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Supplemental Information

Expanding the Structural Diversity of Drimentines by Exploring the Promiscuity of Two *N*-methyltransferases

Tingting Yao, Jing Liu, Enjing Jin, Zengzhi Liu, Huayue Li, Qian Che, Tianjiao Zhu, Dehai Li, and Wenli Li

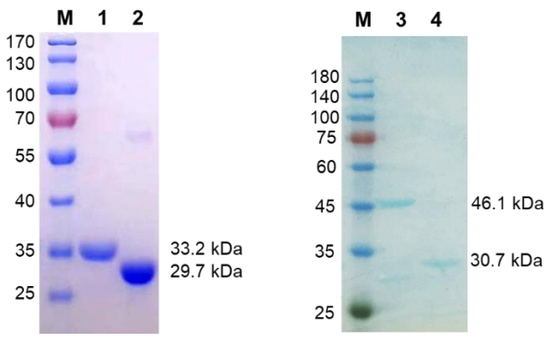

Figure S1. SDS-PAGE gels of DmtMT2-1 (lane 1), DmtMT1 (lane 2), DmtMT2-2 (lane 3) and DmtMT3 (lane 4), Related to Figures 2, and 4-5.

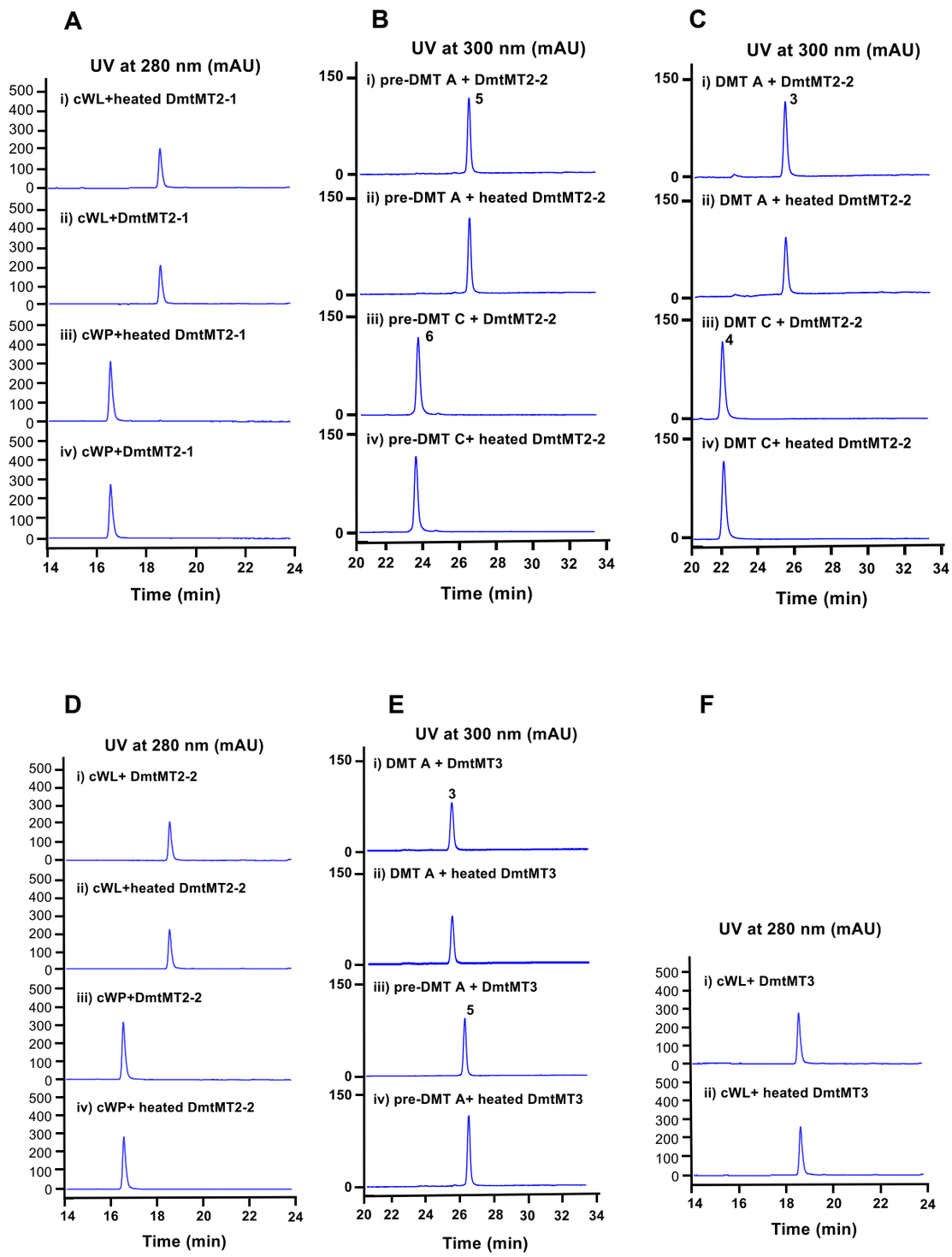

Figure S2. *In vitro* assays of DmtMT2-1, DmtMT2-2 and DmtMT3, Related to Figure 2.

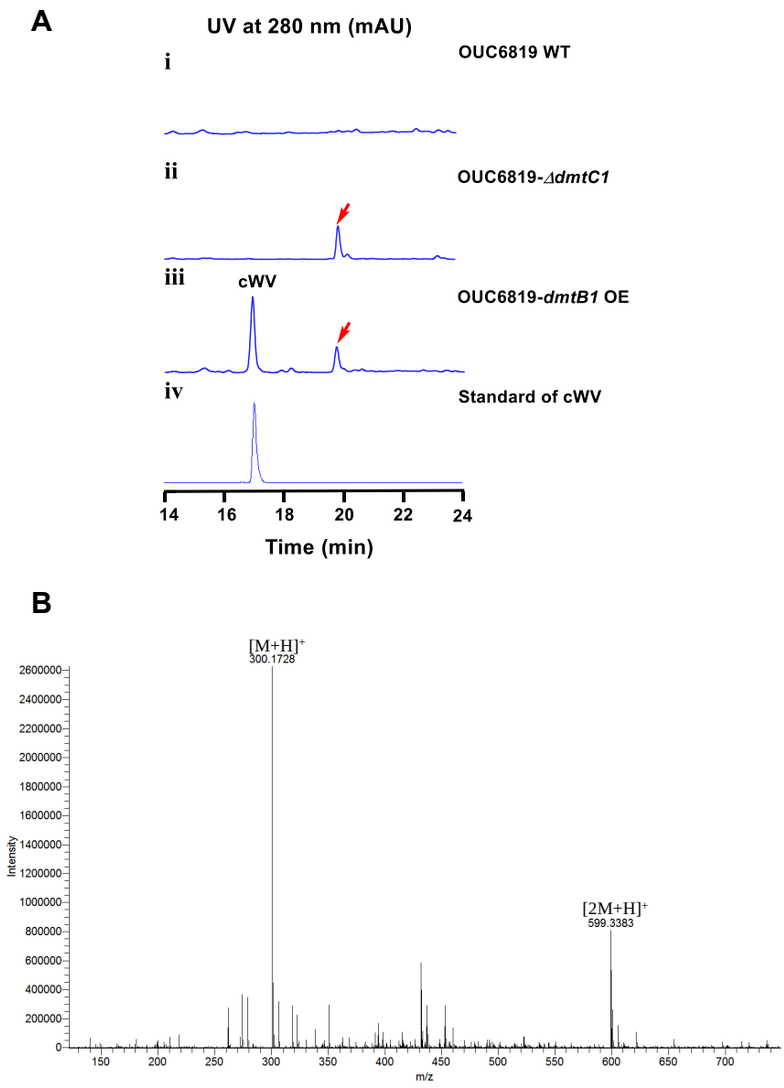

Figure S3. (A) HPLC traces of the fermentation products from (i) the wild-type OUC6819 strain; (ii) *ΔdmtC1*; (iii) overexpression of *dmtB1* in *S. youssoufiensis* OUC6819; (iv) standard of cWV. (B) The HR-ESI-MS spectrum of the compound peak indicated by red arrow, Related to Figure 3.

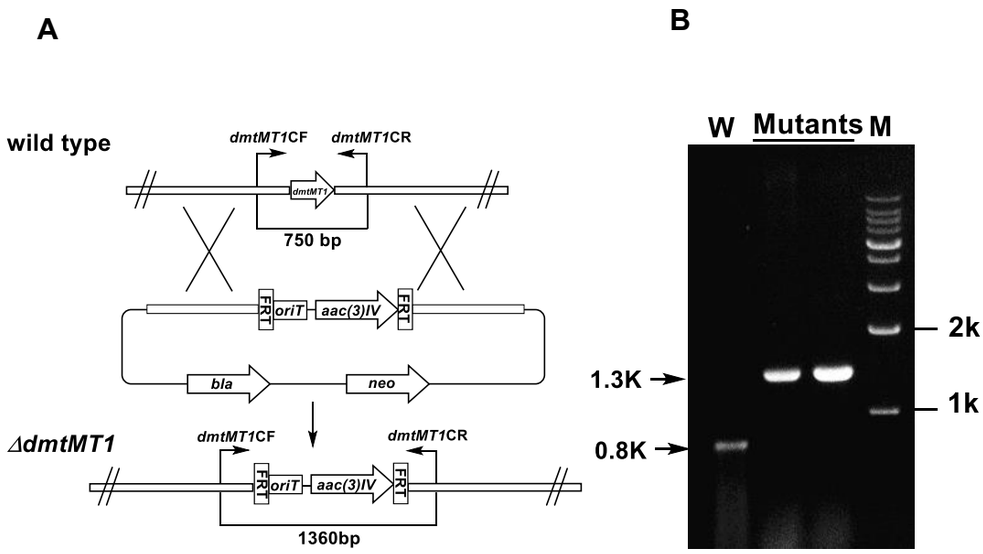

Figure S4. Inactivation of *dmtMT1*. (A) Construction of the Δ*dmtMT1* mutant. (B) PCR confirmation of the Δ*dmtMT1* mutant. M: DNA marker; W: *S. youssoufiensis* OUC6819 wild-type strain; Mutant: the Δ*dmtMT1* mutant, Related to Figure 3.

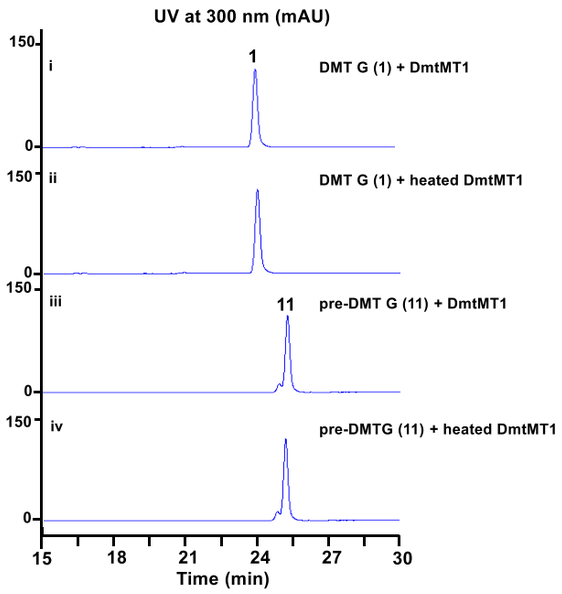

Figure S5. *In vitro* methyltransferase activity of DmtMT1 using DMT G (**1**) and pre-DMT G (**11**) as substrates, Related to Figure 4.

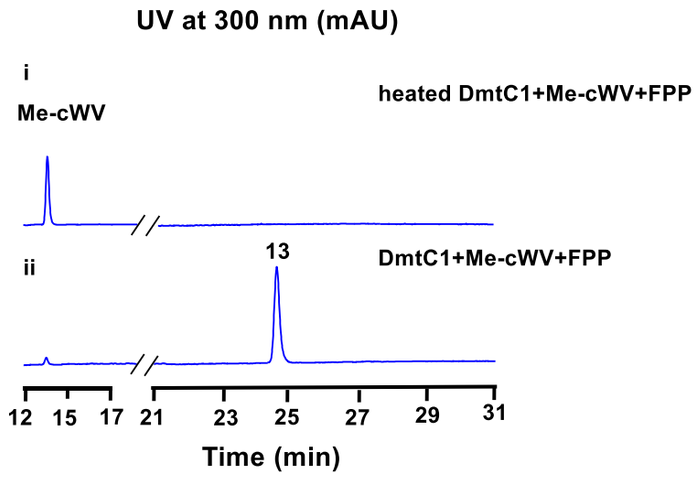

Figure S6. DmtC1-catalyzed reaction using Me-cWV and FPP as substrates, Related to Figure 4.

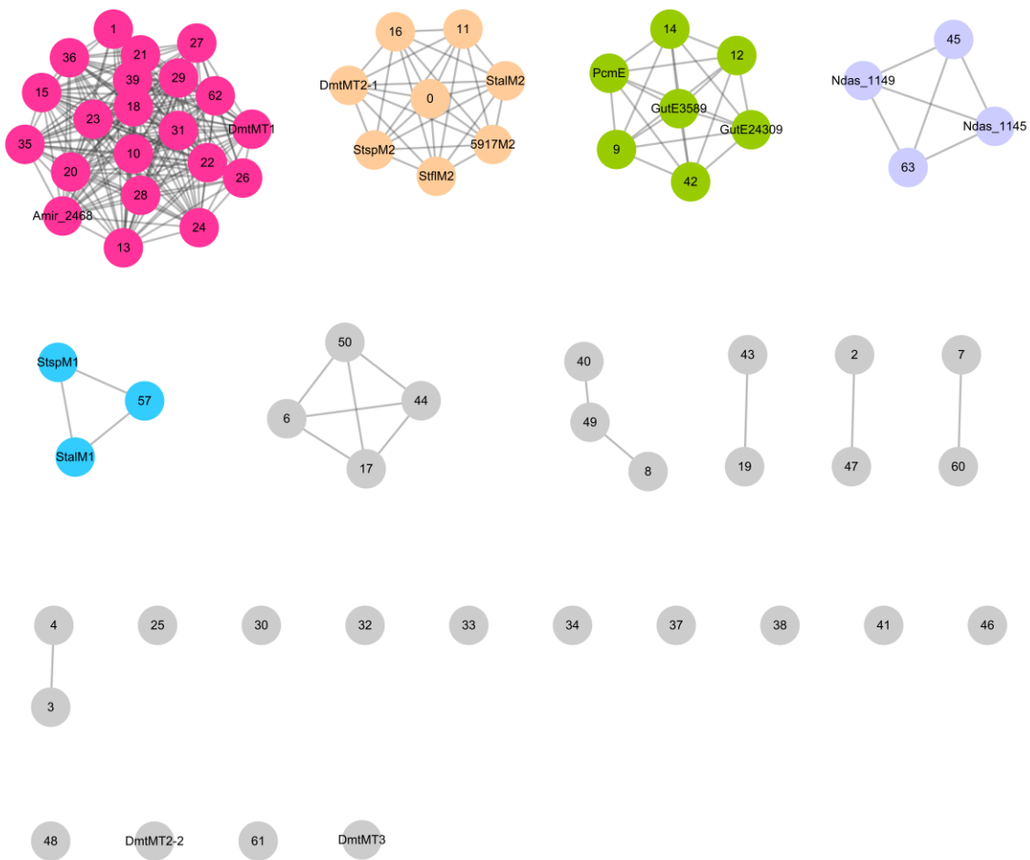

Figure S7. Sequence similarity networks (SSNs) of selected methyltransferases using an alignment score of 30. The details of the above proteins were shown in Data S3. Multiple MTs within this network are grouped with DmtMT1/Amir_4628 involved in the DKP-ring methylation. A couple of MTs clustered with known MTs are also included: four PcmE/GutE homologs (WP_078645497.1, WP_078897103.1, WP_078624486.1, and WP_023552492.1), two Ndas_1149/1145 homologs (WP_017534685.1 and WP_017534689.1), and one StspM1/StalM1 homolog (WP_027751607.1) , Related to Figure 1.

Table S1. Bacteria and plasmids used in this study, Related to Figures 2-6.

Strains or plasmids	Description	Reference or source
Strains		
E. coli Top10	Host strain of cosmid vector SuperCos1	Invitrogen
E. coli DH5*a*	Host strain for general cloning	Invitrogen
E. coli ET12567/pUZ8002	Host strain for conjugation	(Gust et al., 2003)
E. coli BW25113/pIJ790	Host strain for PCR-targeting	(Gust et al., 2003)
E. coli BL21-CodonPlus(DE3)	Protein expression host	Stratagene
E. coli BL21(DE3)	Protein expression host	Invitrogen
Streptomyces youssoufiensis OUC6819	Strain harboring the *dmt1* locus	(Che et al., 2012)
Streptomyces sp. NRRL F-5123	Strain harboring the *dmt2* locus	NRRL[a]
Streptomyces aidingensis CGMCC 4.5739	Strain harboring the *dmt3* locus	CGMCC[b]
ΔdmtMT1	*dmtMT1* inactivation mutant of *S. youssoufiensis* OUC6819	This study
Streptomyces coelicolor M1146	Host strain for heterologous expression	(Gomez‐Escribano and Bibb, 2011)
Plasmids		
SuperCosI	Amp^R, Kan^R, cosmid vector	Stratagene
pIJ773	Apr^R, source of *acc(3)IV-oriT* cassette	(Gust et al., 2003)
pIJ790	Cm^R, λ RED recombination plasmid	(Gust et al., 2003)
pSET152C	pSET152 derivative, with insertion of the *neo* gene from SuperCos1 at the sites of *Apa*I and *Sgr*AI	(Yao et al., 2018)
pIJ10500	Hyg^R, integrative plasmid containing the ϕBT1 integrase gene	(Kieser et al., 2000)
pET28a(+)	Kan^R, expression vector	Novagen
pET32a(+)	Amp^R, expression vector	Novagen
pWLI628	pET28a carrying *dmtMT1*	This study
pWLI629	pET28a carrying *dmtMT2-1*	This study
pWLI630	pET28a carrying *dmtMT3*	This study
pWLI631	pET32a carrying *dmtMT2-2*	This study
pWLI632	cosmid harboring *dmtMT1* gene from *S. youssoufiensis* OUC6819	This study
pWLI633	pWLI632 derivative where *dmtMT1* was replaced with *acc(3)IV-oriT* cassette	This study
pWLI634	pSET152C derivative harboring *dmtMT1* under the control of P_{gapdh}	This study
pWLI635	pSET152C derivative harboring *dmtMT2-1* under the control of P_{gapdh}	This study
pWLI636	pIJ10500 derivative harboring *dmtMT1* under the control of P_{hrdB}	This study
pWLI637	pIJ10500 derivative harboring *dmtMT2-1* under the control of P_{gapdh}	This study
pWLI638	pWLI636 derivative harboring *dmtMT2-1* under the control of P_{gapdh}	This study

a: Agricultural Research Service Culture Collection, NRRL

b: China General Microbiological Collection Center, CGMCC

Table S2. Primers used in this study, Related to Figures 2-6.

Name	Sequence (5'-3')
For DmtMT1, DmtMT2-1, DmtMT2-2 and DmtMT3 protein expression	
DmtMT2-1-FP	GGAATTCCATATGCAGCAGCAGACCACGGCG
DmtMT2-1-RP	CCGCTCGAGTCAGTTCCGCGCGGCGACG
DmtMT2-2-FP	CCGGAATTCATGGCCACTTCCTCGCCCTC
DmtMT2-2-RP	CCGCTCGAGCTAGCTCCGCGCCGCCCGC
DmtMT3-FP	GGAATTCCATATGACCTTCTCCCCCACCCC
DmtMT3-RP	CCGCTCGAGTCAGCGGGAGCGGCCGGTG
DmtMT1-FP	GGAATTCCATATGGGAAGTAAGCAGTACGAC
DmtMT1-RP	CCGCTCGAGCTTCACCGCCTCCAGGAC
For PCR-targeted mutagenesis of *dmtMT1*	
*dmtMT1*MF	GCGTAAGAGAGCGATTGCCGAGCGTGCGGGAAGGCGTGACattccggggatcc gtcgacc
*dmtMT1*MR	TGTCACTTCACCGCGCCACTTCACCGCCTCACTCGACCGCGtgtaggctggag ctgcttc
*dmtMT1*CF	CGTAAGAGAGCGATTGCCGA
*dmtMT1*CR	TCACCGCCTCACTCGACCG
*dmtMT1*EF	GTGGGAAGTAAGCAGTACGAC
*dmtMT1*ER	CTAGTCTAGATCACTTCACCGCCTCCAGGA
For heterologous expression of *dmtMT1* and *dmtMT2-1*	
dmtMT2-1-FP	ATGCAGCAGCAGACCACGG
dmtMT2-1-RP	CTAGTCTAGATCAGTTCCGCGCGGCGAC
dmtMT2-1-FP2	CCGCTCGAGCCGTCGCGGAAAGCTGGCC
dmtMT2-1-RP2	GGAATTCCATATGTCAGTTCCGCGCGGCGACG
dmtMT1-FP	GTGGGAAGTAAGCAGTACGAC
dmtMT1-RP	CCCAAGCTTTCACTTCACCGCCTCCAGG
pHFP	GGGGTACCTCTAGACCGCCTTCCGCCGG
pHRP	GAACAACCTCTCGGAACGTTG
pGFP	CCAATGCATCCGTCGCGGAAAGCTGGC
pGRP	GAACCGATCTCCTCGTTGGTG

Underlined red letters represent restriction sites. The primer pair of DmtMT1-FP/RP was also used for genomic library screening. The 3'-OH of pGRP and pHRP was phosphorylated. pHFP/pHRP and pGFP/RP were used for amplification of promotor P_{hrdB} and P_{gapdh}, respectively.

Table S3. Predicted functions of the four MT genes, Related to Figure 1.

Strain	Protein	Size (aa)	Proposed function	Homologs	
				Protein/Organism	Accession no. (Identity/Similarity %)
6819	DmtMT1	249	methyltransferase	Amir_4628/*Actinosynnema mirum* DSM 43827	ACU38461.1 (33.5/45.0)
F-5123	DmtMT2-1	294	methyltransferase	MitM/*Streptomyces lavendulae*	AAD28459.1 (36.7/49.8)
	DmtMT2-2	275	methyltransferase	UbiE/*Escherichia coli*	YP_026269.1 (16.4/25.3)
4.5739	DmtMT3	275	*N*5-glutamine methyltransferase	PrmC/*Chlamydia trachomatis*	AY600244.1 (24.2/34.6)

Transparent Methods

Strains

Streptomyces Strains

S. youssoufiensis OUC6819 strains were grown at 30 °C on R2YE agar medium. MS agar medium (3% soya flour, 2% mannitol, 2% agar powder) was used for the cultivation of *Streptomyces* sp. NRRL F-5123, *S. aidingensis* CGMCC 4.5739, and *S. coelicolor* M1146 strains. All the above *Streptomyces* strains were cultured in liquid TSBY medium (3% tryptic soya both, 10.3% sucrose, 0.1% tryptone, 0.05% yeast extract) at 30 °C for genomic DNA extraction. For the DMTs production, the strains were incubated in the production medium (1 % soluble starch, 2 % glucose, 4 % corn syrup, 1 % yeast extract, 0.3 % beef extract, 0.05 % $MgSO_4 \cdot 7H_2O$, 0.05 % KH_2PO_4, 0.2 % $CaCO_3$, and 3 % bay salt, pH = 7.0), followed by incubation at 30 °C, 220 rpm for 7 days.

E. coli Strains

E. coli strains including DH5α, BL21, BW25113/pIJ790 and ET12567/pUZ8002 were cultivated at 37 °C in Luria–Bertani (LB) liquid medium or on LB agar. When necessary, the medium was supplemented with 50 μg/mL of apramycin, 25 μg/mL of chloramphenicol, 100 μg/mL of kanamycin, 100 μg/mL of hygromycin, or 50 μg/mL of ampicillin.

DNA Manipulation and Plasmid Constructions

Plasmid extractions and DNA purification were carried out using commercial kits (OMEGA, BIO-TEK). Chromosomal DNA isolation, restriction endonuclease digestion, ligation, and transformation were performed according to standard procedures (Sambrook et al., 1989) or manufacturer's instructions.

For the expressions of *dmtMT1*, *dmtMT2-1*/*2* and *dmtMT3* in *E. coli*, the responding genes were amplified by polymerase chain reaction (PCR) using primer pairs listed in Table S2. *dmtMT1*, *dmtMT2-1*, and *dmtMT3* were digested with *Nde*I and *Xho*I, ligated into the pET28a(+) resulting in pWLI628-630; while *dmtMT2-2* was cloned into the *Eco*RI and *Xho*I sites of pET32a(+) resulting in pWLI631. After confirmation by sequencing, the resulting constructs pWLI628-630 were transformed into *E. coli* BL21 (DE3), and pWLI631 was transformed into *E. coli* BL21-CodonPlus (DE3).

For the combinatorial expressions of *dmtMT1* and *dmtMT2-1* in *Streptomyces* strains, the corresponding gene was put under the control of the constitutive promoter P_{gapdh} or P_{hrdB}. P_{gapdh} was amplified using primer pair of pGFP/3'-OH phosphorylated pGRP (Table S2) and digested with *Nsi*I; P_{hrdB} was amplified using primer pair of pHFP/3'-OH phosphorylated pHRP (Table S2) and digested with *Kpn*I. For expressions in OUC6819, *dmtMT1* and *dmtMT2-1* were respectively amplified with the primer pairs of *dmtMT1*EF/*dmtMT1*ER and *dmtMT2-1*-FP/*dmtMT2-1*-RP (Table S2) followed by digestion with *Xba*I; and then they were respectively ligated with P_{gapdh}, and cloned into the *Nsi*I and *Xba*I sites of pSET152C to give pWLI634-635. For expressions in M1146, *dmtMT1* was ligated with P_{hrdB} followed by insertion into the *Kpn*I and *Xba*I sites of pIJ10500 to give pWLI636; the P_{gapdh}-*dmtMT2-1* fragment was cloned into the *Nde*I and *Xho*I sites of pIJ10500 and pWLI636 to yield pWLI637-638. After confirmation by sequencing, the resulting plasmids pWLI636-638 were passed through *E. coli* ET12567/pUZ8002, and then introduced into *S. coelicolor* M1146/*dmtB1C1* or *S. coelicolor* M1146/*dmtA1B1C1 via* conjugation (Kieser et al., 2000).

Protein Expression and Purification

The expressions of *dmtMT1*, *dmtMT2-1*, *dmtMT2-2* and *dmtMT3* followed the same protocol and were detailed as follows. Overnight culture of *E. coli* harboring the expression plasmid (10 mL) was inoculated into 1 L of LB medium (containing 50 μg/mL of kanamycin, or 25 μg/mL of chloramphenicol and 50 μg/mL of ampicillin) and grown at 37 °C, 220 rpm. Expression was induced at an OD_{600} of approximately 0.6 by addition of isopropyl β-D-thiogalactopyranoside (IPTG) (with final concentration of 0.05 mM), and cultivation was continued for additional 16 hrs at 16 °C**.**

The cells were pelleted by centrifugation (15 min at 8,000 x *g*) and resuspended in 30 mL of binding buffer A (0.05 M Tris-HCl, 0.5 M NaCl, 5.0% glycerol (v/v), pH 7.5, containing cOmplete^{TM} protease inhibitor cocktail). The resuspended cells were lysed by sonication in an ice-water bath with an ultrasonic processors VCX750 (Sonics & Materials Inc, PA, USA), and were centrifuged at 10,000 x *g* for 30 min at 4 °C. The resulting supernatant was applied to a HisTrap HP column (1 mL, GE Healthcare) and the His-tagged protein was eluted with a linear gradient of imidazole (30–500 mM) in the binding buffer using an ÄKTA Purifier system. After SDS–PAGE analysis, fractions containing pure protein were pooled, concentrated and exchanged to Tris buffer (0.025 M Tris-HCl, 0.02 M NaCl, and 10.0% glycerol, pH 7.5) by using Amicon Ultra-15 30-kDa cutoff centrifugal concentrator (Millipore).

***In Vitro* Assays**

For *in vitro* experiments, each of the recombinant DmtMT1, DmtMT2-1/2, and DmtMT3 (10 μM) was incubated with 0.5 mM DKPs/pre-DMTs/DMTs (Sun et al., 2013) and 0.5 mM SAM in Tris buffer [50 mM Tris (pH 8.0) and 0.1 mM DTT] at 30 °C for 12 hrs. Reactions were stopped by the addition of equal volume of methanol and mixed by vortexing. For the detection of pre-DMTs/DMTs, the mixtures were subjected to HPLC analysis, using a YMC-Pack ODS-AQ C18 column (150 mm × 4.6 mm, particle size of 5 μm, pore size of 120 Å) under the program: phase A consisting of 0.1% (v/v) formic acid and ddH_2O, phase B consisting of 0.1% (v/v) formic acid and acetonitrile; 50% B (0–5 min), 50% to 100% B (5–30 min), 100% B (30–45 min), at a flow rate of 1 mL min^{-1} and UV detection at 300 nm. For the detection of DKPs, the program was set as follows: 10% B (0–5 min), 10% to 50% B (5–25 min), 100% B (25–35 min), at a flow rate of 1 mL min^{-1} and UV detection at 280 nm. For probing substrate promiscuity of DmtMT1, different DKPs (0.5 mM) were tested as described above and were analyzed with HPLC-MS^2; the enzymatic reactions were performed in triplicate, and all rates were calculated with their peak areas at 280 nm.

The assay of DmtMT1 with cWV was scaled up and subsequently subjected to a semi-preparative HPLC column (YMC-Pack ODS-AA C18 column, 120 Å, 250×10 mm, 5 μm) for purification. The resulting Me-cWV was stored at -20 °C until use. The enzymatic assay of DmtC1 was carried out in 50 mM Tris-HCl buffer (pH 8.0) with 2.5 mM $MgCl_2$, containing 10 μM DmtC1, 1 mM Me-cWV, and 0.2 mM FPP at 30 °C. After 1 hr, the reaction was quenched by the addition of equal volume of methanol and mixed by vortexing. The mixture was centrifuged at 17,000 x *g* for 20 min to remove proteins. The supernatant was then applied to YMC-Pack ODS-AQ C18 column with UV detection at 300 nm under the program: 10% B (0–5 min), 10% to 50% B (5–15 min), 80% to 100% B (15–25 min), at a flow rate of 1 mL min^{-1} (phase A, 0.1% formic acid in ddH_2O; phase B, 100% acetonitrile supplemented with 0.1% formic acid).

Gene Inactivation, Complementation, and Overexpression in *S. youssoufiensis* OUC6819

Inactivation of *dmtMT1* in *S. youssoufiensis* OUC6819 was performed using the REDIRECT Technology according to the literature protocol (Yao et al., 2018). The genomic library was screened using the primer pair of DmtMT1-FP/ DmtMT1-RP, giving positive cosmid pWLI632. The *aac(3)IV-oriT* resistance cassette was amplified with primer *dmtMT1*MF/ *dmtMT1*MR (Table S2) using pIJ773 (Gust et al., 2003) as template and was transformed into *E. coli* BW25113/pIJ790 containing pWLI632 to replace an internal region of *dmtMT1*, resulting in

mutant cosmid pWLI633. pWLI633 was passed through *E. coli* ET12567/pUZ8002 and was then introduced into *S. youssoufiensis* OUC6819 by intergenic conjugation using mycelia as recipients. The mutant *ΔdmtMT1* was selected by the apramycin-resistant and kanamycin-sensitive phenotype and was further confirmed by PCR using the primer pair of *dmtMT1*CF/*dmtMT1*CR (Table S2). For overexpression, pWLI634-635, containing intact *dmtMT1* and *dmtMT2-1*, were respectively passed through *E. coli* ET12567/pUZ8002 and introduced into the wild-type *S. youssoufiensis* OUC6819 *via* conjugation. pWLI634 was further introduced into *ΔdmtMT1* for genetic complementation.

Production and Analyses of DMTs

Spores of *Streptomyces* strains were incubated into 50 mL of production medium in 250 mL Erlenmeyer flasks fitted with glass beads, at 30 °C and 220 rpm for 7 days. The supernatants were extracted twice with an equal volume of ethyl acetate, and the combined ethyl acetate extracts were concentrated *in vacuo* to afford residue A. The precipitated mycelia were extracted twice with acetone. The extracts were combined, and acetone was evaporated *in vacuo* to yield residue B. The combined residues were dissolved in methanol and filtered through a 0.2 μm filter. The resulting fermentation products derived from *ΔdmtMT1*, genetic complementation and overexpression strains were subjected to HPLC analysis, eluting with a linear gradient of B/A in 40 min (phase A, 0.1% formic acid in ddH_2O; phase B, 100% acetonitrile supplemented with 0.1% formic acid; flow rate: 1 mL min^{-1}; wavelength: 300 nm) using a YMC-Pack ODS-AQ C18 column. The fermentation products of heterologous expression strains were detected under the identical conditions used for analyzing the DmtMT2-1-cataylzed reactions using pre-DMTs/ DMTs as substrates.

Isolation and Characterization of the Methylated pre-DMTs and DMTs

For isolation of DmtMT2-1 enzymatic products, the *in vitro* assays contained pre-DMTs/DMTs (0.5 mM) and DmtMT2-1 (30 μM) were scaled up to 30 mL. The reactions were extracted three times with an equal volume of ethyl acetate and concentrated *in vacuo*. The extracts were further purified by eluting with linear gradient from 80 to 100% B/A (phase A: ddH_2O; phase B: acetonitrile, 1.5 mL min^{-1}, UV detection at 300 nm) in 40 min using a semi-preparative HPLC column (YMC-Pack ODS-AA C18 column, 120 Å, 250×10 mm, 5 μm). For isolation of pre-DMT F (**13**), the reaction consisted of cWV (0.5 mM), SAM (1 mM), FPP (0.5 mM), DmtMT1 (50 μM), DmtC1 (30 μM), and $MgCl_2$ (2.5 mM) in Tris-HCl buffer (50 mM, pH 8.0) was performed, the mixture was treated in the same way as that of the DmtMT2-1-catalyzed reactions.

To isolate compound **16**, The *S. youssoufiensis* OUC6819 expressing *dmtMT2-1* was fermented in a total volume of 15 L. The fermentation cultures were treated as described above. The residues were applied to reversed-phase C18 open column, eluting with a gradient eluent of 20%–100% methanol to give five fractions (Fr.1~Fr.5) for each fermentation culture. Compound **16** was obtained by further separation of the Fr.4, eluting with the identical program used for isolation of DmtMT2-1 enzymatic products. The structures of the above compounds were characterized by HR-ESI-MS carried out on Thermo LTQ Orbitrap XL mass spectrometer, and NMR spectroscopy recorded with Bruker Avance III 600 spectrometers. All spectra were processed with MestReNova.6.1.0 (Metrelab), and chemical shifts were referenced to those of the solvent DMSO-d_6 signals.

Bioinformatic Analysis

ORF assignments and their proposed functions were accomplished by using FramePlot4.0 beta (Ishikawa and Hotta, 1999) (http://nocardia.nih.go.jp/fp4). Sequence comparisons and database searches were accomplished with BLAST programs (McGinnis and Madden, 2004) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). 71 CDPS-associated MTs listed in Data S3 were extracted from the NCBI database (https://www.ncbi.nlm.nih.gov/protein); and Enzyme Function

Initiative-Enzyme Similarity Tool (EFI-EST) (Gerlt et al., 2015) was used to construct the sequence similarity network using an alignment score of 30. The network was visualized in Cytoscape (Shannon et al., 2003).

Supplemental References

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