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Evidence from ^{18}O feeding studies for hydroxyl group donor in the reaction catalyzed by cytidylate hydroxymethylase MilA

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5-Hydroxymethylcytosine (5hmC) was present in T-even phage and mammalian DNA. 5hmC in phage is formed by hydroxymethylation of the cytosine base in deoxycytidylate (dCMP) by deoxycytidylate hydroxymethylase (CH), which uses the solvent water as the hydroxyl group donor. By contrast, 5hmC is formed in mammal zygotes by the oxidation of 5-methylcytosine (5mC). 5hmC was also present in a nucleoside antibiotic mildiomycin and its formation is governed by a cytidylate hydroxymethylase MilA. However, the catalytic mechanism remains unknown. In the present study, we purified His-tagged MilA and fed its *in vitro* reaction with $H_2{}^{18}O$. The LC-MS analysis of the product revealed that ^{18}O was incorporated into the hydroxymethylated CMP (HmCMP), and the secondary MS result of ^{18}O-labeled HmCMP indicated that ^{18}O was incorporated into the cytosine of HmCMP. The results demonstrate that MilA uses solvent water as the hydroxyl group donor like CH. Moreover, Thr102 of MilA was predicted as potential critical amino acid anchoring one molecule of water for hydroxylation. Finally, organizational context comparison in microbial genomes reveals that six homologous ORFs originally annotated as putative thymidylate synthase (TS) are more likely to be CMP hydroxymethylase.

5-hydroxymethylcytosine, hydroxymethylase, $H_2{}^{18}O$ feeding, mildiomycin

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Since the discovery of the "sixth base", 5-hydroxymethylated cytosine (5hmC) in mammalian DNA, the enzymology behind the formation of this new base has drawn world-wide attention recently. Such an attention stems from Rao and coworkers' discovery via bioinformatics analysis that 5hmC is formed post-replicatively by Tet1-catalyzed oxidation of 5-methylated cytosine (5mC) [1]. 5hmC in DNA could be further oxidized by Tet3 dioxygenases *in vitro* to 5-carboxylcytosine that is specifically recognized and excised by thymine-DNA glycosylase (TDG), and therefore Tet3-mediated DNA hydroxylation is involved in epigenetic reprogramming of the zygotic paternal DNA following natural fertilization [2]. At present, all known TET enzymes (TET1-3) are Fe(II)- and 2-ketoglutarate-dependent oxidases, which efficiently convert 5mC to 5hmC both *in vitro* and *in vivo* [3].

Aside from functioning as the epigenetic regulator in the mammals, 5hmC was first identified in bacteriophage genomes as a strategy to evade bacterial restriction endonucleases [4]. Moreover, the hydroxymethylation is catalyzed by dCMP hydroxymethylase (CH), which transfers methylene group from methylenetetrahydrofolate (CH_2THF) to C_5 of dCMP and then uses water molecule to hydrate the methylene group to form hydroxymethyl-dCMP (HmdCMP), one precursor for phage DNA replication [5].

Besides the occurrence of 5hmC on the chromosomal

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DNA, several nucleotide-derived nucleoside antibiotics also feature modified base such as hydroxymethyl pyrimidine in polyoxin, and hydroxymethyl cytosine in mildiomycin. SAV_4805 of *Streptomyces avermitilis*, showing similarity to thymine-7-hydroxylase, was proved responsible for the hydroxyl group incorporation *in vivo* [6].

Recently, we identified a CMP hydroxymethylase named MilA in the biosynthetic pathway of nucleoside mildiomycin in *Streptoverticillum rimofaciens* ZJU5119. *In vitro*, it can convert CMP in the presence of tetrahydrofolate (THF) to 5-hydroxymethyl-CMP (HmCMP) that is immediately hydrolyzed into free 5hmC by MilB (Figure 1) [7]. As the THF is always the carrier of the methyl group for the methylation, *in vitro* assay of MilA with CMP could not reveal the mechanism by which 5-mCMP is oxidized to 5-hmCMP by MilA. In mammalian cells, 5hmC was formed by combined function of Tet proteins and methyltransferase. Tet proteins are responsible for the oxidation of 5mC to 5hmC after DNA replication. In contrast, CH and MilA can individually catalyze dCMP and CMP to 5-hmCMP and 5-hmdCMP, respectively [7,8]. The oxidation at C_5 of the cytosine for the above two enzymes was believed to share similar mechanism. However, MilA and CH share only 26% amino acid identity, despite the subtle difference in the substrates' structure. We investigated and here reported the mechanism of hydroxylation of CMP by feeding experiment *in vitro*.

E. coli BL21(DE3) pLysE cells carrying pJTU2955 (*milA* on pET28a with His-tag at carboxyl end) [7] were grown overnight at 37°C in LB media supplemented with chloramphenicol (34 μg/mL) and kanamycin (50 μg/mL). The seed cultures (10 mL) were used to inoculate 1 L production cultures of LB with the corresponding antibiotics. The cells were grown at 37°C to an optical density at OD_{600} of 0.5–0.6 and then induced with isopropyl-β-D-thiogalactopyranoside (IPTG, 0.8 mmol/L). The cultures were then grown for an additional 5 h at 30°C. After centrifugation, the cells were resuspended in Binding Buffer (40 mL, 20 mmol/L Tris-HCl, 0.5 mol/L NaCl, pH 7.4) and lysed by sonication in an ice bath (120×5 s at 20 W with 10 s pauses). After centrifugation (12000×*g* for 45 min at 4°C), the supernatant was purified with Ni-NTA His Bind Resin (Novagen, USA) as described by the manufacturer.

Briefly, 2 mL of His Bind Resin was transferred to a 10 mL polypropylene column and settled under gravity flow. The resin was washed with 3 mL of 0.2 mmol/L Ni_2SO_4 and subsequently equilibrated with 4 mL Binding Buffer. The column was loaded with 30 mL prepared cell extract, and then washed by 8 mL Wash Buffer (20 mmol/L Tris-HCl, 0.5 mmol/L NaCl, 120 mmol/L imidazole, pH 7.4). Finally, the target protein was eluted from column with 8 mL Elute Buffer (20 mmol/L Tris-HCl, 0.5 mmol/L NaCl, 300 mmol/L imidazole, pH 7.4). The column was washed with 8 mL 500 mmol/L imidazole and 8 mL deionized water and filled with 20% ethanol. The purified His-tagged MilA was desalted and concentrated with centrifugal filter devices (Amicon Ultra-15, 10000 MWCO, Merck Millipore Corp, Germany) and stored in Tris-HCl buffer (50 mmol/L, pH 7.5) with glycerol (20%) at −80°C. The expression and purification of His- tagged MilA were analyzed by 12% SDS-PAGE, and protein concentration was determined using the Bradford Protein Assay Kit (Bio-Rad, USA).

The induced and non-induced expression products were analyzed by SDS-PAGE. It revealed that the recombinant protein His-tagged MilA was overexpressed and migrated as an intense band of 38 kD (Figure 2(a), while non-induced cells did not contain the new protein). Results showed that His-tagged MilA was purified efficiently by using the His Bind Kit. Most of the target protein was eluted by Elute Buffer containing 300 mmol/L imidazole, while most of non-specifically bound protein was washed out by Wash Buffer containing 120 mmol/L imidazole. And 500 mmol/L imidazole can only wash out little target protein (Figure 2(b)).

The standard *in vitro* reaction assay was started by adding purified MilA-C-His (1.6 μmol/L) to the reaction mixture (100 mmol/L Tris-HCl buffer, pH 7.5, 15 mmol/L paraformaldehyde, 50 mmol/L 2-mercaptoethanol, 2 mmol/L tetrahydrofolate and 1 mmol/L CMP, adding normal water or ^{18}O labeled water to 100 μL). After incubation for 1 h at 37°C, the reaction was terminated by Trichloroacetic acid (4%).

The reaction was analyzed by LC-MS (Agilent 1100 series LC/MSD Trap system, Agilent, Santa Clara, CA, USA).

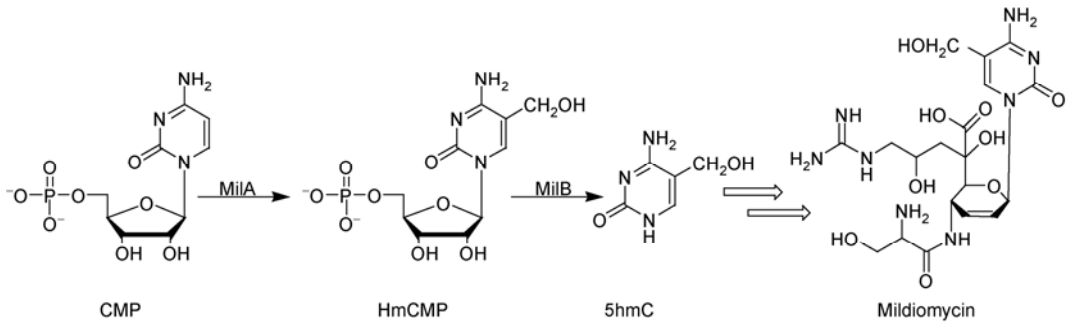

Figure 1 Initial steps of mildiomycin formation catalyzed by MilA and MilB.

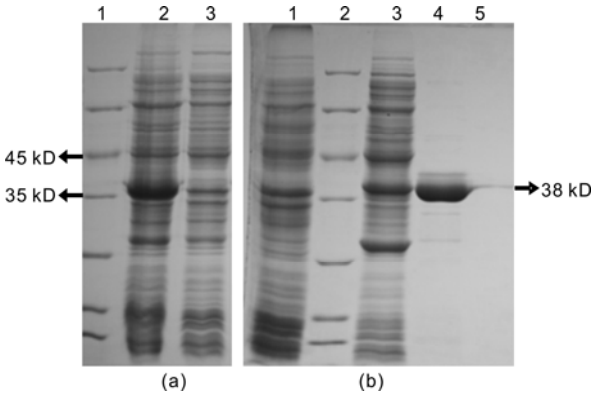

Figure 2 SDS-PAGE of the expressed product of MilA-C-His. (a) 1, Protein molecular weight marker, from the top: 116.0, 66.2, 45.0, 35.0, 25.0, 18.4, 14.4 kD; 2, pJTU2955 transformed BL21 (DE3) pLysE, induced; 3, pJTU2955 transformed BL21 (DE3) pLysE, non-induced. (b) 1, Cell extract through the column; 2, protein molecular weight marker, from the top: 116.0, 66.2, 45.0, 35.0, 25.0, 18.4, 14.4 kD; 3, protein washed out by 120 mmol/L imidazole; 4, target protein washed out by 300 mmol/L imidazole; 5, residue protein washed out by 500 mmol/L imidazole.

The HPLC was operated at a flow rate of 0.3 mL/min on an Agilent TC-C18 (4.6 mm×250 mm, 5-Micron) column. Solvent A was 20 mmol/L ammonium acetate, and solvent B was acetonitrile. The program used 2% solvent B over 30 min. The ion trap mass spectrometer was operated in the positive ion mode. Drying gas flow rate was 8 L/min, and nebulizer pressure was 206.8 kPa. Drying gas temperature was 350°C, and the fragmentation was between 1.0 to 1.8 V.

To test if the hydroxyl group of HmCMP comes from water molecule like CH, we added $H_2{}^{18}O$ to final concentration of 50% (v/v) into the reaction system of MilA, while in the control reaction only the same amount of $H_2{}^{16}O$ was added. The HPLC-MS result showed that in both reactions, HmCMP ($[M+H]^+$=354) was produced to a similar extent. In the control reaction, a relatively small naturally occurring isotope peak ($[M+H]^+$=356, indicated by an dashed arrow) of HmCMP is detected (Figure 3(b)). But in the $H_2{}^{18}O$-fed reaction, we detected a large peak with a molecular mass $[M+H]^+$ at *m/z* of 356 (Figure 3(c)). The high relative abundance suggests that this peak is not the naturally occurring isotope peak of HmCMP, and the hydroxyl group from ^{18}O labeled-H_2O is incorporated into HmCMP.

In the secondary MS fragmentation profile of HmCMP, there was a fragment of 142 ($[M+H]^+$) corresponding to hydroxymethylated cytosine, while the ^{18}O incorporated HmCMP resulted in a peak of 144 ($[M+H]^+$) with a high relative abundance, which is two protons larger than that of hydroxymethyl cytosine (Figure 4). This result indicated that the ^{18}O was incorporated in the base moiety of HmCMP. Taking together, we conclude that MilA uses H_2O as hydroxyl donor for the hydroxyl group formation.

Since MilA have similar mechanism as CH in hydroxyl group formation, it may belong to the superfamily of thymidylate synthase (TS) and cytidylate hydroxymethylase

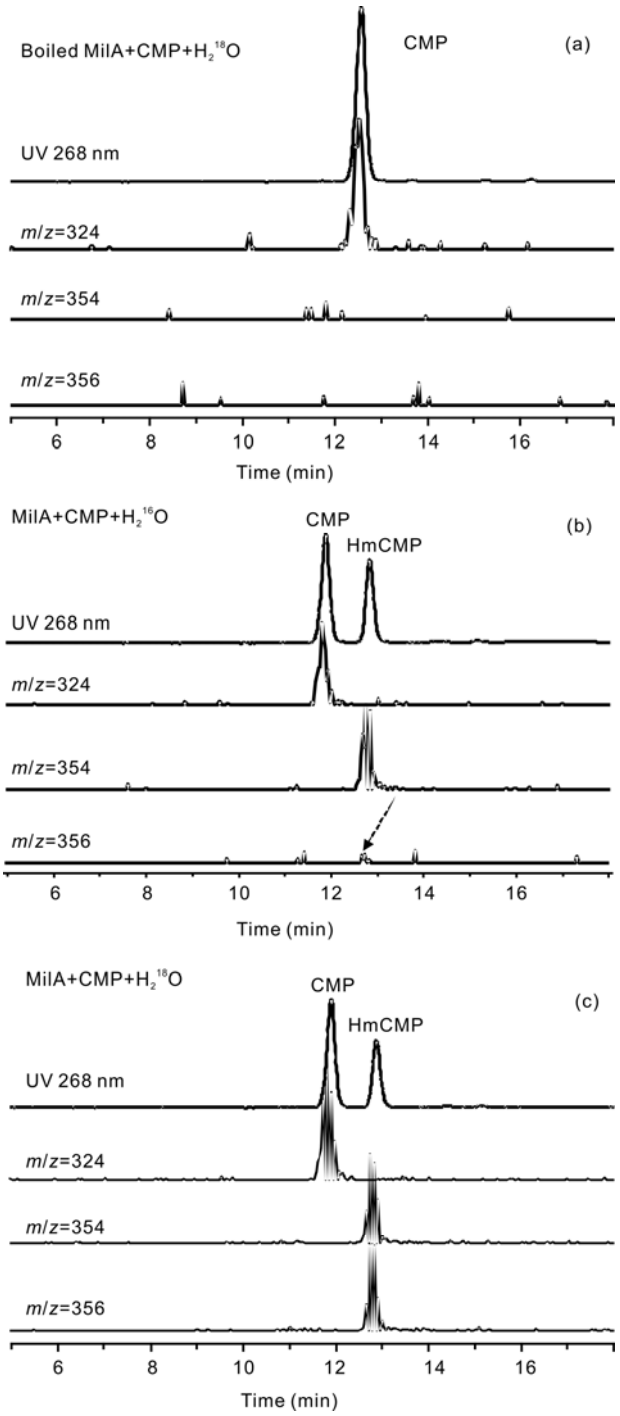

Figure 3 LC-MS analysis of reactions of MilA with CMP fed by $H_2{}^{16}O$ (b) or $H_2{}^{18}O$ (c) respectively. Extracted Ion Chromatogram at *m/z* 324 stands for the substrate CMP, 354 for normal product HmCMP and 356 for ^{18}O labeled HmCMP. The black dotted arrow indicates the naturally occurring isotope peak of HmCMP. In (a), boiled MilA can not react with CMP.

(CH). TS is one of the most conserved enzymes in nucleotide metabolism across species and phyla. It catalyzes the conversion of dUMP and CH_2THF to dTMP and dihydrofolate (DHF), which plays an important role in the *de novo* pathway for thymidylate synthesis and, hence, DNA syn-

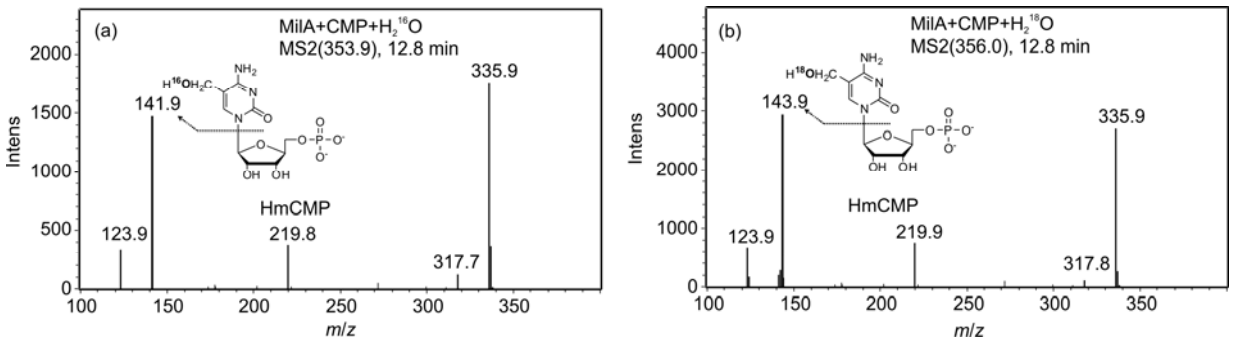

Figure 4 Secondary mass spectrum of HmCMP (a) and ^{18}O labeled-product HmCMP (b) respectively.

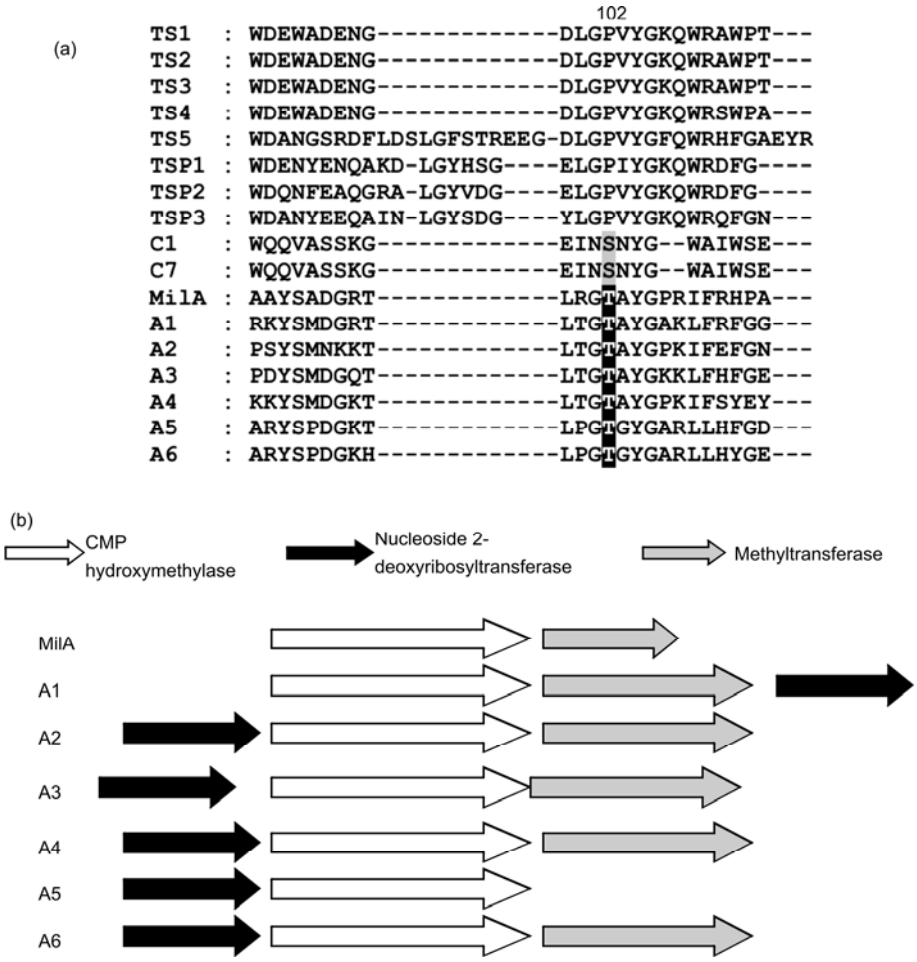

Figure 5 (a) Alignment of a segment of MilA and its homologous thymidylate synthases and cytidylate hydroxymethylases. Identical residues are indicated by black background. Residue Thr102 in black background is considered as critical in the hydroxylation process. Numbered residues correspond to MilA. TS1, *Escherichia coli*, GI: 15803346; TS2, *Shigella flexneri*, GI: 24114106; TS3, *Citrobacter youngae*, GI: 283835406; TS4, *Salmonella enterica*, GI: 16766303; TS5, *Enterobacter cloacae*, GI: 295097375; TSP1: *Enterobacteria* phage T4, GI: 9632831; TSP2, *Shigella* phage, GI: 308814595; TSP3, Aeromonas phage, GI: 37651687; C1, *Enterobacteria* phage T4, 9632636; C2, *Shigella* phage, GI: 330858570; MilA, *Streptomyces rimofaciens*, 167996982; A1, *Stigmatella aurantiaca*, 310823030; A2, *Bacillus amyloliquefaciens*, 154685031; A3, *Leuconostoc mesenteroides*, GI: 116618549; A4, *Clostridium botulinum*, GI: 170755095; A5, *Burkholderia pseudomallei*, GI: 167906492; A6, *Burkholderia oklahomensis*, GI: 167565755. (b) Representation of genomic organizational context of *milA* and its homologs in several different bacterial species. A1–A6 are the same as in (a).

thesis. Indeed, according to the crystal structure of CH from *Enterobacteria* phage T4, Ser94 was proposed to anchor the water molecule which would be incorporated into the substrate later. We compared MilA and its known homologous thymidylate synthases and cytidylate hydroxymethylases by MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/) to iden-

tify the presence of critical amino acid for hydroxylation in MilA. The multiple sequence alignment results show that Ser94 in CH was substituted by Thr102 in MilA and its homologs (Figure 5(a)) [9]. Like Ser, Thr also has a reactive hydroxyl group on the side chain and can also participate in hydrogen bonding with a water molecule. The conserved Ser and Thr in the hydroxymethylase may help us distinguish possible hydroxymethylase from methylase. In addition, MilA and its homologs containing Thr at this location all have a similar genomic organization (Figure 5(b)). Their genes were linked with *milB* and its homologs (nucleoside 2-deoxyribosyltransferase). This again supports the hypothesis that all of the MilA's homologs containing Thr corresponding to Ser94 in CH may be hydroxymethylase. Future site-directed mutation experiments are needed to confirm this hypothesis.

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