaA   304   KEALEGGIDV LAPGCGIAPM TPLENVKALV AARDEFYA-- -------
Mace_MtaA    304   KQALADGVDV LAPGCGIAPM TPLENIKAMV EARNEFYA-- -------
Mbark_MtaA   242   KKVLDAGVGL LTVGCGTVSM TPTVNLQKMI ECAKSHTY-- -------
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Fig. 4. Multiple sequence alignment of the protein encoded by *orf2632* of contig 309 of *M. thermoacetica* genome with MtaAs from *M. mazei* Go 1 (accession no. NP_633094), *Methanosarcina acetovorans* C2A (accession no. NP_619241), and *M. barkeri* (accession no. S62369). Conserved residues were shown in bold face.

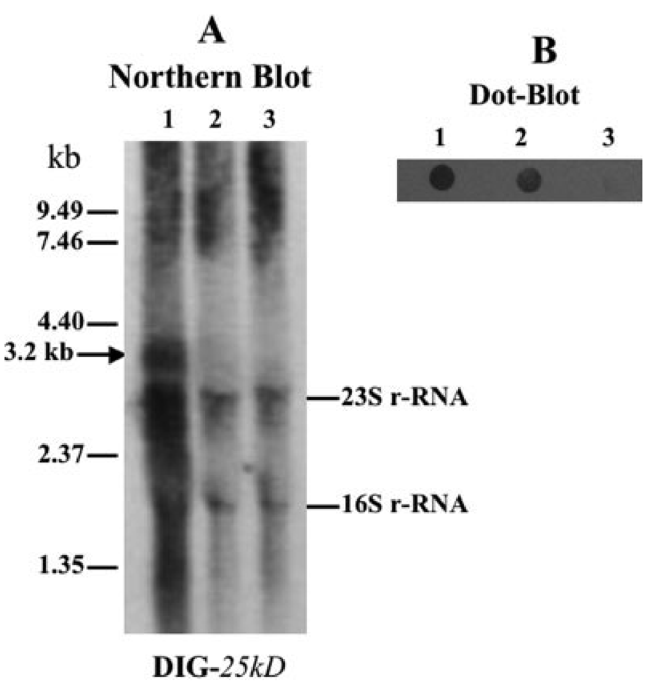

Fig. 5. Northern blot (A) and the dot blot (B) analysis of *M. thermoacetica* total RNA after hybridization with the DIG-labeled 462 bp PCR product amplified from *orf1948*. RNA was isolated from the bacterium grown on methanol (lane/dot 1), glucose (lane/dot 2) and glucose plus nitrate (lane/dot 3) as described in Materials and Methods.

glucose plus nitrate-grown cells. To check for any non-specific hybridization between the probe and the RNA, total RNA from these cells were also hybridized with DIG-labeled PCR-probe amplified from house-keeping gene *atpD* encoding the β subunit of F_1-ATPase.[39] Comparable hybridization signals were observed with RNA from methanol-, glucose-, and glucose plus nitrate-grown cells (not shown). These results suggest induction and strong activation of the corrinoid cluster by methanol.

The Expression of the 25 kDa Protein Under Different Growth Conditions

Figure 6 shows higher level of expression of the 25 kDa protein in methanol-grown cells than in glucose-grown cells, and both nitrate and cyanide completely inhibited the expression of the protein. We compared the level of expression of the 25 kDa protein with that of other Wood/Ljungdahl pathway enzymes including the corrinoid protein C/Fe-S, and CODH and Mtr. Cyanide completely inhibited the expression of CODH, and significantly reduced the expression of C/Fe-S, Mtr, and the 25-kDa protein (see Fig. 7). In comparison to cyanide, nitrate had negligible effect on the expression of CODH, C/Fe-S, and Mtr in *M. thermoacetica*, also reported by Frostl et al.[57] The level of expression of the 25 kDa-protein was much higher in methanol-grown cells than in glucose-grown cells, which correlates well with the

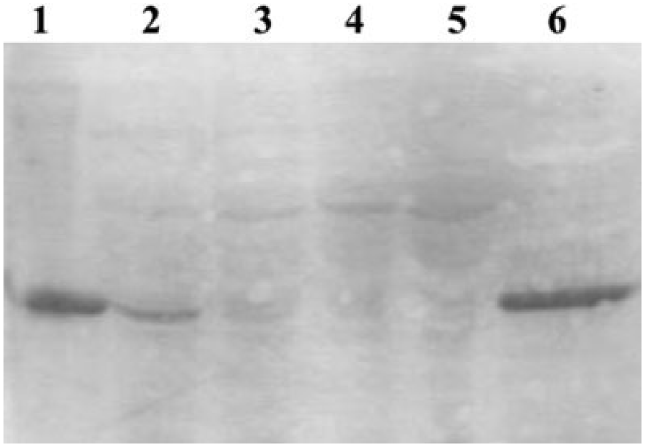

Fig. 6. Expression of the 25 kDa protein under different growth conditions. The purified 25 kDa protein (10 μg, lane 6) and whole cell extracts (40 μg per lane) of *M. thermoacetica* grown on methanol (lane 1), glucose (lane 2), glucose plus potassium cyanide (500 μ*M*, lane 3), glucose plus 5 m*M* KNO_3 (lane 4), and glucose plus 15 mmKNO_3 (lane 5) were subjected to SDS-PAGE, the proteins were transblotted from the SDS-gel onto PVDF membranes, and probed with antibodies against the 25 kDa protein by Western blotting experiments (details in the Materials and Methods).

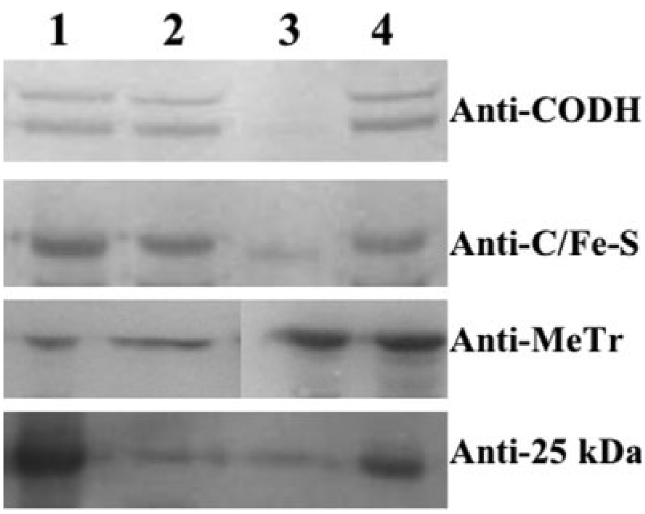

Fig. 7. Expression of the Wood/Ljungdahl pathway enzymes CODH/ACS (CO dehydrogenase/acetyl-CoA synthase), C/Fe-S (corrinoid iron-sulfur protein), and Mtr (methyltransferase) in *M. thermoacetica* grown on methanol (lane 1), glucose plus KNO3 (15 m*M*, lane 2), glucose plus KCN (500 μ*M*, lane 3) and glucose only (lane 4). Whole cell extracts (40 μg per lane) were subjected to SDS-PAGE and the proteins were transblotted onto PVDF membranes as described in the legends to Fig. 8. Western blotting experiments were carried out with antibodies raised the 25 kDa protein, CODH/ACS, C/Fe-S, and Mtr as described in the Materials and Methods.

results of Northern hybridization experiments (see Fig. 5) suggesting a role of this protein in the methanol metabolism of *M. thermoacetica*. Having the similarities of the 25 kDa protein and those encoded by *orf1949* and *orf2632* with MtaC, MtaB, and MtaA, respectively, of the methanol:CoM Mtr methyltransferase system of methanogenic archaea, it is likely that *orf1948*, *orf1949,* and *orf2632* could function as a Mtr system in *M. thermoacetica*. Winter-Ivey and Ljungdahl[58] suggested that the synthesis of acetyl-CoA from methanol could occur via direct transfer of the methyl group of methanol to CODH/acetyl-CoA synthase. However, this interpretation of the results needs verification at the enzyme level.

Analysis of the Crystallographic Data and the Structural Configuration of the 25 kDa Protein

The cobalt ion of the corrinoid facilitated protein structure determination by acting as an anomalous scatterer during diffraction. The preparation of a heavy atom derivative was therefore not required. Using a data set obtained from a copper X-ray source, the cobalt ion could indeed be located and initial phases calculated using the SAS method.[59] Crystals contained an N-terminal truncation[31] of the full-length protein and residues 85–209 were located in the electron density map (see Fig. 8). The model was refined using data to 1.7 Å resolution and showed both good fit to the experimental data and reasonable geometry (Table II). At 30% sequence identity over the alignment region, the overall structure aligns with residues 743–871 of PDB entry 1BMT,[60] the model of methionine synthetase from *E. coli*, with an RMSD of 1.1 Å.[61] The location of the corrinoid is outlined in good detail by strong electron density (see Fig. 9). Differentiation from cobalamin is unambiguous due to strong density for the 5-methoxy group and the lack of density in the 4-position of the benzamidazole moiety of the corrinoid protein. The benzimidazole moiety is displaced from the cobalt ion by the imidazole moiety of residue His-101 (see Fig. 9) resulting in the "base-off" configuration of the corrinoid. Additional polar interactions between the corrinoid and the protein have been identified and are listed in Table III including the interactions of some of the amino acid residues that belong to the so-called the corrinoid signature motif of the polypeptide.

DISCUSSION

Based on the primary structure the 25 kDa protein appears to be a homolog of MtaC, the corrinoid of the methanol:CoM Mtr system of methane-producing archaea.[20] The latter enzyme system consists of two additional enzymes MtaB and MtaA. In the *M. thermoacetica* genome homologs of MtaB and MtaA were found to be encoded by *orf1949* of contig 303 and *orf2632* of contig 309, respectively. In methanogenic arcahea *mtaC* and *mtaB* are present in a polycistronic operon while *mtaA* in a separate loci. A similar organization is also found for the corresponding genes in *M. thermoacetica* (see Fig. 1). The 25 kDa polypeptide has no homology to either Mtr or the corrinoid iron-sulfur protein C/Fe-S of the Wood/Ljungdahl pathway which catalyzes methyl transfer reactions from methytetrahydrofolate to synthesize acetyl-CoA.[5] The expression of the 25 kDa protein was induced and activated by methanol (see Fig. 6) suggesting a role of this protein in the methanol metabolism of *M. thermoacetica*. In methanogenic archaea, methane

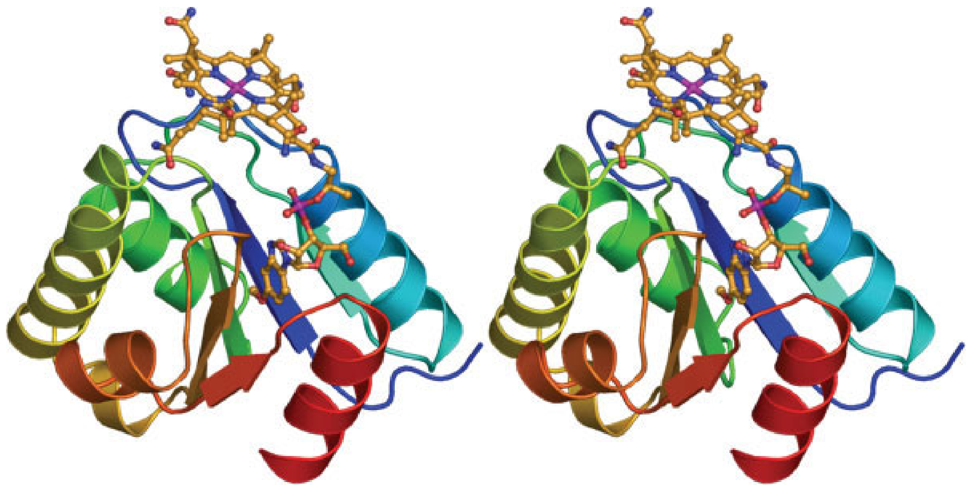

Fig. 8. Stereo view of the crystal structure of the 25 kDa corrinoid protein from *M. thermoacetica*. The image of the crystals was generated with software program PYMOL (DeLano, 2002).

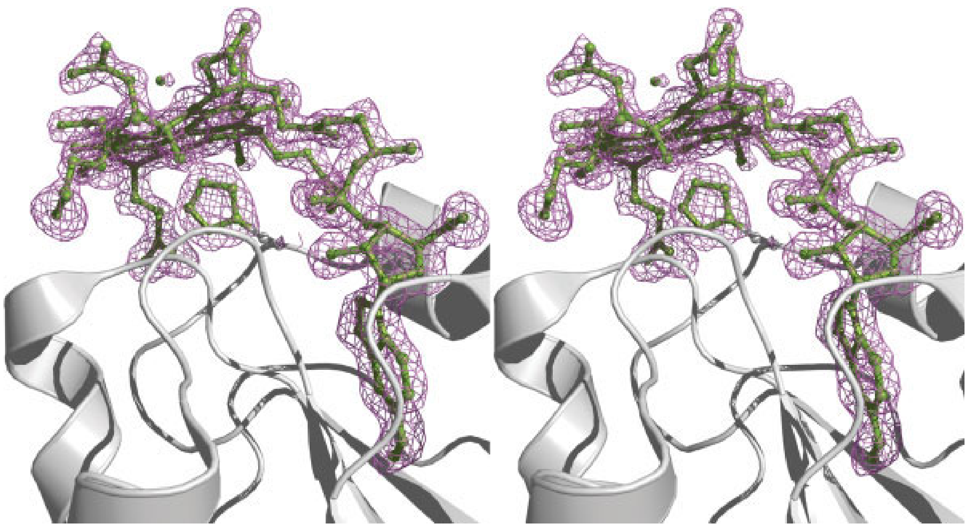

Fig. 9. Coordination of the corrinoid-bound cobalt ion with the ligand of the 25 kDa protein. The corrinoid is seen in the "base-off" configuration where Nϵ2 of His-101 displacing the benzimidazol moiety of the corrinoid as the ligand. Atoms shown in green were omitted in the calculation of a model-phased Fo-Fc electron density map shown here in magenta contoured at 2σ around the omitted atoms.

TABLE II. Statistics for the Protein Model

Resolution range (Å)	41.63–1.70
Total number of HKLs used (free)	12184 (528)
R_{work} (R_{free})[a]	17.4% (21.0%)
Mean/Wilson B factor (Å^2)	14.1/14.0
Total number of refined atoms (water)	1081 (72)
RMSD from ideal bond lengths (Å)	0.015
RMSD from ideal bond angles (°)	2.0

[a] $R_{\text{work}} = \Sigma||F_{\text{obs(work set)}}| - k|F_{\text{call}}||/\Sigma|F_{\text{obs(work set)}}|$; $R_{\text{free}} = \Sigma||F_{\text{obs(test set)}}| - k|F_{\text{call}}|| /\Sigma|F_{\text{obs(test set)}}|$.

TABLE III. Bond Distances for Polar Protein–(solvent)–Corrinoid Interactions

Residue			Residue			
Type	ID	Atom	Type	ID	Atom	Distance (Å)
Gly	104	N	Wat	401	O	2.90
Wat	401	O	B1M	301	O4	2.61
B1M	301	O7R	Ala	179	N	3.13
Ser	146	OG	B1M	301	N3B	2.87
B1M	301	CO	His	101	NE2	2.42
B1M	301	CO	Wat	458	O	2.66

is produced from methanol catalyzed by MtaA, MtaB and MtaC.[20] Since acetate, not methane, is the final product of methanol metabolism in *M. thermoacetica* (Das and vanHoek, unpublished) it is likely that the methyl transfer reactions from methanol catalyzed by the proteins encoded by the corrinoid cluster could be coupled to acetate biosynthesis. The 25 kDa protein was poorly expressed under nonacetogenic conditions for example in the presence of nitrate or cyanide (Figs. 6 and 7) which block acetogenesis in *M. thermoacetica*.[56,57] On the other hand, CODH, C/Fe-S and Mtr were all expressed in the presence of nitrate but not in the presence of cyanide (see Fig. 7). These results suggest a specific role of the 25 kDa protein in acetate biosynthesis.

Naidu and Ragsdale[27] reported an inducible three component aromatic *O*-demethylase system similar to that of methanol:CoM Mtr of methanogenic archaea in *M. thermoacetica*.[20] The three components of the *M. thermoacetica O*-demethylase system designated MtvA, MtvB and MtvC, were shown to catalyze direct transfer of the *O*-methyl group from methoxylated aromatic compounds for example syringate to the one carbon carrier tetrahydrofolate. The corrinoid protein of the aromatic *O*-demethylase from *M. thermoacetica* was shown to be MtvC. It was shown that MtvB catalyzed the transfer of the methyl group from phenylmethyl ether to the cobalt center of MtvC, and MtvA catalyzed the transfer of the methyl group from MtvC to tetrahydrofolate forming methyltetrahydrofolate. Methyltetrahydrofolate then served as the methyl donor in the synthesis of acetyl-CoA from CO and CoA catalyzed by Mtr, C/Fe-S, and CODH/ACS.[8] The N-terminal sequence of MtvC was reported to be MLTDTL(S)KAMAELEEEQ(V)LA which did not match the N-terminal sequence of the 25 kDa protein but matched exactly with the N-terminal sequence of the polypeptide encoded by *orf223* of contig270 of the *M. thermoacetica* genome. The *orf223* is preceded by two additional ORFs, *orf221* and *orf222*, neither of which has similarity to *mtaA* and *mtaB* of methanogenic archaea or *orf1949* and *orf2632* of *M. thermoacetica*. The predicted amino acid sequence of MtvC deduced from *orf223* shared 33% identical residues with that of the 25 kDa protein including the highly conserved corrinoid-binding motif Asp-X-His-X-X-Gly-X_{41}-Ser-X-Leu-$X_{26–28}$-Gly-Gly (not shown). Based on these similarities it is assumed that the functions of *orf1949, orf1948*, and *orf2632* could mimic that of the aromatic *O*-demethylase system of *M. thermoacetica*.[20] Therefore, ORF1949 (equivalent to MtvB) could catalyze the transfer of the methyl group from methanol to the 25 kDa protein (equivalent to MtvC) while ORF2632 (equivalent to MtvA) catalyzes the transfer of the methyl group from 25 kDa protein to tetrahydrofolate forming methyltetrahydrofolate. Finally acetyl-CoA could be formed from methytetrahydrofolate following condensation with CO and CoA catalyzed by Mtr, C/Fe-S, and CODH/ACS as previously described.[8] All the enzymes, essential for the synthesis of acetyl-CoA from methyltetrahydrofolate including CODH/ACS, C/Fe-S, and Mtr were expressed in methanol-grown cells (see Fig. 7), suggesting their involvement in the acetate biosynthesis from methanol. Analysis of the *M. thermoacetica* genome revealed several genes encoding putative corrinoid proteins, the function of which could be coupled methyl transfer reactions from a variety of naturally occurring compounds. This metabolic potential of *M. thermoacetica* and other acetogens led them to inhabit virtually any anoxic environment in nature.[1]

ACKNOWLEDGMENTS

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