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Catalytic promiscuity of a bacterial α-N-methyltransferase

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Abstract

The posttranslational methylation of N-terminal α-amino groups (α-N-methylation) is a ubiquitous reaction found in all domains of life. Although this modification usually occurs on protein substrates, recent studies have shown that it also takes place on ribosomally synthesized natural products. Here we report an investigation of the bacterial α-N-methyltransferase CypM involved in the biosynthesis of the peptide antibiotic cypemycin. We demonstrate that CypM has low substrate selectivity and methylates a variety of oligopeptides, cyclic peptides such as nisin and haloduracin, and the ε-amino group of lysine. Hence it may have potential for enzyme engineering and combinatorial biosynthesis. Bayesian phylogenetic inference of bacterial α-N-methyltransferases suggests that they have not evolved as a specific group based on the chemical transformations they catalyze, but that they have been acquired from various other methyltransferase classes during evolution.

Keywords

α-N-methyltransferase; peptide antibiotic; lantibiotic; catalytic promiscuity; evolution

1. Introduction

Posttranslational methylation of N-terminal α-amino groups (α-N-methylation) is a ubiquitous reaction found in both bacteria and eukarya [1–10]. These reactions occur usually on protein substrates that function as part of macromolecular complexes, including the ribosome, chromatin, respiratory chains, photosynthetic complexes, myofibrils and bacterial pili [4]. Although the exact role and the enzymology of α-N-methylation remains largely unknown, recent studies have revealed that the N-terminal methylation of Ran guanine nucleotide exchange factor RCC1 catalyzed by NRMT (N-terminal RCC1 methyltransferase) is critical for normal bipolar spindle formation and chromosome segregation in the mitotic phase of mammalian cells [7,8]. NRMT was shown to be promiscuous and is able to methylate protein substrates other than RCC1, such as SET (also known as TAF-I or PHAPII) and the retinoblastoma protein RB [8]. An ortholog of NRMT in *Drosophila melanogaster*, dNTMT, was shown to catalyze methylation of histone H2B [10]. For bacteria, methylation of the ribosomal protein L11 has been relatively well

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Appendix A. Supplementary data Supplementary data associated with this article can be found in the online version at xxxxxxxxxxx

characterized. This reaction is catalyzed by PrmA, a versatile enzyme that methylates not only the N-terminal α-amino group but also the ε-amino group of Lys residues [11,12].

In addition to protein substrates, ribosomally synthesized peptides also serve as substrates for α-N-methylation. The recently-characterized thiazole/oxazole-modified peptide plantazolicin B, for example, is N-terminally methylated to produce plantazolicin A containing an N,N-dimethylarginine residue (Fig. 1) [13–15]. The N,N-dimethylalanine (Me_2-Ala) residue in cypemycin and its structural variant grisemycin also results from α-N-methylation [16,17] (Fig. 1). Cypemycin is a posttranslationally modified peptide antibiotic produced by *Streptomyces sp.* OH-4156 with potent in vitro activity against mouse leukemia cells [18]. Historically, it has been grouped in the lantibiotic family because of its dehydrobutyrine (Dhb) residues and (Z)-2-aminovinyl-cysteine (Avi-Cys) moiety (Fig. 1), which are also found in some lantibiotics [19,20]. However, cypemycin does not contain lanthionine or methyllanthionine rings, and the Dhb and Avi-Cys residues are proposed to be introduced by a pathway distinct from that of lantibiotic biosynthesis [16]. CypM, an enzyme of the methyltransferase 11 family, is responsible for the synthesis of the N-terminal Me_2-Ala residue, as deletion of *cypM* from the cypemycin biosynthetic gene cluster resulted in a demethylated cypemycin derivative, which could be methylated by CypM in vitro to produce cypemycin (Fig. 1) [16]. Interestingly, the antimicrobial activity of demethylated cypemycin was almost completely abolished. A similar result was found for plantazolicin, as plantazolicin B was devoid of activity against *B. anthracis* whereas plantazolicin A displayed potent activity [15]. These results demonstrated a new role of α-N-methylation in the biological activity of natural products.

Given the essentiality of α-N-methylation for the antibiotic activity and the catalytic versatility found for NRMT and PrmA, α-N-methyltransferases might possibly serve as a useful tool for structural diversification and functional optimization of peptide antibiotics. Modification of the N-terminus is an often-used strategy for synthetic peptides to decrease the susceptibility to aminopeptidases. Here we probed the catalytic specificities of CypM using various synthetic and natural peptides. Our data show that CypM has low substrate specificity and can methylate a series of structurally distinct substrates, suggesting that catalytic promiscuity might be a common property among α-N-methyltransferases and demonstrating a potentially useful tool for future combinatorial biosynthesis studies. Phylogenetic analysis using the Bayesian Markov chain Monte Carlo (MCMC) method [21] indicates that bacterial α-N-methyltransferase may not have evolved from a common ancestor, but were likely acquired several times from other ancient methyltransferases.

2. Materials and Methods

2.1. Chemicals, biochemicals, plasmids and strains

This information is provided in the **Supplementary Methods**.

2.2. Synthesis of oligopeptides

Peptides were synthesized using standard fluorenylmethyloxycarbonyl (Fmoc) based solid phase peptide synthesis (SPPS) techniques using a Rainin PS3 peptide synthesizer. Preloaded resin (either Wang or 2-chlorotrityl; 0.1 mmol) was first swollen in dimethylformamide (DMF) (3 × 5 mL × 10 min). Fmoc-amino acids (0.4 mmol, 4 equiv) were coupled using O-(1H-6-chlorobenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HCTU, 165 mg, 0.4 mmol, 4 equiv) as coupling reagent and 0.4 M N-methyl morpholine (NMM) as activating reagent (45 min). Fmoc deprotection was performed with piperidine (3 × 5 mL × 3 min; 20% in DMF). After completion of the

coupling of the final amino acid, the Fmoc group was removed to generate the free amino group.

Peptides were cleaved from the resin by adding a solution of trifluoroacetic acid (TFA) (5 mL), triisopropylsilane (TIPS) (100 μL), and H_2O (100 μL) to the resin (0.1 mmol), and stirring the solution for 2 h at room temperature. The solution was concentrated by purging with a nitrogen stream and peptides were precipitated with cold diethyl ether. The crude peptides were dissolved in 0.1% aqueous TFA, lyophilized, and purified by preparative RP-HPLC on a Waters Delta-PakTM C18 column (2.5 cm × 10.0 cm) employing a water-acetonitrile solvent system. HPLC fractions containing products as confirmed by (MALDI) mass spectrometry (MS) were collected and lyophilized.

2.3. In vitro CypM assays

Detailed procedures for overexpression and purification of C-terminally hexa His-tagged CypM are described in the Supplementary Methods. For in vitro methylation assays, peptide substrates (100 μM) were incubated with CypM-His_6 (20 μM) in a reaction buffer containing 50 mM HEPES (pH 7.2), 0.5 mM *S*-adenosylmethionine (SAM), 1 mM DTT, 100 mM NaCl and 0.01 U of *S*-adenosylhomocysteine hydrolase (from rabbit erythrocytes, Sigma-Aldrich). The reactions were incubated at 37 °C for 5 h. Reactions were quenched by 5% TFA and the protein precipitate was removed by centrifugation. Samples were analyzed directly by liquid chromatography electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) as described in the Supplementary Methods. For MALDI-TOF MS analysis, samples were either purified by reverse phase (C4) solid phase extraction or desalted using ZipTipC18 before analysis.

2.4. Phylogenetic analysis

The protein sequences in this study were obtained from the GenBank database; their accession numbers and the source organisms are listed in Supplementary Table S2. The sequences were aligned in ClustalX [22] using default parameters with iteration at each alignment step, and the alignments were manually fine-tuned afterwards. Bayesian inference was used to calculate posterior probability of clades utilizing the program MrBayes (version 3.1) [23]. Final analyses consisted of two sets of eight chains each (one cold and seven heated), run for about 3 million generations with trees saved and parameters sampled every 100 generations. Analyses were run to reach a convergence with standard deviation of split frequencies <0.005. Posterior probabilities were averaged over the final 75% of trees (25% burn in). The analysis utilized a mixed amino acid model with a proportion of sites designated invariant, and rate variation among sites modeled after a gamma distribution divided into eight categories, with all variable parameters estimated by the program based on BioNJ starting trees. The figures of the Bayesian phylograms were prepared by using TreeView [24].

3. Results and Discussion

3.1. CypM-catalyzed methylation of short oligopeptides

Cypemycin biosynthesis follows a common paradigm of ribosomal natural product maturation, involving posttranslational modifications of the C-terminal core region of a precursor peptide and subsequent proteolytic removal of an N-terminal leader sequence [25]. CypM methylates the α-amino group of an N-terminal Ala residue that is released by removal of the leader peptide. Because the leader peptide is typically indispensable for precursor peptide modifications [25], α-N-methylation of Ala1 probably occurs as the last step of cypemycin biosynthesis. Accordingly, the natural substrate of CypM would be a peptide containing the Dhb residues and a C-terminal Avi-Cys moiety (Fig. 1).

We synthesized a series of short oligopeptides resembling the N-terminal sequence of cypemycin, in which the Dhb residues were replaced with Ala, Ser or Thr. As shown in Table 1, in the presence of S-adenosylmethionine (SAM) these peptides (entries 1–5) were methylated by CypM (Table 1 and Supplementary Fig. S1–S5). In contrast to the reaction with native substrate in which only di-methylated product (cypemycin) was observed [15], mono- and di-methylated products were both identified in most cases, reflecting the relatively inefficient methylation of these short oligopeptides as compared to the native substrate. However, the tolerance of CypM to Thr- and Ser- containing peptide indicates the rather relaxed substrate specificity of the enzyme, as the N-terminus as well as the overall scaffold of cypemycin is highly hydrophobic (Fig. 1). Moreover, the methylation is not confined to N-terminal Ala. Peptides with N-terminal Gly, Ser, and Met residues were also modified by CypM (Table 1, entries 6–8), albeit with reduced efficiencies (Supplementary Fig. S6–S8). Collectively, these results indicate that CypM has a relaxed substrate specificity and can methylate a series of short oligopeptides that mimic the N-terminal sequence of cypemycin.

The ability of PrmA to methylate not only the N-terminal α-amino group but also the ε-amino group of Lys led us to investigate whether CypM can also methylate the ε-amino group of Lys, which could be useful in histone research. We synthesized several oligopeptides similar to peptide 1 (AAPAAPA) but with Lys replacements in various positions (peptides 9–11). As shown in Table 1, peptides with Lys in the first or second position from the N-terminus (entries 9–10) were not modified by CypM, but a peptide with a Lys at the C-terminus (entry 11) was methylated, with both di- and tetra-methylated products detected in the reaction mixture (Supplementary Fig. S11). Detailed MS-MS analysis clearly showed that di-methylation occurred almost entirely on the ε-amino group of Lys instead of the N-terminus (Supplementary Fig. S12), suggesting that the ε-amino group of Lys might be preferred over the N-terminal amino group provided it is not near the N-terminus. We note that cypemycin does not contain any Lys residues.

Cypemycin and many α-N-methylated proteins contain Pro residues within a few amino acids of the N-termini [4,8,16], which might be important for enzyme recognition. Indeed, a peptide in which Pro3 was replaced by Ala (entry 12) was not modified by CypM. CypM also did not methylate a peptide with an N-terminal APK motif (entry 13) that is the target of NRMT; CypM and NRMT share no sequence similarity.

3.2. Methylation of other peptide antibiotics

We next interrogated whether CypM could methylate structurally unrelated peptide antibiotics. Nisin (Fig. 2A) represents the best studied member of the lantibiotic family and has been widely used in the food industry because of its potent antimicrobial activity and unique mode of action [26,27]. There is interest in expanding its use to clinical applications in both human and animal health products [28]. As nisin possesses an unmodified Ile residue at the N-terminus, the α-N-methylation of this residue may potentially improve the stability and the pharmacological property of the molecule by preventing proteolytic degradation by aminopeptidases. Incubation of CypM with nisin resulted in addition of three methyl groups (Fig. 2B). Detailed MS-MS analysis revealed that one methylation occurred on the N-terminal Ile with the other two methylations on Lys12 (Supplementary Fig. S15). The MIC value of the methylated nisin A against *Lactococcus Lactis* HP (ATCC11602) and *Bacillus subtillis* 168 (ATCC6633) was determined to be 1.0 μM and 4 μM, 8-fold and 4-fold higher than that of nisin A, respectively, indicating that the methylated nisin is still a potent antibiotic. CypM may thus serve a methylating tool for diversification of peptide natural products.

We also evaluated haloduracin as substrate for CypM. Haloduracin is a two-component lantibiotic containing two distinct peptides halduracin α (Halα) and β (Halβ) (Fig. 3A), which act synergistically to exhibit potent antibiotic activity [29,30]. Halα contains a Cys residue at its N-terminus, which links to Cys8 by a disulfide bond and forms a macrocyclic system, and Halβ possesses an N-terminal methyllanthionine ring (Fig. 3A). Intriguingly, both Halα and Halβ were methylated by CypM, and the mono-methylated product was found to be the major product in both cases (Fig. 3B). MS-MS spectrometric analysis indicated that, although both Halα and Halβ contain Lys residues, methylation occurred on the N-terminal amino group in both cases (Supplementary Fig. S16–17). Given the considerable structural difference between the native substrate of CypM (demethyl-cypemycin) and nisin and haloduracin, the catalytic promiscuity of CypM demonstrated herein is surprising. The factors that determine methylation of either α-N-amino or Lys ε-amino groups in different structural contexts, however, is currently unclear and requires structural studies.

3.3. Evolution of bacterial α-N-methyltransferases

A search of CypM homologs in the National Center for Biotechnology Information (NCBI) sequence database revealed that except for GrmM, involved in the biosynthesis of grisemycin (a cypemycin structural variant), proteins with homology to CypM share only modest sequence identities with CypM. These homologous proteins include the α-N-methyltransferase PznL for plantazolicin A biosynthesis (Fig. 1), LmbJ and CcbJ that are involved in the biosynthesis of lincomycin and celesticetin, respectively [31,32], and several proteins that are designated as ubiquinone/menaquinone methylase UbiE [33]. However, most proteins with sequence homology to CypM in the NCBI database are functionally uncharacterized.

We used the Bayesian MCMC method to construct the phylogenetic inference of CypM and other bacterial methyltransferases [34]. Several UbiE enzymes identified from the ubiquinone/menaquinone biosynthetic gene clusters of different genera and three methyltransferases proposed to be involved in producing cypemycin-like natural products [16] were included in the analysis. Several RsmE proteins that methylate the imide nitrogen N3 of a unique uridine (U1489 for *E. coli*) of 16S rRNAs [35,36] were also included for comparative analysis. The Bayesian MCMC tree shown in Fig. 4 clearly indicates that bacterial α-N-methyltransferases did not evolve together, as CypM, PnzL and PrmA fall into different clades with strong posterior probability support. CypM and GrmM, LmbJ and CcbJ, and other methyltransferases for putative cypemycin-like natural product biosynthesis form a distinct clade. Given the close phylogenetic relationship and sequence similarities of CypM with UbiE enzymes, it is likely that CypM and other enzymes in the same clade have evolved from ancient UbiE enzymes despite the latter being C-methyltransferases [33]. PrmA [12,37,38] seems to have a closer relationship with RsmE than with CypM, suggesting that these two α-N-methyltransferases evolved from different ancestors. Although PznL is an α-N-methyltransferase involved in ribosomal natural product biosynthesis like CypM, it is phylogenetically distant from CypM and falls into a distinct clade (Fig. 4). The phylogenetic analysis demonstrates that α-N-methyltransferases did not evolve based on the chemical reaction of methylating N-terminal amino groups, but rather were acquired through convergent evolution from diverse precursors with different functions. It has previously been suggested that some plant O-methyltransferases might also have undergone convergent evolutionary processes [39].

In conclusion, we demonstrate that CypM, an α-N-methyltransferase involved in cypemycin biosynthesis, has high catalytic flexibility and can act on a series of structurally distinct substrates, including the lantibiotic nisin. Combined with the studies of NRMT [8] and PrmA [12,37,38], it seems that catalytic promiscuity is a common property of α-N-

methyltransferase enzymes, which appear to have evolved by distinct pathways. The flexibility of α-N-methytransferases may find utility in methylation of therapeutic peptides that are metabolized by aminopeptidases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Dha	dehydroalanine
Dhb	dehydrobutyrine
Avi-Cys	S-[(Z)-2-aminovinyl]-cysteine
SAM	S-adenosylmethionine
SAH	S-adenosylhomocysteine
NRMT	N-terminal RCC1 methyltransferase
MCMC	Markov chain Monte Carlo
SPPS	solid phase peptide synthesis
DMF	dimethylformamide
NMM	N-methyl morpholine
MALDI	matrix assisted laser desorption ionization
Abu	2-aminobutyric acid

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Highlights

- CypM can methylate both α-N-terminal amino groups and lysine ε-amino groups
- CypM methylates a variety of cyclic peptides such as nisin and haloduracin
- α-N-methyltransferases did not evolve as a specific clade

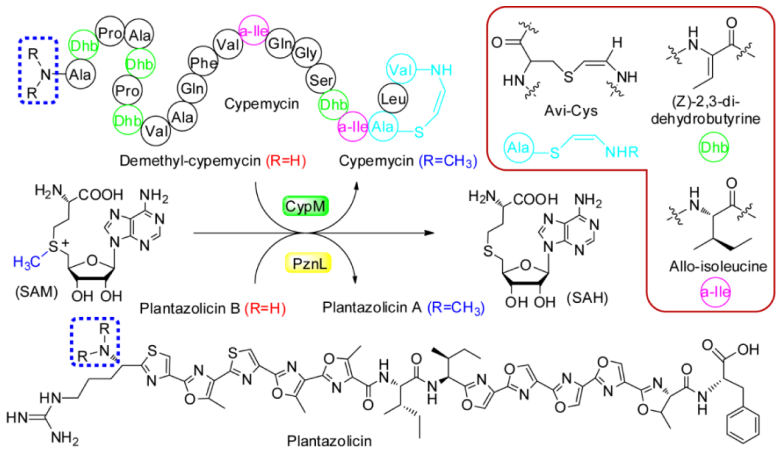

Fig. 1.

N-Methylation of cypemycin and plantazolicin A catalyzed by CypM and PznL, respectively. The amino groups on which methylation occurs are highlighted by dashed boxes. Dhb, dehydrobutyrine; a-Ile, *allo*-isoleucine; Avi-Cys, S-[(Z)-2-aminovinyl]-cysteine; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine. A shorthand notation for these structures is shown below each chemical structure in the red box.

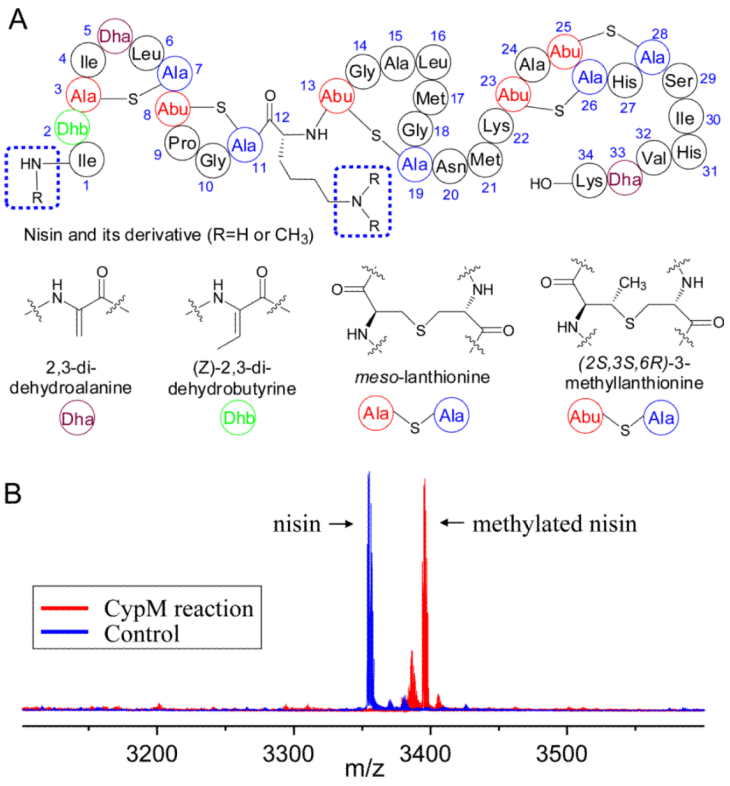

Fig. 2.

(A) Structure of nisin and its methylated derivatives. The amino groups on which methylation occurred are highlighted by dashed boxes. Abu, 2-aminobutyric acid; Dha, dehydroalanine; Dhb, dehydrobutyrine. For lanthionine and methyllanthionine structures, the segments derived from Ser/Thr are in red and those derived from Cys are in blue. A shorthand notation for these structures used in the natural product drawings is shown below each chemical structure (B) MS spectra of nisin A and nisin A in vitro modified by CypM.

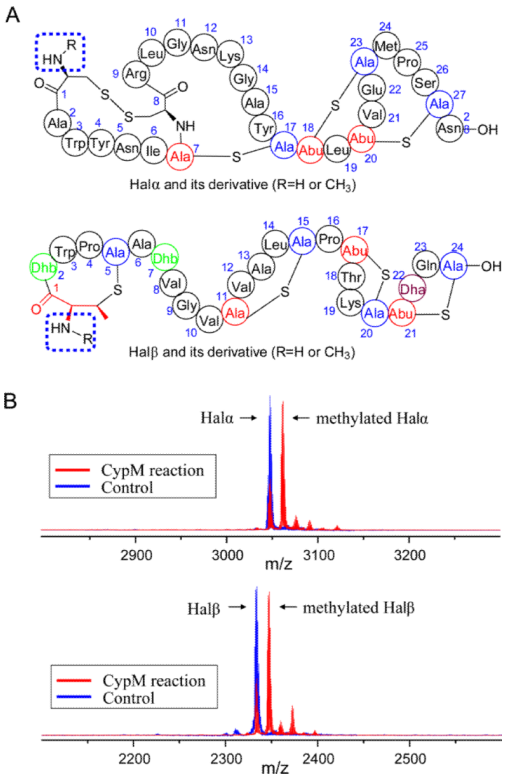

Fig. 3.

(A) Structure of Halα, Halβ and their methylated derivatives. The amino groups on which methylation occurred are highlighted by dashed boxes. Structures are similarly represented as in Fig. 2. (B) MS spectra of Halα and Halβ modified by CypM.

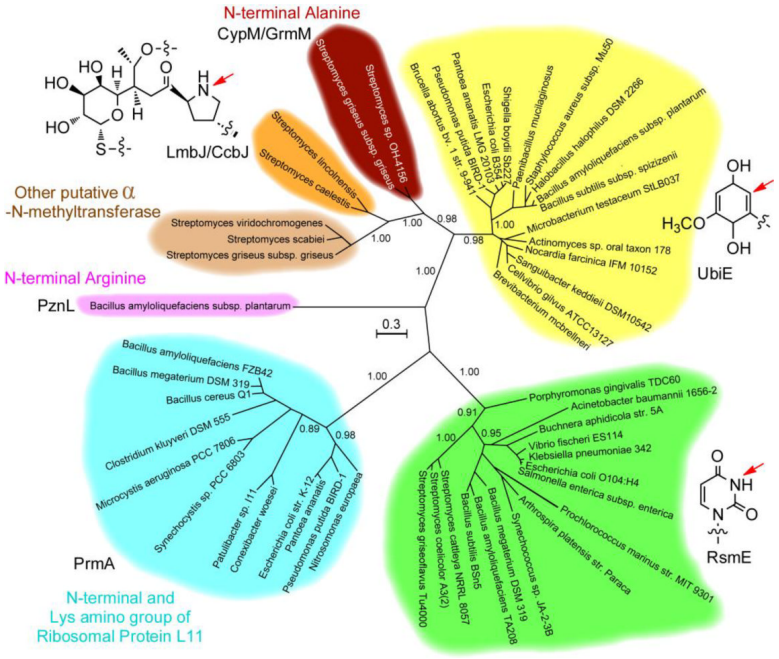

Fig. 4.

Unrooted tree of selected bacterial methyltransferases generated by Bayesian MCMC method. Support for the major clades is indicated by posterior probability values. Substrates for each clade of enzymes are shown, and the red arrows indicate the methylation sites.

Table 1

Methylation of short oligopeptides by CypM.

Peptide	Sequence	Observed methylation
1	AAPAAPA	Mono-, Di-
2	AAPAAPS	Di-
3	ASPAAPA	Di-
4	ATPAAPA	Mono-, Di-
5	ATPATPA	Mono-, Di-
6	GAPAAPA	Di-
7	SAPAAPA	Di-
8	MAPAAPA	Di-
9	KAPAAPA	No reaction
10	AKPAAPA	No reaction
11	AAPAAPK	Di-, Tetra-
12	AAAATPT	No reaction
13	APKAAPA	No reaction