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Isolation and Expression of a cDNA Encoding Methylmalonic Aciduria Type A Protein from *Euglena gracilis* Z

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Abstract: In animals, cobalamin (Cbl) is a cofactor for methionine synthase and methylmalonyl-CoA mutase (MCM), which utilizes methylcobalamin and 5′-deoxyadenosylcobalamin (AdoCbl), respectively. The *cblA* complementation class of inborn errors of Cbl metabolism in humans is one of three known disorders that affect AdoCbl synthesis. The gene responsible for *cblA* has been identified in humans (*MMAA*) as well as its homolog (*meaB*) in *Methylobacterium extorquens*. Recently, it has been reported that human *MMAA* plays an important role in the protection and reactivation of MCM *in vitro*. However, the physiological function of *MMAA* is largely unknown. In the present study, we isolated the cDNA encoding *MMAA* from *Euglena gracilis* Z, a photosynthetic flagellate. The deduced amino acid sequence of the cDNA shows 79%, 79%, 79% and 80% similarity to human, mouse, *Danio rerio MMAAs* and *M. extorquens MeaB*, respectively. The level of the MCM transcript was higher in Cbl-deficient cultures of *E. gracilis* than in those supplemented with Cbl. In contrast, no significant differences were observed in the levels of the *MMAA* transcript under the same two conditions. No significant difference in MCM activity was observed between *Escherichia coli* that expressed either MCM together with *MMAA* or expressed MCM alone.

Keywords: cobalamin; *Euglena gracilis*; methylmalonyl-CoA mutase; *MMAA*; vitamin B_{12}

1. Introduction

Vitamin B_{12}, or cobalamin (Cbl), is an organometallic cofactor that supports the activities of enzymes in organisms, ranging from bacteria to humans [1,2]. In animals, Cbl is the cofactor for cytosolic methionine synthase (EC 2.1.1.13) and mitochondrial methylmalonyl-CoA mutase (MCM, EC 5.4.99.2), more specifically, methylcobalamin (MeCbl) and 5′-deoxyadenosylcobalamin (AdoCbl), respectively. Inhibition of Cbl transport or biochemical modifications of Cbl usually causes severe diseases [3]. The *cblA* complementation class of inborn errors of Cbl metabolism is one of three known disorders that affect AdoCbl synthesis without affecting the synthesis of MeCbl [4]. The gene responsible for *cblA* has been identified through the examination of prokaryotic gene arrangements and is called *MMAA* [3]. The deduced amino acid sequences of human or mouse MMAA show homology with the periplasmic protein kinase ArgK of *Escherichia coli* [3]. ArgK is a coupled ATPase of the lysine, arginine and ornithine (LAO) transport system [5], suggesting the possibility that MMAA is a component of a transporter or an accessory protein that is involved in the translocation of Cbl into mitochondria [3].

MCM and a homolog of *MMAA* (*MeaB*) are present in the genome of the methylotrophic bacterium *Methylobacterium extorquens* AM1 [6]. This bacterium synthesizes AdoCbl and utilizes AdoCbl as the cofactor for MCM and *meaA* (ethylmalonyl-CoA mutase). The mutation in *meaB* has no effect on the function of *meaA*. However, total MCM activity was significantly reduced in *meaB* mutants compared with that of the wild-type, although the *meaB* mutants synthesized AdoCbl [7]. These findings suggest that *meaB* is not involved in the biosynthesis of AdoCbl or the transport of Cbl in *M. extorquens*.

Diol and glycerol dehydratases, Cbl-dependent enzymes, undergo concomitant irreversible inactivation by glycerol during catalysis [8]. This inactivation involves irreversible cleavage of the Co-C bond of AdoCbl, forming 5′-deoxyadenosine and an alkylcobalamin-like species. The Cbl species that is formed remains tightly bound to the enzyme, inactivating the enzyme irreversibly. Diol or glycerol dehydratase-reactivating factor participates in reactivation of the inactivated holoenzymes by mediating ATP-dependent exchange of the modified coenzyme for free intact coenzyme [9–12].

Korokova and Lidstrom [7] found that *meaB* forms a complex with MCM and stimulates MCM activity *in vitro*. However, neither cyanocobalamin (CNCbl) nor AdoCbl is released from MCM in the presence of *meaB*. Further, purified *meaB* did not restore MCM activity in the extracts prepared from the *meaB* mutant. Therefore, they proposed that *meaB* does not function as a reactivating factor, but is involved in protection of MCM from suicide inactivation by possibly providing a stabilization function. However, recently, it has been reported that *meaB* involved in AdoCbl trafficking to MCM functions as an editor, discriminating between inactive and active cofactor forms and permitting transfer only of AdoCbl, in a process that is gated by GTP hydrolysis [13]. Furthermore, the editing function of *meaB* is also used for reactivating inactivated MCM formed occasionally during turnover. Takahashi-Íñiguez *et al.* [14] have reported that human *MMAA* plays as two important roles as both the "protectase" and reactivase of MCM. In *E. coli*, sleeping beauty mutase (sbm; MCM homolog) interacts with *ygfD* (the *MMAA* or *meaB* homolog) [15].

Euglena gracilis Z, a photosynthetic flagellate, possesses characteristics of both plant and animal cells [16] and requires Cbl for its growth [17,18]. It has been reported that *E. gracilis* has Cbl-dependent methionine synthase and ribonucleotide redactase [19,20]. Furthermore, the purification

of aquacobalamin reductase form *E. gracilis* was reported [21]. Recently, we reported that *E. gracilis* MCM is a homodimer and is present in mitochondria [22]. Further, we isolated a full-length *E. gracilis* MCM cDNA whose deduced amino acid sequence indicates that it is a mitochondrial protein. These findings suggest the possibility that *E. gracilis*, human and *M. extorquens* MCMs share molecular properties. To explore the function of *MMAA*, we isolated a cDNA encoding *MMAA* from *E. gracilis* and measured MCM activity in *E. coli* that expressed MCM alone or together with *MMAA*.

2. Results and Discussion

2.1. Isolation and Characterization of a cDNA Encoding MMAA

To isolate a cDNA encoding *E. gracilis MMAA*, a BLASTP search was performed against the *Euglena* EST data base [23] using the amino acid sequence of human *MMAA* [3] as a query. This search revealed an EST clone (cluster ID ELL00001639) encoding a putative *E. gracilis MMAA*. Similar to other *MMAA* or *meaB*, this EST clone is annotated as a putative periplasmic protein kinase ArgK. The corresponding full-length cDNA was cloned using the rapid amplification of cDNA ends method, and its sequence was submitted to the DDBJ/GenBank/EMBL data base (accession number AB772316). In *E. gracilis*, the short sequences present at the 5′ end of small cytoplasmic mRNAs [24–26] are transferred to pre-mature mRNAs by a *trans*-splicing mechanism. The presence of a spliced leader sequence at the 5′ end of *E. gracilis MMAA* cDNA (5′-TTTTTTTTCG-3′) indicated that full-length *MMAA* cDNA was obtained. The cDNA contains 1,400 bp with an open reading frame of 1,056 bp predicted to encode 352 amino acids residues (with a calculated molecular mass of 39113.9 Da).

The deduced amino acid sequence of the *E. gracilis MMAA* cDNA shows 79%, 79%, 79% and 80% similarity to human (NP_758454), mouse (NP_598584), *Danio rerio* (NP_001098582) *MMAAs* and *M. extorquens* (AAL86727) *meaB*, respectively (Figure 1). The *E. gracilis MMAA* cDNA encodes a predicted protein of 352 amino acid residues and includes Walker A and Walker B motifs, as well as a GTP-binding sequence [3]. The presence of a signal peptide and cleavage sites was predicted by the TargetP prediction program [27]. However, the cleavage site is present in the relatively well-conserved region among the *MMAAs* of vertebrates (Figure 1), suggesting the possibility that the *MMAA* cleavage site in *E. gracilis* is located closer to its N-terminal region.

### 2.2. Expression of MMAA by E. gracilis

Although it is known that the levels of MCM protein and total MCM activity in the livers of Cbl-deficient rats are higher than those in the livers of rats whose diet was supplemented with Cbl, the level of the MCM transcript in the livers of rats fed a Cbl-deficient diet was lower than that in the livers of rats fed Cbl-supplemented diets [28]. To explore the regulation of the expression of *MCM* and *MMAA*, we analyzed the levels of their respective transcripts in response to Cbl in Cbl-deficient *E. gracilis*.

Cells cultured (1 mL) for one week were transferred to CNCbl-free medium, cultured for another week and then transferred (1 mL) to CNCbl-free medium. After three days, CNCbl (5 µg/mL) was added, and the cells were cultured for another three days (closed circles; Figure 2). Under the Cbl deficient condition, the growth of *E. gracilis* cells was highly suppressed (open circles; Figure 2). The addition of CNCbl increased the cell growth.

Figure 1. Comparison of the deduced amino acid sequences of *MMAA* from *E. gracilis*, human, mouse, *D. rerio* and *meaB* from *M. extorquens*. Sequences were aligned using ClustalW [29]. Sequence motifs defining the G3E family of proteins are indicated, including the GxxGxGK[S/T] Walker A and the DHbHbHbHbE Walker B motif. Hb denotes hydrophobic residues and an [N/T]KxD GTP-binding motif, as described by Leipe *et al.* [30], is underlined. Red letters indicate the mitochondrial leader sequence. Amino acids that are identical in all species are shaded gray.

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E.gracilis    ----------------------------------------------------------
human         --MPMLLPHPHQHFLKGLLRAPFRCYHFIFHSSTHLGSGIPCAQPFNSLGLHCTKWMLLS 58
mouse         MTISTLLLSPNRRLLTCLSRVPS---PWLLHSS-HPAPGPPGALP-NCFGHHCTKRVLLS 55
D.rerio       -------MRPSR-VFSILHHVAA-------FSSIHSTTALRHSLCKPSVNLRYAS--LCT 43
M.extorquens  ----------------------------------------------------------

E.gracilis    --------MQRRPCKNLVHMGQRLRCTSAELYARLQAGERRALGQALTLVESNHMEHRQQ 52
human         DGLKRKLCVQTTLKDHTEGLSDKEQRFVDKLYTGLIQGQRACLAEAITLVESTHSRKKEL 118
mouse         DGFRRTLCVQATLKDHTEGLSDKEQRFVDRLYTGLVKGQRACLAEAITLVESTHTRKREL 115
D.rerio       TQQTRSLSTETAISHHISDLTDREKRLLTKLYDGLIGGRRAALAESITLVETQHPRKKEL 103
M.extorquens  ---------------MSATLPD-----MDTLRERLLAGDRAALARAITLAESRRADHRAA 40

E.gracilis    ADALLLKALECERR----------CLRLGISGPPGAGKSTFIDAMGMYLVSLGLRVGVFA 102
human         AQVLLQKVLLYHREQEQSNKGKPLAFRVGLSGPPGAGKSTFIEYFGKMLTERGHKLSVLA 178
mouse         AQVLLQRVLALQREQELRNQGKPLTFRVGLSGPPGAGKSTFIECFGKMLTEQGHRLSVLA 175
D.rerio       AQVLLQRVLAFRQEQERSGGKPVAFRVGLSGPPGAGKSSFIEVIGKMLTGKGHKVSVLA 163
M.extorquens  VRDLIDAVLPQTGR----------AIRVGITGVPGVGKSTTIDALGSLLTAAGHKVAVLA 90
                                            Walker A

E.gracilis    VDPTSVKHGGSVLGDKTRMHRLAQEPRAFVRPSPSKGHLGGVTANAYESTLLFEVAGYDV 162
human         VDPSSCTSGGSLLGDKTRMTELSRDMNAYIRPSPTRGTLGGVTRTTNEAILLCEGAGYDI 238
mouse         VDPSSCTSGGSLLGDKTRMIELSRDMNAYIRPSPTSGTLGGVTRTTNEAIVLCEGGGYDI 235
D.rerio       VDPSSCTTGGSLMGDKTRMTELSRDMNAYIRPSPTSGTLGGVTRTTNEAIVLCEGAGYDI 223
M.extorquens  VDPSSTRTGGSILGDKTRMARLAIDRNAFIRPSPSSGTLGGVAAKTRETMLLCEAAGFDV 150
                                                                      Walker B

E.gracilis    VLIETVGVGQSEVAVADLADCFLFLIPPSSGDELHGIKKGIVEVSDILCITKAD-GKTLP 221
human         ILIETVGVGQSEFAVADMVDMFVLLLPPAGGDELQGIKRGIIEMADLVAVTKSD-GDLIV 297
mouse         ILIETVGVGQSEFAVADMVDMFVLLLPPAGGDELQGIKRGIIEMADLVVITKSD-GDLIV 294
D.rerio       VLVETVGVGQSEFAVADMVDMFVLLIPPAGGDELQGIKRGIIEMADLVVVTKSD-GELVV 282
M.extorquens  ILVETVGVGQSETAVADLTDFFLVLMLPGAGDELQGIKKGILELADMIAVNKADDGDGER 210
              Walker B                                       GTP binding

E.gracilis    QAEKALDQYSSAMRLLYSTGDKWEKRVLLTSAKEPALLKQVWEEVQRHQEVMRQEGEFAR 281
human         PARRIQAEYVSALKLLRKRSQVWKPKVIRISARSGEGISEMWDKMKDFQDLMLASGELTA 357
mouse         PARRIQAEYVSALKLLRRRSEVWRPKVIRISARSGEGITEMWDTMREFQHQMLASGELAA 354
D.rerio       PARKIQAEYTSALKLLRKKSKVWKPKVVRISSQTGQGVPELWETMLHFREEMLSSGELQV 342
M.extorquens  RASAAAASEYRAALRILTPPSATWTPPVVTISGLHGKGLDSLWSRIEGHRSKLTATGEIAG 270

E.gracilis    RREAQRQKQLWNNIQAEVVHRLQTLPSVQGVMGQLERQVLRGTVTPRRACYQILDLPLRP 341
human         KRRKQQKVWMWNLIQESVLEHFRTHPTVREQIPLLEQKVLIGALSPGLAA----DFLLKA 413
mouse         KRQTQHKVWMWNLIQENVLEHFKTHPSIREQIPLMERKVLSGALSPGRAA----DLLLKA 410
D.rerio       RRQTQQKVWMWSLIQENALRHFQQHPAVRAELPELESRVTCGEISPGLAA----DLLLKV 398
M.extorquens  KRREQDVKWMWALVHERLHQRLVGSAEVRQATAEAERAVAGGEHSPAAGA----DAIATL 326

E.gracilis    LRQAMMEGCSL 352
human         FKSRD       418
mouse         FKSRH       415
D.rerio       FTDTQ       403
M.extorquens  IGL         329
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Figure 2. Effect of the addition of CNCbl on the growth of Cbl-deficient *E. gracilis* cells. Open circles are Cbl-deficient cells. Closed circles are Cbl-supplemented cells. The data are the mean value ± SD of three individual experiments. Asterisks indicate that the mean values are significantly different compared with those of Cbl-deficient *E. gracilis* cells ($p < 0.05$).
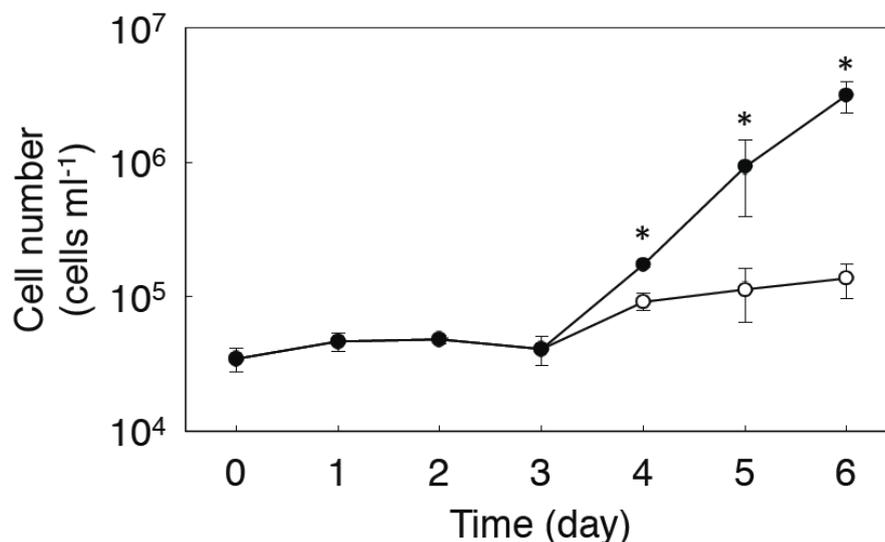
In three-day cultured Cbl-deficient *E. gracilis* cells, total MCM activity was 8.54 nmol min^{-1} mg^{-1} protein. At three days after the addition of Cbl to Cbl-deficient cells, total MCM activity was decreased to 38%, while that in the Cbl-deficient cells remained high (89%). In Cbl-deficient *E. gracilis* cells, the level of the *MCM* transcript was 2.3-fold higher than that in the Cbl-supplemented *E. gracilis* cells (Figure 3).

In contrast, no significant differences in the levels of the *MMAA* transcript were observed between the Cbl-deficient and Cbl-supplemented *E. gracilis*, suggesting the possibility that *MMAA* is constitutively expressed these cells.

Figure 3. Semi-quantitative real-time (RT)-PCR analysis of the levels of *MCM* and *MMAA* transcripts in (1) Cbl-deficient and (2) Cbl-supplemented *E. gracilis* cultures. Fragments corresponding to *MCM*, *MMAA*, and *EF1-α* were amplified using PCR.
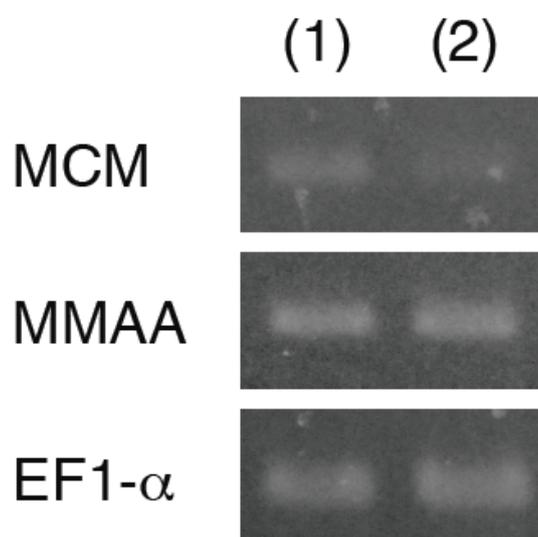
2.3. Effect of Coexpression of MMAA on MCM Activity in E. coli

As noted above, *MCM* activity is significantly decreased in *meaB* mutants of *M. extorquens* [7]. To examine the effect of the expression of *MMAA* on *MCM* activity, the expression vectors pET/Eg.MCM and pCDF/Eg.MMAA were transformed together or individually in *E. coli* using the expression vectors described below. In cells transformed with either the combinations of pET-16b and pCDF/Eg.MMAA or pET-16b and pCDF-1b, *MCM* activity was below the detection limit of the assay (5 pmol min^{-1} mg^{-1} protein) (Table 1). No significant difference in *MCM* activity was detected between cells expressing *MCM* alone or together with *MMAA*. We checked *E. gracilis* MMAA protein in *E. coli* cells using anti His-tag antibody. *E. gracilis* MMAA protein was slightly expressed in the soluble fraction in the pCDF-1b/Eg.MMAA transformed *E. coli*, suggesting that the expression level of *E. gracilis* MMAA protein was very low.

Table 1. *MCM* activity in *E. coli* expressing *MCM* or *MMAA* together or individually.

Vector pairs	MCM activity (nmol min^{-1} mg^{-1} protein)
pET/Eg.MCM - pCDF-1b	12.1 ± 2.0
pET-16b - pCDF-1b/Eg.MMAA	< 0.005
pET-16b - pCDF-1b	< 0.005
pET/Eg.MCM - pCDF/Eg.MMAA	11.6 ± 1.8

Data are mean values ± standard deviation from three assays.

E. coli sbm and *ygfD* genes are contained in an operon comprising *sbm-ygfD-ygfG-ygfH* [31]. *E. coli sbm* forms a homodimer [14] as its *E. gracilis* homolog. Further, the deduced amino acid sequence of *E. coli sbm* is 89% similar to that of *E. gracilis MCM*. These findings suggest the possibility that *ygfD* functions to protect *E. gracilis MCM* from inactivation and acts to reactivate it in *E. coli*. Previous findings, and our presented data suggests the possibility that the function of *E. gracilis MMAA* was similar to those of human *MMAA* and *meaB*.

In *Caenorhabditis elegans*, the suppression of *MMAA*, *MMAB* [co(I)balamin adenosyltransferase] or *MCM* expression using RNAi technology caused the increase of methylmalonic acid **[32]**. To clarify the Cbl metabolism, including the function of *MMAA* in *E. gracilis*, analyses using recombinant proteins and RNAi technology are necessary.

3. Experimental Section

3.1. E. gracilis and Culture Conditions

E. gracilis strain *Z* was grown in Koren–Hutner medium under continuous illumination at a photosynthetic photon flux density of 24 μmol m^{-2} s^{-1} at 26 °C until the cells reached stationary phase (6 days) [33]

3.2. RT-PCR-Amplification and Sequence Analysis

Based on analysis of the *Euglena* EST database using the Protest EST Program [23], a gene-specific primer was designed to amplify the missing 3′ and 5′ ends of the *MMAA* EST sequence

(ELL0001639). Total RNA was isolated from *E. gracilis* using Sepasol-RNA I (Nakalai Tesque, Kyoto, Japan). The primers 5′-TGGTGGAGTCAAACCACATG-3′ and 5′- TCAGACACTTCCACAATGCC -3′ were used sequentially for 3′-rapid amplification of cDNA ends (3′-RACE) or 5′-RACE with a GeneRacer^TM Kit (Life Technologies, Carlsbad, CA, USA). Amplified fragments were cloned into pSTBlue-1, and the nucleotide sequences of the 3′- or 5′-extended fragments were determined using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The full-length coding sequence of *Euglena MMAA* was amplified using PCR primed by Eg.MMAA-F (5′-ATTCCATGCAACGCCGCCCA-3′) and Eg.MMAA-R (5′-CCACGTATGCTTTCCTCCTT-3′). PCR amplification was carried out using PrimeSTAR (Takara Shuzo, Kyoto, Japan). A single-stranded cDNA prepared from 3-day *Euglena* cultures was used as template. The amplified product was cloned into pZErO-2 (Life Technologies), and the sequence was verified using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

3.3. Semi-Quantitative RT-PCR Analysis

Semi-quantitative RT-PCR analysis was performed according to Ishikawa *et al.* [22]. Primer pairs were as follows: EF1-α-F (5′-GTTGACCCTCATTGGTGCTT-3′); EF1-α-R (5′-CTTGGTCACCTTCCCAGTGT-3′); Eg.MCM-F (5′-GTCCATCGACAACACCATTG -3′); Eg.MCM-R (5′-CGACTTCAGCTCGTTGATGA -3′); and Eg.MMAA-F (5′-ATTCCATGCAAC GCCGCCCA-3′); Eg.MMAA-R (5′-TCAGACACTTCCACAATGCC-3′). The experiments were repeated at least three times with cDNA prepared from three batches of *E. gracilis* cultures. The quantitative intensity was determined by applying densitometry to images of the blots [34].

3.4. Construction of MMAA *and* MCM *Expression Plasmids*

To construct *MMAA* and *MCM* expression vectors, their open reading frames were amplified from the first strand cDNAs, using the primer sets as follows: MMAA-*Pma* CI-F (5′-TCACCACC ACCATCACGTGATGCAACGCCGCCCATGCAA-3′), MMAA-*Pma* CI-R (5′-TCGAACCGGTAC CCACGTGCTACAGGCTGCAGCCCTCCA-3′), MCM-*Nde* I-F (5′-CATATGATCGACCTTCCA CCAAAGTG-3′) and MCM-*Bam* HI-F(5′-GGATCCTCAGAGTTTGTTCAGGACCT-3′). The amplified DNA fragments were ligated into the vector pZErO-2 (Life Technologies), and the sequence was verified using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). The resulting constructs were digested with *Pma* CI for *MMAA* or *Nde* I/*Bam* HI for *MCM* and ligated into the expression vectors pCDF-1b (Novagen, Madison, WI) or pET16b (Novagen) to produce histidine-tagged proteins. The resulting constructs were designated pCDF/Eg.MMAA and pET/Eg.MCM, respectively.

3.5. Expression of Recombinant MMAA or MCM Proteins

To explore the effect of the expression of Eg.MMAA on MCA activity in *E. coli*, four plasmids sets (pET16-b and pCDF-1b, pET/Eg.MCM and pCDF-1b, pET16-b and pCDF/Eg.MMAA and pET/Eg.MCM and pCDF/Eg.MMAA) were introduced into *E. coli* strain BL21 Star (DE3) (Life Technologies). Transformed *E. coli* were cultured in 50 mL LB medium supplemented with ampicillin (50 µg mL^{-1}) and chloramphenicol (34 µg mL^{-1}) at 37 °C overnight. The culture was then

transferred to Luria–Bertani medium (1 l). When the culture reached an absorbance of 0.6 at 600 nm, 0.4 mM isopropyl β-D-thiogalactopyranoside was added, and the bacteria were grown for another 6 h at 37 °C. The cells were harvested by centrifugation at $6,000 \times g$ for 10 min, and the pellets were kept frozen at −20 °C.

3.6. Enzyme Extraction and Assay

E. coli pellets were resuspended in 100 mM Tris-HCl, pH 7.5, sonicated (Tomy, Tokyo, Japan) (10 kHz) using 20 s strokes with 30 s intervals and centrifuged at $15,000 \times g$ for 15 min. *MCM* activity was assayed by a modified HPLC method described by Gaire *et al.* (1999). Briefly, the assay mixture (0.15 mL) for determining total *MCM* activity contained 100 mM potassium phosphate buffer (pH 7.5), 30 μM AdoCbl, 0.15 mM (*R, S*)-methylmalonyl-CoA and enzyme. The components, except for (*R, S*)-methylmalonyl-CoA, were mixed in microcentrifuge tubes in the dark, and the temperature was equilibrated by incubation in a heating bucket (e-Heating Bucket EHB, TAITEC Cop., Saitama, Japan) maintained at 3 °C. The reaction mixture was preincubated for 5 min, and the reaction was started by the addition of (*R, S*)-methylmalonyl-CoA and maintained for 5 min. The enzyme reaction was stopped by the addition of 50 μL of 10% (w/v) trichloroacetic acid. The reaction mixture was filtered through a 0.45 μM membrane filter (Millex Syringe Driven Filter Unit, LH-type, Milli-pore, USA). An aliquot (20 μL) of the filtrate was analyzed using a Shimadzu HPLC apparatus (two LC-10ADvp pumps, DGV-12A degasser, SCL-10Avp system controller, SPD-10Avvp ultraviolet-visible detector, CTO-10Avp column oven, 100 μL sample loop and C-R6A Chromatopac Integrator). The sample (20 μL) was loaded onto a reversed-phase HPLC column (Cosmosil 5C18-AR-II, Φ3.0 × 150 mm) equilibrated with 50% solvent A (100 mM acetic acid in 100 mM potassium phosphate buffer, pH 7.0) and 50% solvent B [18% (v/v) methanol in solvent A]. (*R, S*)-methylmalonyl-CoA and succinyl-CoA were eluted with a linear gradient of methanol (50%–100% solvent B) for 7.0 min at 40 °C and monitored by measuring the absorbance at 254 nm. The flow rate was 1.0 mL min^{-1}. *MCM* activity was calculated from the amount of succinyl-CoA formed. Protein concentrations were determination using a Quant-iT™ Protein Assay and a Qubit fluorometer (Life Technologies).

4. Conclusion

E. gracilis MMAA cDNA contained an open reading frame encoding the protein of 352 amino acids with a calculated molecular mass of 39113.9 Da, preceded by a putative mitochondrial targeting signal consisting of 41 amino acid residues. No significant differences in the levels of the *MMAA* transcript were observed between the Cbl-deficient and Cbl-supplemented *E. gracilis*. No significant difference in MCM activity was observed between *E. coli* that expressed either MCM together with MMAA or expressed MCM alone. Previous findings, and our presented data suggests the possibility that the function of *E. gracilis MMAA* was similar to those of human *MMAA* and *meaB*.

Conflict of Interest

The authors declare no conflict of interest.

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