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A Trojan-Horse Peptide-Carboxymethyl-Cytidine Antibiotic from *Bacillus amyloliquefaciens*

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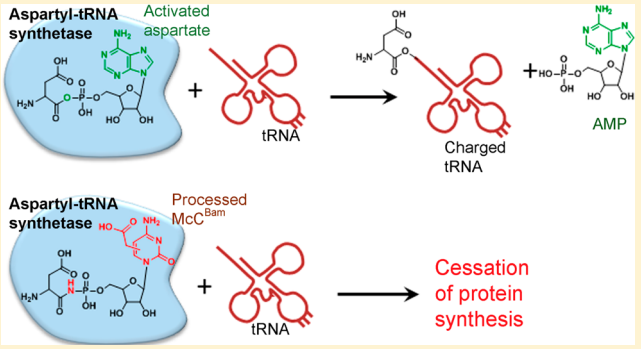
Supporting Information

ABSTRACT: Microcin C and related antibiotics are Trojan-horse peptide-adenylates. The peptide part is responsible for facilitated transport inside the sensitive cell, where it gets processed to release a toxic warhead—a nonhydrolyzable aspartyl-adenylate, which inhibits aspartyl-tRNA synthetase. Adenylation of peptide precursors is carried out by MccB THIF-type NAD/FAD adenylyltransferases. Here, we describe a novel microcin C-like compound from *Bacillus amyloliquefaciens*. The *B. amyloliquefaciens* MccB demonstrates an unprecedented ability to attach a terminal cytidine monophosphate to cognate precursor peptide in cellular and cell free systems. The cytosine moiety undergoes an additional modification—carboxymethylation—that is carried out by the C-terminal domain of MccB and the MccS enzyme that produces carboxy-SAM, which serves as a donor of the carboxymethyl group. We show that microcin C-like compounds carrying terminal cytosines are biologically active and target aspartyl-tRNA synthetase, and that the carboxymethyl group prevents resistance that can occur due to modification of the warhead. The results expand the repertoire of known enzymatic modifications of peptides that can be used to obtain new biological activities while avoiding or limiting bacterial resistance.



INTRODUCTION

Ribosomally synthesized and post-translationally modified peptides (RiPPs) with attached adenine residues form a family of Trojan-horse antibiotics encoded by gene clusters present in diverse bacteria.[1] Here, we show that one such RiPP cluster actually directs biosynthesis of an unprecedented cytidylated peptide, which is biologically active and has modifications that allow it to overcome a resistance mechanism that effectively detoxifies related adenylated peptide antibiotics. The data underscore how limited is our knowledge about diversity of RiPP modifications performed by evolutionarily related enzymes. This information can be used for creation of new antibiotics while avoiding or limiting bacterial resistance.

Trojan-horse antibiotic microcin C (McC) is a peptide-adenylate produced by some strains of *Escherichia coli*. McC-producing cells carry a plasmid-borne six-gene *mcc* biosynthesis/self-immunity cluster (Figure 1A). The ribosomally synthesized McC precursor heptapeptide MRTGNAN, the product of $mccA^{Eco}$ gene, is adenylated by $MccB^{Eco}$, a THIF-type NAD/FAD adenylyltransferase that attaches AMP to the C-terminal Asn residue of $MccA^{Eco}$ via a phosphoramidate bond.[2] The reaction proceeds through formation of a succinimide intermediate and requires two ATP molecules.[3] As a result of modification the terminal asparagine residue of the peptide is converted to an aspartate-adenylate with phosphoramidate linkage. The products of the $mccD^{Eco}$ and $mccE^{Eco}$ genes jointly are responsible for an additional decoration of the phosphate of adenylated $MccA^{Eco}$ with aminopropyl group,[4] increasing the bioactivity.[5] McC is exported from the producing cell by the $MccC^{Eco}$ pump.

The peptide part of McC is responsible for the import into sensitive *E. coli* cells through the YejABEF transporter.[6] Inside the host cells McC undergoes proteolysis by cellular aminopeptidases, which causes the release of processed McC—a nonhydrolyzable aspartyl-adenylate that binds aspartyl-tRNA synthetase (Asp-RS) and inhibits it, leading to cessation of protein synthesis.[7] The products of $mccE^{Eco}$ and $mccF^{Eco}$

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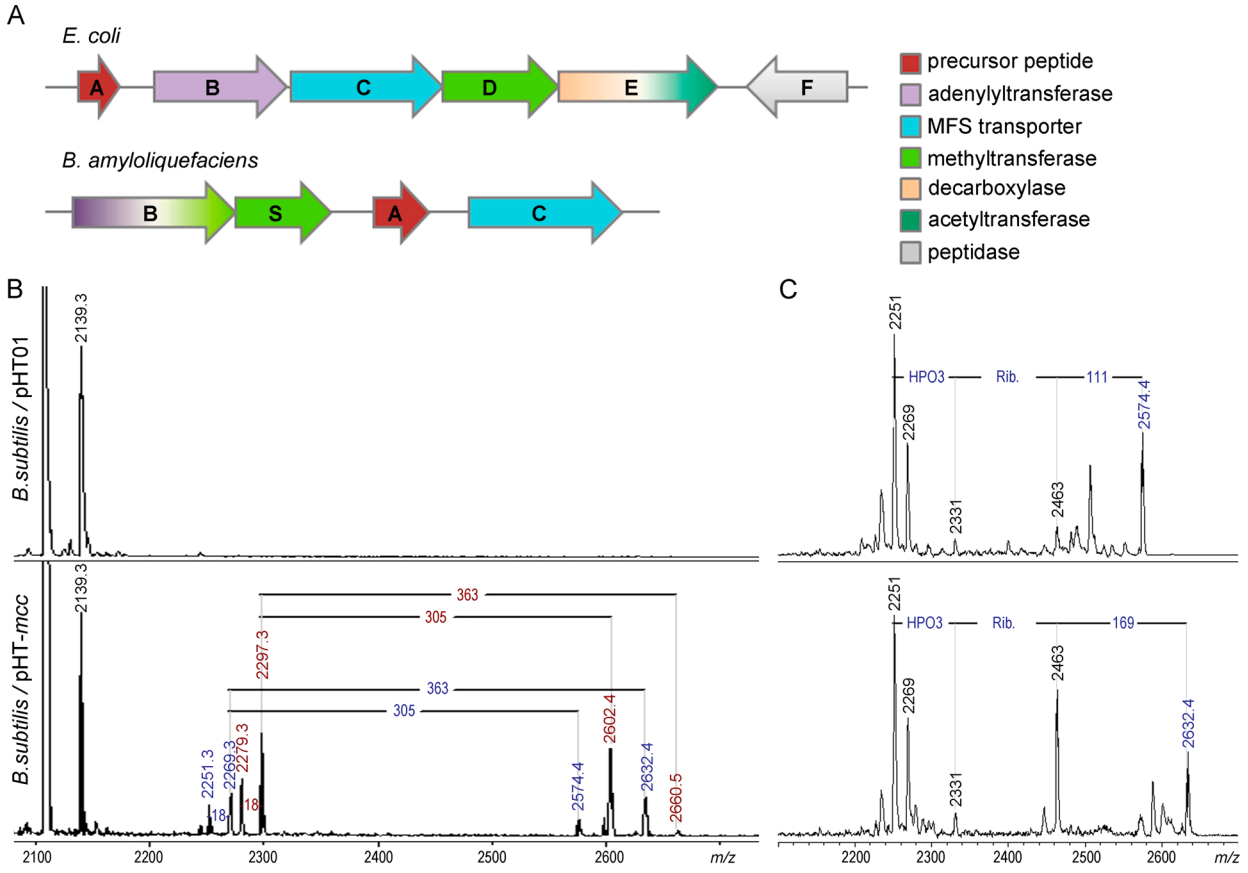

Figure 1. The *mcc*-like operon from *Bacillus amyloliquefaciens* DSM7 and its products. (A) The structure of *mcc* clusters from *E. coli* and *B. amyloliquefaciens* is schematically presented. Arrows represent genes and are colored according to validated (*E. coli*) or predicted (*B. amyloliquefaciens*) functions of their products. (B) MALDI MS analysis of *B. subtilis* cell lysates. Upper panel shows the background signals from *B. subtilis* cells bearing empty pHT01 plasmid. Lower panel shows the additional difference mass peaks detected in *B. subtilis* cells carrying plasmid-borne mcc^{Bam} cluster. Series of peaks derived from N-terminally formylated $MccA^{Bam}$ are marked in red; peaks, corresponding to deformylated $MccA^{Bam}$ peptide and its post-translationally modified forms are marked in blue. *m/z* 2251.3 and 2279.3 correspond to peptide-succinimides, *m/z* 2269.3 and 2297.3 to unmodified $MccA^{Bam}$, *m/z* 2574.4 and 2602.4 correspond to cytidylated peptide, *m/z* 2632.4 and 2660.5 correspond to $MccA^{Bam}$ modified with an unidentified nucleotide. (C) Fragments of MS/MS spectra of parent mass-ions 2574.4 and 2632.4 shown in (B) are presented. Both spectra contain *m/z* 2269 peaks corresponding to the peptide part of the compounds and additional peaks indicating nucleotide monophosphate modification on its C-end terminus. Mass shift of 111 Da on the upper pannel (*m/z* 2574 to *m/z* 2463) matches cytosine while mass shift of 169 Da on the lower panel (*m/z* 2632 to *m/z* 2463) does not correspond to any known nucleobase. "HPO3" and "Rib." stand for phosphate and ribose loss, respectively.

contribute to self-immunity by acetylating processed McC and cleaving the amidate bond connecting the terminal aspartate and AMP, respectively.[8,9] Stand-alone cellular homologues of MccE and MccF that are not part of *mcc* operon also contribute to basal levels of McC resistance.[10,11]

Bioinformatics searches revealed *mcc*-like operons in diverse Gram-negative and Gram-positive bacteria.[12,13] Some of predicted MccB-MccA pairs were subsequently validated in vitro by demonstrating that recombinant MccB proteins adenylate cognate precursor peptides and the resulting compounds inhibit bacterial growth by the same mechanism as *E. coli* McC.[14] Many of the validated *mcc* clusters contain just three genes, *mccABC*. Such minimal arrangement is apparently sufficient to produce a toxic adenylated peptide without additional decorations and export it outside the producing cells.

The *mccD*, *mccF*, and *mccE* homologues are detected in only a few of non-*E. coli mcc* clusters. On the other hand, some *mcc* operons contain diverse additional genes that are absent from the prototypic *E. coli* operon. These genes may contribute to self-immunity or be responsible for additional modifications of the basic peptide-adenylate scaffold.

A group of microcin C-like RiPP clusters from *Bacillus amyloliquefaciens* DSM7, *Streptococcus bovis* JB1, and various strains of *Yersinia sp.* and *Serratia sp.* is characterized by an extended *mccB* gene and the presence of an additional gene *mccS*. The simplest of these gene clusters mcc^{Bam}, from *B. amyloliquefaciens* DSM7, consists of four genes, $mccA^{Bam}$, $mccB^{Bam}$ (BAMF_RS40475), $mccS^{Bam}$ (BAMF_RS29790), and $mccC^{Bam}$ (BAMF_RS29795) (Figure 1A). Here we show that $MccB^{Bam}$ performs an unprecedented reaction of cytidylation of the terminal residue of cognate $MccA^{Bam}$. We further show that $MccS^{Bam}$ cooperates with the C-terminal methyltransferase domain of $MccB^{Bam}$ to attach carboxymethyl to the base of the cytidine moiety. We finally show that peptide-cytidylates inhibit aspartyl-tRNA synthetase, leading to cessation of cell growth. The presence of carboxymethyl modifications inactivates the self-immunity/resistance mechanism mediated by MccE. Our results underscore the diversity of modifications carried out by RiPP biosynthesis machinery and may provide new tools for rational design of novel antibacterial compounds.

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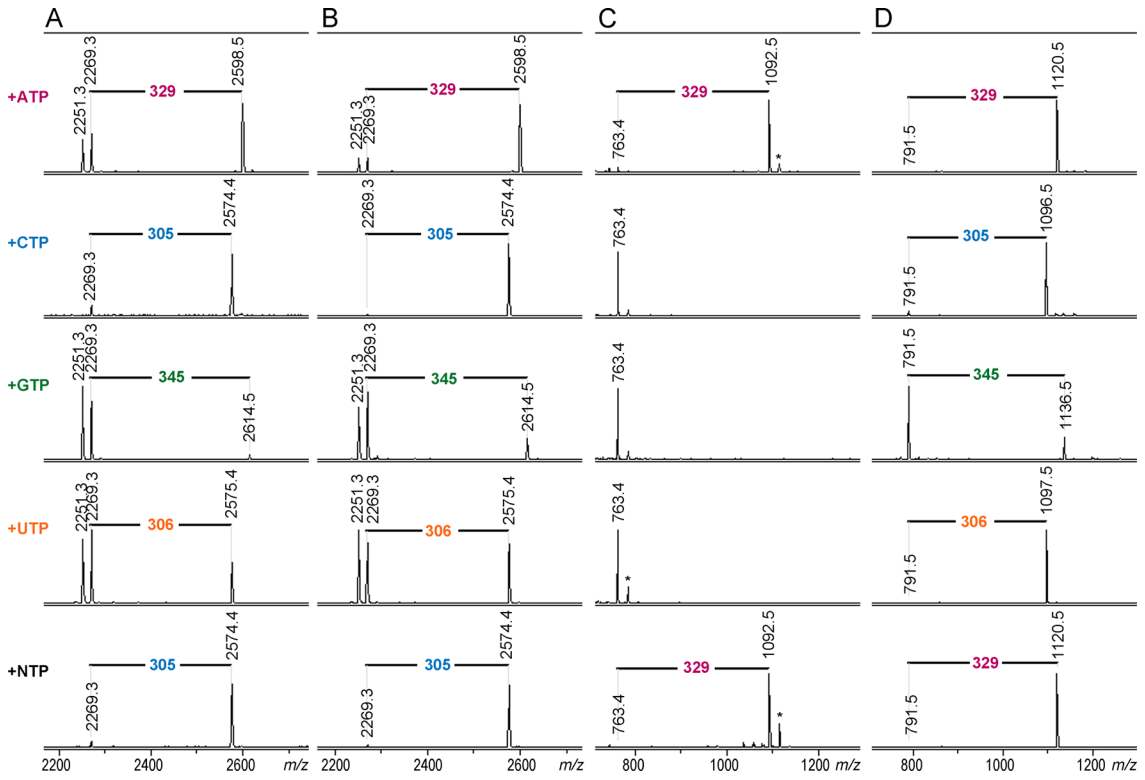

Figure 2. Nucleotide specificity of *E. coli* and *B. amyloliquefaciens* MccB nucleotidyltransferases in in vitro reactions. (A) $MccB^{Bam}$ was combined with $MccA^{Bam}$ (m/z 2269.3) in the presence of indicated individual NTPs or an equimolar mixture of four NTPs ("+NTP"). The reaction products were analyzed by MALDI MS. On mass spectra shown, the differences between the parental peptide mass peak (m/z 2269.3) and peptidyl-nucleotide products are indicated. The m/z 2251.3 peak is the parental peptide carrying terminal succinimide. (B). As in (A) but $MccB_{NTD}^{Bam}$, an $MccB^{Bam}$ derivative lacking the C-terminal domain was used instead of $MccB^{Bam}$. (C) Reaction products formed in the presence of $MccB^{Eco}$ and $MccA^{Eco}$ (m/z 763.4). (*D*) As in (C) but using formylated $MccA^{Eco}$ (m/z 791.5). The peaks of sodium salt of $MccA^{Eco}$ and its peptydyl-adenilates are marked with asterisks.

RESULTS

Prediction and Validation of the *mcc* Gene Cluster from *Bacillus amyloliquefaciens* DSM7. The $MccB^{Eco}$ homologue from *B. amyloliquefaciens* DSM7 was identified earlier.[13] Unlike most other MccB proteins, $MccB^{Bam}$ contains, in addition to the N-terminal adenylyltransferase part, a ∼30 kDa C-terminal extension ($MccB_{CTD}^{Bam}$) homologous to SAM-dependent methyltransferases. The $mccB^{Bam}$ gene is followed by $mccS^{Bam}$, whose product is homologous to carboxy-*S*-adenosyl-L-methionine (cxSAM) synthetase CmoA of *E. coli*. A gene encoding the export pump of the Major Facilitator Superfamily (MFS) $mccC^{Bam}$ is located downstream of $mccS^{Bam}$. In most validated *mcc* clusters the precursor peptide-encoding *mccA* genes are located upstream of *mccB* to ensure an optimal ratio of production of the peptide and its cognate adenylyltranferase[14] (Figure 1A). However, no *mccA*-like gene upstream to $mccB^{Bam}$ was found. Visual inspection of the extended *mccS-mccC* intergenic region identified a short open reading frame encoding a 19-amino acid peptide MLKIRKVKIVRAQ-NGHYTN. This peptide contains a terminal asparagine and may be a putative $MccA^{Bam}$.

To check if any McC-like compounds are produced by *B. amyloliquefaciens* DSM7 we performed mass-spectrometric analysis of cell lysates and conditioned media after growth under various conditions. A mass-ion of predicted $MccA^{Bam}$ peptide (m/z 2269.3) or its adenylated form (m/z 2598.4) along with mass-ions corresponding to species with N-formylated peptide and, possibly, forms carrying additional modifications were expected. However, no such peaks were observed. We therefore next cloned the entire mcc^{Bam} cluster into multicopy pHT01 plasmid and introduced it into a surrogate host *B. subtilis* 168. Mass-spectrometric analysis of transformed cells revealed a series of peaks some of which could have been the products of the *B. amyloliquefaciens mcc* cluster (Figure 1B). There were $[MH^+]$ signals corresponding to predicted $MccA^{Bam}$ peptide in N-formylated (2297.3) and deformylated (2269.3) forms. There were also peaks of corresponding succinimide intermediates (2251.3 and 2279.3) that should be produced at the first step of MccB-catalyzed reaction.[3] However, peaks corresponding to adenylated $MccA^{Bam}$ (329 Da shift from unmodified peptide peaks) were not observed. Instead, peaks with 305 and 363 Da shifts were present. MS/MS analysis of these compounds unambiguously showed that they contain terminally modified $MccA^{Bam}$ (Figure 1C). Fragmentation of the m/z 2574.4 compound resulted in major daughter ions corresponding to unmodified $MccA^{Bam}$ (m/z 2269) and its dehydrated form (m/z 2251). Signals matching $MccA^{Bam}$-phosphate (m/z 2331) and $MccA^{Bam}$-phosphate-ribose (m/z 2463) were also clearly seen. Therefore, the putative nucleobase of the m/z 2574.4 compound has a molecular weight of 111 Da. Analysis of fragmentation spectrum of the m/z 2602.4 compound, which produces a 2279 daughter ion matching N-formylated $MccA^{Bam}$, led to the same conclusion. In contrast, fragmentation of the m/z 2632.4 compound suggested a 169 Da putative nucleobase mass. While neither calculated mass difference matched adenine (135 Da), the 111 Da mass shift matches cytosine. The 169 Da mass

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change might correspond to an unknown or modified known nucleobase.

MccB^{Bam} Exhibits Unusual Nucleotide Specificity. To check if MccB^{Bam} indeed modifies the precursor peptide with CMP, recombinant MccB^{Bam} protein was prepared and combined with chemically synthesized MccA^{Bam} peptide in the presence of ATP, CTP, UTP, or GTP. Mass-spectrometric analysis revealed the presence of the peptide (m/z 2269.3) and succinimide intermediate (m/z 2251.3) peaks in all four reactions (Figure 2A). Peaks corresponding to peptide−nucleotides were also detected. The apparent efficiency of in vitro modification decreased in the order CTP ≫ ATP ∼ UTP ≫ GTP. If the reaction was carried out in the presence of all four nucleotide triphosphates, only the MccA^{Bam}-CMP product was detected (Figure 2A, lower panel). The observed preference for CTP is consistent with detection of MccA^{Bam}-CMP in the *B. subtilis* surrogate production host.

The unexpected nucleotide preference of MccB^{Bam} could have been caused by the presence of an additional domain MccB$_{CTD}^{Bam}$ that is absent from the *E. coli* and most other homologues. The N-terminal fragment of MccB^{Bam} lacking the methyltransferase domain was prepared and its ability to modify cognate MccA peptide was investigated. The mutant, MccB$_{NTD}^{Bam}$, variant exhibited the same promiscuity toward NTP substrates (Figure 2B), indicating that the C-terminal domain is not involved in substrate specificity modulation.

Intrigued by substrate promiscuity of the MccB^{Bam} we tested if MccB^{Eco} also can modify its cognate peptide MccA^{Eco} MRTGNAN with various nucleotides. Under our reaction conditions, only adenylated peptide was observed (Figure 2C). Yet, when we tested formylated MccA^{Eco}, which should be considered as a bona fide ribosomally synthesized MccB^{Eco} substrate, modification products by each of the four individual nucleotides were observed (Figure 2D). However, the preference to ATP was retained when the enzyme was combined with an equimolar mixture of all four nucleotides (Figure 2D, lower spectrum).

MccS^{Bam} and MccB$_{CTD}^{Bam}$ Are Responsible for Additional Modification of Peptidyl-Cytidylate. MccS^{Bam} is a homologue of the *E. coli* cxSAM-synthase CmoA. CmoA uses SAM and prephenate to produce cxSAM,[15,16] which is then used by the second enzyme, CmoB, as a donor of carboxymethyl group in the reaction of carboxymethylation of 5-hydroxyuridine in wobble positions of various tRNAs.[15,17] We hypothesized that MccS^{Bam} and MccB$_{CTD}^{Bam}$ can also act as a synthase-transferase pair and modify the peptide cytidylated by MccB$_{NTD}^{Bam}$. Indeed, the observed mass difference of 58 Da between cytidylated MccA^{Bam} peptide (m/z 2574.4) and another peptide−nucleotide (m/z 2632.4) detected in *B. subtilis* (Figure 1B) matches carboxymethylation. To test this hypothesis, we determined whether (i) MccS^{Bam} can produce cxSAM from SAM and prephenate and (ii) MccB$_{CTD}^{Bam}$ can use cxSAM to carboxymethylate MccA^{Bam}-CMP.

Upon incubation of MccS^{Bam} or *E. coli* CmoA with SAM and prephenate, a new peak appeared during HPLC separation of reaction products that was absent from the mixture of SAM and prephenate incubated without added proteins (compare the trace shown in Figure 3A with those shown in Figures 3BC). The same peak was observed among the multiple products of a reference reaction of where cxSAM was chemcially synthesized from *S*-adenosylhomocysteine and iodoacetic acid[15,18] (Figure 3D). In all cases, these HPLC peaks contained a substance with [MH+] of 443.2, which matches cxSAM. The main signals in

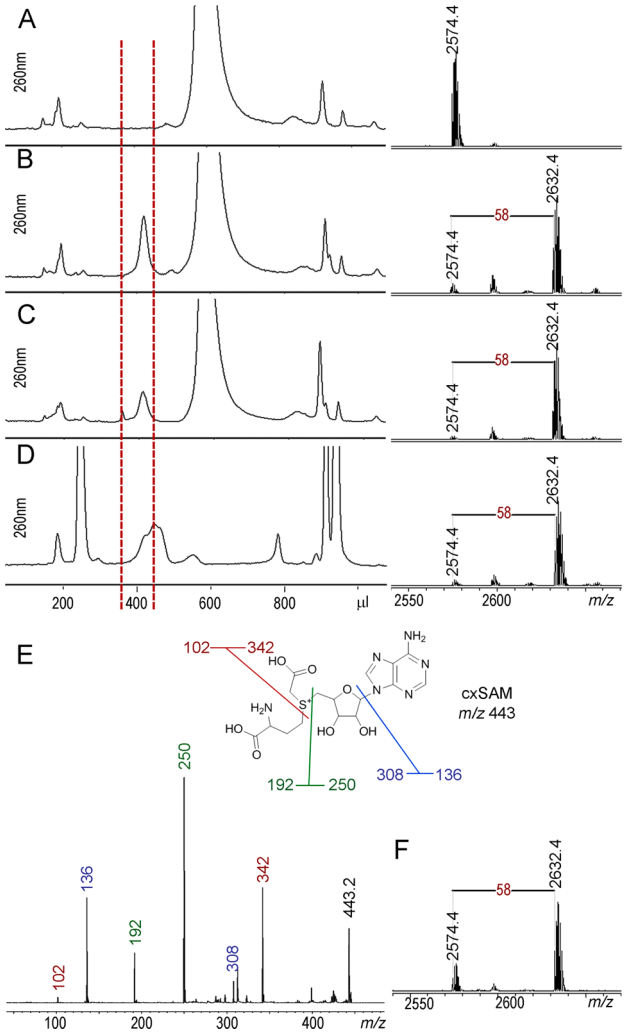

Figure 3. MccS^{Bam} synthesizes cxSAM which is used as a donor of carboxymethyl group during modification of MccA^{Bam}-CMP by MccB$_{CTD}^{Bam}$. Left sides of panels (A−D) show HPLC traces of cxSAM synthesis reactions products. (A) A mixture of SAM and prephenate without enzyme addition. (B) SAM and prephenate incubated with MccS^{Bam}. (C) SAM and prephenate incubated with *E. coli* CmoA. (D) Chemical synthesis of cxSAM from SAH and iodoacetic acid. Collected HPLC fractions used for in vitro modification of MccA^{Bam}-CMP with MccB^{Bam} are marked with dashed lines. On the right of each panel MALDI MS spectra of reactions products obtained after incubation of MccA^{Bam}-CMP (m/z 2574.4) with recombinant MccB^{Bam} and material from the indicated HPLC fractions are shown. The newly appearing m/z 2632.4 mass peak matches that obtained in *B. subtilis* cells carrying plasmid-borne *mcc*^{Bam} cluster (Figure 1B). (E) A representative fragmentation spectrum of m/z 443.2 parental ion from HPLC fractions indicated in (B−D). m/z peaks of complementing cxSAM fragments are shown in same colors. For details of peaks interpretation see text. (F) MALDI MS analysis of MccA^{Bam}-CMP carboxymethylation reaction by recombinant MccB$_{CTD}^{Bam}$ in the presence of cxSAM.

fragmentation spectra of these mass-ions were those of adenine (m/z 136) and its complementary part (m/z 308); adenosine (m/z 250) and its complementary part (m/z 192); and the 3-amino-3-carboxypropyl tail of methionine (m/z 102) and its complementary part (m/z 342) (Figure 3E). Since the adenosine moiety of the [MH+] 443.2 compound was not modified, the methionine part must have been carboxylated. Since 3-amino-3-carboxypropyl tail was not modified, the

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carboxylic group must be attached to the S-methyl group of the methionine moiety. We therefore conclude that $MccS^{Bam}$ indeed synthesizes carboxy-*S*-adenosyl-L-methionine from SAM and prephenate, and is therefore a functional analog of *E. coli* CmoA.

We next tested if $MccB^{Bam}$ can use cxSAM to modify the cytidine moiety of $MccA^{Bam}$-CMP. When purified peptidyl-cytidylate and full-sized $MccB^{Bam}$ were combined with enzymatically prepared or chemically synthesized cxSAM, an m/z = 2632.4 mass-ion appeared indicating that conversion of the $MccA^{Bam}$-CMP to $MccA^{Bam}$-carboxymetylated CMP (-cxCMP) had taken place (Figure 3B–D, right panels). Fragmentation patterns of the newly synthesized compounds matched that of m/z 2632.4 compound produced in *B. subtilis* (Figure 1C, lower panel), suggesting that carboxymethylation of peptidyl-cytidylate takes place both in vivo and in vitro.

We tested if $MccB_{CTD}^{Bam}$ alone is sufficient for transferring the carboxymethyl moiety from cxSAM to peptidyl-cytidylate. As can be seen from Figure 3F, $MccB_{CTD}^{Bam}$ successfully caboxymethylated the purified $MccA^{Bam}$-CMP in the presence of cxSAM. When $MccA^{Bam}$-AMP, $MccA^{Bam}$-UMP, and $MccA^{Bam}$-GMP were tested in reactions with $MccB_{CTD}^{Bam}$ and cxSAM, no modification was observed (data not shown), indicating that this modification is specific for cytidine.

The entire biosynthesis pathway of $MccA^{Bam}$-cxCMP was reconstituted in vitro in the presence of $MccA^{Bam}$, $MccB^{Bam}$, $MccS^{Bam}$, CTP, SAM, and prephenic acid (Figure S1). Omission of either component led to the disappearance of the $MccA^{Bam}$-cxCMP product peak. The $MccA^{Bam}$-cxCMP was, however, present, when *E. coli* cxSAM synthase CmoA was used instead of $MccS^{Bam}$ in the coupled $MccA^{Bam}$ modification reaction (Figure S1). The peak corresponding to $MccA^{Bam}$-cxCMP was absent when $MccB_{NTD}^{Bam}$ was used instead of full-sized $MccB^{Bam}$. Overall, the data clearly show that $MccB_{CTD}^{Bam}$ is carboxymethyl transferase that uses cxSAM produced by MccS to modify $MccA^{Bam}$ cytidylated by $MccB_{NTD}^{Bam}$.

In Vivo and In Vitro Activity of Peptide-Cytidylates. Attempts to detect biological activity of in vitro synthesized McC^{Bam}, with or without the carboxymethyl modification, were unsuccessful. Neither *B. subtilis* 168 nor several *E. coli* strains appeared to be sensitive to these compounds. At least in the case of *E. coli*, the result could be due to poor intracellular transport, lack of processing, or the absence of appropriate target. The serendipitous observation about decreased nucleotide specificity of $MccB^{Eco}$ when presented with formylated cognate peptide substrate allowed us to obtain, in addition to $fMccA^{Eco}$-AMP, the $fMccA^{Eco}$-CMP, $fMccA^{Eco}$-UMP, and $fMccA^{Eco}$-GMP compounds. An $MccA^{Eco}$-cxCMP variant was also prepared by in vitro carboxymethylation of $fMccA^{Eco}$-CMP with the $MccB_{CTD}^{Bam}$-$MccS^{Bam}$ pair (Figure S2). The resulting compounds contained the *E. coli* fMccA transport peptide that in the context of AMP modification is recognized by the YejABEF transporter[6] and is efficiently processed by intracellular aminopeptidases.[19] Each of the compounds was tested against a wild-type *E. coli* strain and isogenic *yej* mutant in the spot bioactivity test. An *sbmA* mutant lacking a transporter that is not involved in McC^{Eco} transport was included as a control. As can be seen in Figure 4A, $MccA^{Eco}$-AMP, $MccA^{Eco}$-CMP, and $MccA^{Eco}$-cxCMP inhibited the growth of wild-type and *sbmA* strains. The MIC values determined using spot bioactivity test were 1, 40, and 20 μM for $MccA^{Eco}$-AMP, $MccA^{Eco}$-CMP, and $MccA^{Eco}$-cxCMP, respectively. The growth of the *yej* mutant was unaffected.

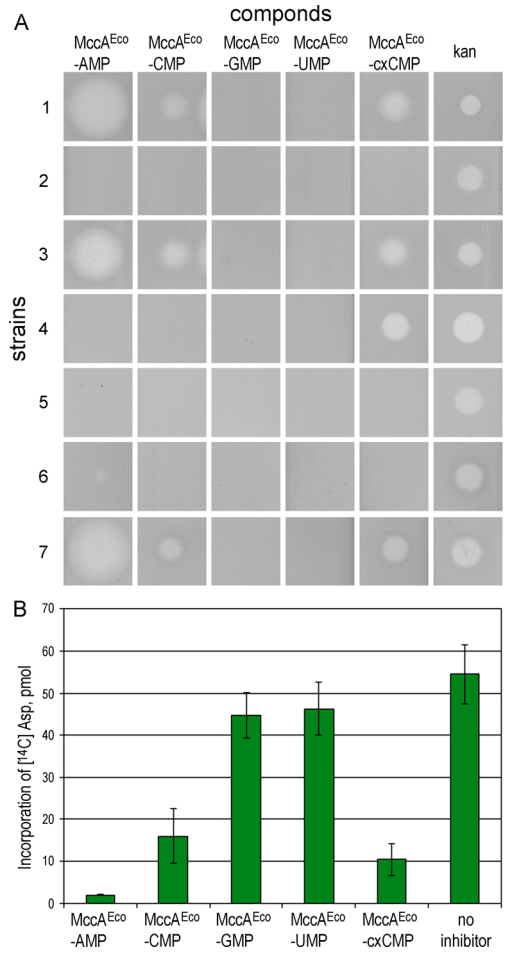

Figure 4. Biological activity of peptidyl-cytidylates. (A) Five microliters of 50 μM solutions of HPLC-purified peptidyl-nucleotides were deposited onto lawns of *E. coli* B cells. Compounds tested are indicated on the top. Panels are arranged in rows showing results obtained with different tester strains: (1) wild type *E. coli* B; (2) *E. coli* B Δ*yejB*; (3) *E. coli* B Δ*sbmA;* (4) *E. coli* B overexpressing MccE; (5) *E. coli* B overexpressing MccF; (6) *E. coli* B overexpressing Asp-RS; and (7) *E. coli* B overexpressing Pro-RS. Growth inhibition zones are seen as clear circles on the turbid surface of cell lawn. (B) In vitro inhibition of aminoacylation of $tRNA^{Asp}$ by peptidyl-nucleotides. S30 extracts prepared from wild-type *E. coli* cells were supplied with HPLC-purified compounds and incorporation of radioactive aspartate was measured. Water was used as a blank control. Error bars show standard deviations calculated from three independent measurements.

E. coli cells that express MccE become resistant to McC due to acetylation of processed McC;[8] cells that express MccF become resistant due to cleavage of intact or processed McC.[9] Cells expressing MccF were resistant to both $MccA^{Eco}$-CMP and $MccA^{Eco}$-cxCMP. In contrast cells expressing MccE were resistant to $MccA^{Eco}$-CMP but remained sensitive to $MccA^{Eco}$-cxCMP (Figure 4A). Thus, carboxymethylation of cytidine moiety allows to overcome one of the two resistance mechanisms to McC-like peptide–nucleotide antibiotics.

Cells expressing Asp-RS but not Pro-RS became resistant to $MccA^{Eco}$-AMP, $MccA^{Eco}$-CMP, and $MccA^{Eco}$-cxCMP (Figure 4A). To directly demonstrate that peptidyl-cytidylates target Asp-RS, in vitro $tRNA^{Asp}$ aminoacylation reactions were carried out. An S30 *E. coli* extract was incubated with $MccA^{Eco}$-derived peptide–nucleotides to preprocess the peptide part of antibiotics and $tRNA^{Asp}$ aminoacylation reaction was next carried

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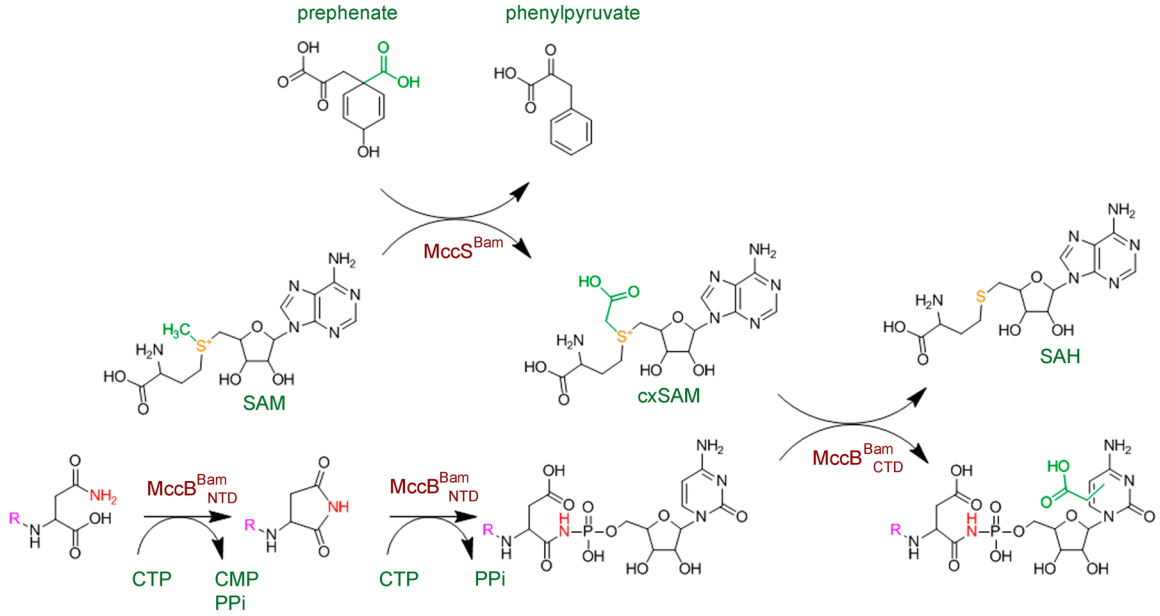
Scheme 1. Proposed McC^{Bam} Biosynthetic Pathway[a]



[a]N-terminal residues of MccA (MLKIRKVKIVRAQNGHYT) are denoted as R for clarity.

out. As can be seen from Figure 4B, MccA^{Eco}-AMP, MccA^{Eco}-CMP, and MccA^{Eco}-cxCMP inhibited incorporation of radioactive Asp in acid-insoluble fraction, while MccA^{Eco}-UMP and MccA^{Eco}-GMP were inactive. A mimic of processed MccA^{Eco}-AMP aspartyl sulfamoyl adenosine (DSA) and aspartyl sulfamoyl cytidine (DSC), a mimic of processed MccA^{Eco}-CMP, were also tested (Figure S3). Aminoacylation reactions were carried out immediately after DSA or DSC addition, since no processing is required. In both cases, inhibition of aminoacylation was observed. Both with peptidyl-nucleotides and with aminoacyl sulfamoyl nucleotides, adenylated compounds were better inhibitors than cytidylate ones.

DISCUSSION

There are roughly a dozen of known natural cytosine-containing antibiotics,[20] all of them being the products of nonribosomal synthesis. In this work we describe the first bioactive RiPP modified with cytidine. During the synthesis of *E. coli* microcin C the attachment of the nucleotide moiety to the peptide part is carried out by adenylyltransferase MccB^{Eco} via a two-step mechanism:[3,21] first, carboxyl group of the C-terminal Asn residue of MccA is activated with ATP forming a liable peptidyl-AMP intermediate, which undergoes intramolecular cyclization resulting in the formation of peptidyl-succinimide; second, a new molecule of ATP is used for generation of stable peptidyl-nucleotide with nonhydrolyzable N−P bond. Our work reveals, surprisingly, that the MccB enzymes have relaxed specificity toward the nucleobase moiety of NTP substrate at both stages of the reaction. Thus, the nucleotide-binding site of MccB enzymes should be able to accommodate different nucleoside triphosphates substrates. It remains to be seen how such plasticity is accomplished structurally. MccB^{Bam} has a clear preference for CTP at both stages of the reaction. In contrast, MccB^{Eco} prefers ATP. Interestingly, the specificity of MccB^{Eco} is strongly modulated by the presence of the formyl group located at the other end of the peptide substrate. The mechanism of this modulation remains unknown but can be caused by more avid binding of formylated MccA to the enzyme, allowing the presumably slower reaction of modification with less preferred nucleotide to occur. MccB proteins are part of a very large family of THIF-type NAD/FAD adenylyltransferases. While many enzymes from the family were shown to function as adenylyltransferases, most have not been studied. Our work suggests that many enzymes of the family may be transferring nucleotides other than AMP, raising a possibility that there remains a very large class of post-translational modifications that remain to be discovered.

At the last stage of McC^{Bam} maturation, the C-terminal domain of MccB^{Bam} transfers the carboxymethyl group to the nucleobase of peptidyl-cytidylate using the cxSAM as a donor (Scheme 1). The cxSAM cofactor was discovered during analysis of the *E. coli* CmoA-CmoB enzyme pair. CmoA^{Eco} participates in modification of uridine to 5-oxyacetyl uridine (cmo5U) at the wobble position of anticodon loop of tRNAs by generating cxSAM used by the CmoB enzyme.[15] CmoA-like enzyme MccS^{Bam} encoded in the *mcc*^{Bam} cluster also synthesizes cxSAM. MccS^{Bam} can be substituted by CmoA^{Eco} during McC^{Bam} biosynthesis.

To the best of our knowledge, our study reveals only the second instance of the use of cxSAM in living cells. Linked genes encoding CmoA-CmoB homologues are characteristic for *Proteobacteria* and some *Cyanobacteria*,[17] where they likely perform a function similar to that in *E. coli*, but are extremely rare in Gram-positive bacteria. The C-terminal domain of MccB^{Bam} does not show any significant similarity to CmoB from *E. coli*. Since it uses cxSAM for modification of cytosine instead of 5-hydroxy uridine it might employ a different mechanism for carboxymethyl transfer. In contrast to CmoB^{Eco}, MccB$_{CTD}^{Bam}$ has high specificity to cxSAM since no alkyl transfer was detected in the presence of SAM. Genes encoding CmoA homologues without neighboring *cmoB* homologues are common and our work suggests that such enzymes may produce cxSAM for nucleobase modification in bioactive compounds unrelated to tRNA.

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$MccB_{CTD}^{Bam}$ appears to lack specificity toward the peptide part of peptide-cytidylates, which allowed us to produce carboxymethylated cytidylate of *E. coli* MccA. The exact position of carboxymethylation in the nucleobase remains to be determined. The modification is functionally important for while it has a marginal effect on target inhibition, it inactivates one of the mechanisms of resistance to McC-like compounds, clearly a beneficial trait for the producing organism.

Nonhydrolyzable aminoacyl-adenylates are potent inhibitors of tRNA aminoacylation reactions that proceed through an obligatory activated aminoacyl-adenylate intermediate. Thus, it was an expected result that the common product of proteolytic processing of various peptide-adenylates related to *E. coli* microcin C inhibits Asp-RS.[7] Processed peptide-cytidylates studied in this work also inhibit Asp-RS. The result is not a consequence of general promiscuity of Asp-RS to the nucleotide moiety of aspartyl-nucleotides, since introduction of uridine or guanosine at the terminus of the MccA peptide had no inhibitory effect either in vivo or in vitro. The specificities of other aminoacyl-tRNA synthetases may be different, since a study of chemically synthesized isoleucyl-sulfamoyl nucleoside analogues attributed the highest inhibitory activity toward Ile-RS to uridylated followed by adenylated- and cytidylated compounds.[22] While the group of organisms naturally targeted by *B. amyloliquefaciens* DSM7 producing McC^{Bam} is presently unknown, one may hypothesize that the Asp-RS from these bacteria is inhibited more efficiently by aspartyl-cytidylates than by the adenylate.

Several other natural pyrimidine-containing compounds that bind to the catalytic center of aminoacyl tRNA synthetases are known. One is a natural Trojan-horse antibiotic, albomycin δ_2, produced by *Streptomyces* sp. It comprises of the vehicle siderophore part and the inhibitor part, an N4-carbamoyl-3-methylcytosine attached to diseryl-thioxylofuranosyl moiety.[23,24] Processed albomycin δ_2 inhibits Ser-RS, though its structure is completely different from that of processed McC^{Bam}.

Bioinformatics analysis predicts that *mcc* operons from evolutionary distant bacteria likely produce cytidylated, rather than adenylated RiPPs. Some of these operons also contain additional genes, distinct from functionally characterized *mcc* genes of *E. coli* and the *B. amyloliquefaciens* genes characterized in this work.[13] The products of these genes may be carrying out additional decorations that increase the activity of basic peptidyl-nucleotides and/or decrease resistance of targeted bacteria. The reasons why a particular operon evolves to produce a particular kind of peptide−nucleotide and additional decoration sets presents an interesting evolutionary and functional problem that remains to be solved. In the interim, the ability to attach various modified nucleotides to peptides capable of facilitated transport into cells presents an attractive strategy for design of novel antibacterial compounds.

EXPERIMENTAL SECTION

Bacterial Strains and Media. *B. amyloiquefaciens* DSM7 was purchased from German Collection of Microorganisms and Cell Cultures (DSMZ). All cloning was performed in *E. coli* DH5a. *B. subtilis* 168 was used as a heterologous host for *B. amyloliquefaciens mcc* cluster expression. Protein expression and in vivo McC activity tests were done in *E. coli* B strain. *E. coli* BW25113 Δ*rimI* Δ*rimL* Δ*rimJ*[11] was used for in vitro Asp-RS inhibition assay. Bacteria were grown on LB medium supplemented with antibiotics as required. M9 minimal medium complemented with 0.1% casamino acids, 0.5% glycerol, and either 0.01% yeast extract (for *E. coli*) or 5 mg/L of L-tryptophan (for *B. subtilis*) was used where indicated.

Cloning, Protein Expression, and Purification. Sequences of all primers are listed in Table S1. *B. amyloliquefaciens mcc* gene cluster including its 5′ intergenic region was amplified with Phusion DNA polymerase and primers Bam_F and Bam_R. PCR product was cloned into pHT01 shuttle vector between *Kpn*I and *Bam*HI to create pHT-*mcc*. This removes plasmid-borne pGrac promoter and introduces the *mcc* natural promoter. pHT-*mcc* plasmid was transformed into *B. subtilis* 168 by electroporation and clones were selected on LB agar plates with 10 μg/mL chloramphenicol. McC^{Bam} production by the heterologous *B. subtilis* host was tested by MALDI MS of cells grown for 8 h (OD_{600} = 1) in LB broth supplemented with 10 μg/mL chloramphenicol.

Full length $MccB^{Bam}$ from *B. amyloliquefaciens* and $MccB^{Eco}$ from *E. coli* were expressed as fusion proteins with N-terminal leaderless MalE. For this, the corresponding PCR products were introduced between *Bam*HI and XhoI restriction sites of pET22MBP.[13] DNA fragments spanning 1−348 codons and 348−612 codons of *B. amyloliquefaciens mccB* were cloned into *Bam*HI and XhoI restriction sites of pET22MBP to obtain MBP-fused $MccB_{NTD}^{Bam}$ and $MccB_{CTD}^{Bam}$ correspondingly. Coding sequences of *E. coli cmoA* and *B. amyloliquefaciens mccS* were cloned into pET28 between *Bam*HI and XhoI sites to create N-terminally fused His tag. *E. coli aspS* and *proS* were cloned into pBAD-His between NcoI and XhoI sites to create pBAD-AspRS and pBAD-ProRS plasmids.

For protein expression *E. coli* BL21(DE3) were transformed with the proper plasmids, grown on LB medium supplemented with either kanamycin or ampicillin at 37 °C until OD_{600} reached 0.6 and induced with 1 mM isopropyl β-D-1-thiogalactopyranoside. Cells were allowed to grow for an additional 11−16 h at 20 °C before harvesting. Pelleted cells were washed with loading buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM DTT) and resuspended in the same buffer supplemented with 0.2 mM phenylmethane sulfonyl fluoride and 1 mg/mL lysozyme. After sonication the lysates were cleared by centrifugation at 15 000 rcf for 30 min. At this stage for His-tagged proteins purification imidazole was added up to 10 mM. His-tagged proteins were loaded to HiTrap Chelating HP column (GE Healthcare) precharged with Co^{2+} and equilibrated with the loading buffer. Proteins were eluted with the 10 to 500 mM linear gradient of imidazole. MBP-fused proteins were purified on MBPTrap HP (GE Healthcare) and eluted with 10 mM maltose in the loading buffer. Purity of proteins was checked on Coomassie-stained SDS gels.

In Vitro Nucleotidyl Transferase Assay. Synthetic peptides $MccA^{Bam}$ (MLKIRKVKIVRAQNGHYTN) and $MccA^{Eco}$ (MRTGNAN) with and without N-terminal formyl group were purchased from GenScript (USA). Reactions contained 100 μM peptide, 2 μM of the corresponding enzyme, and 5 mM of one of the NTPs in 100 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT. For the nucleotide selection test all four NTPs were added to the reaction at the 2 mM final concentration. Reaction mixtures were incubated for 1 to 16 h at 32 °C depending on the required yield of the peptide−nucleotide. When necessary, reaction was quenched with 0.1% trifluoroacetic acid (TFA), precipitated protein was removed by centrifugation and soluble peptide−nucleotides were purified using Eclipse Plus C18 column (4.6 × 250 mm, particle size 5 μm) (Agilent Technologies) at flow rate of 1 mL/min. A 10 min (approximately 2.5 column volumes) linear gradient from 5 to 15% acetonitrile in 0.1% TFA was applied. Elution of substances was monitored by absorption at 260 nm. Fractions corresponding to the peptide−nucleotides were dried in a rotary evaporator and redissolved in water. Published extinction coefficients for nucleotide monophosphates were used to calculate peptidyl nucleotide concentrations.

cxSAM Synthesis. For enzymatic cxSAM production 1 mM SAM and 1 mM prephenate were incubated with either 10 μM CmoA or 10 μM $MccS^{Bam}$ in 100 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM DTT for 2 h at 32 °C. Chemical synthesis of cxSAM was performed as described in Kim et al., 2013.[15] In brief, 30 mg of 2-iodoacetic acid was added to 1 mL of the 5 mg/mL suspension of *S*-Adenosyl-L-homocysteine in 300 mM ammonium bicarbonate and incubated at 37

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°C for 20 h with constant agitation. Both enzymatically and chemically synthesized cxSAM was partially purified using RP-HPLC. Analytical chromatography was performed on ProntoSil-120−5-C18 AQ column (particle size 5 μm, 2.0 × 75 mm column dimensions) (Econova, Russia) at a flow rate of 0.2 mL/min with UV detection at 260 nm. The column was equilibrated with 10 mM Na_2HPO_4, pH 5.9. cxSAM was eluted in a linear gradient from 0 to 25% acetonitrile in the presence of 10 mM Na_2HPO_4 in 8 min. Preparative purification was performed using C18 Eclipse Plus (4.6 × 100 mm, 5 μm particle size) (Agilent Technologies) column and eluted using the same buffer system. Neutral pH and low acetonitrile content in the eluted cxSAM samples allowed their direct use in the carboxymethylation reactions.

In Vitro Carboxymethylation. Reaction mixture contained 100 μM purified $MccA^{Eco}$-CMP or $MccA^{Bam}$-CMP peptidyl-cytidylates, ∼0.5 mM cxSAM, and 2 μM $MccB_{CTD}$ in 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM DTT. Reaction was carried out for 6 h at 32 °C then quenched with 0.1% TFA, and centrifuged to remove protein precipitate. Compounds were purified the same way as peptidyl-nucleotides. Fractions containing carboxymethylated peptide-cytidylates were dried in a rotary evaporator and redissolved in water.

MALDI MS and MS/MS Analysis. 1-μL aliquot of in vitro reaction mixture or ∼0.2 μL of cells were diluted in 10 μL of 0.5% TFA. One μL of the diluted sample was mixed with 0.5 μL of 2,5-dihydroxybenzoic acid solution (20 mg/mL in 30% acetonitrile, 0.5% TFA) and left to dry on the stainless steel target plate at room temperature. MALDI-TOF MS analysis was performed on UltrafleXetreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonik, Germany) equipped with Nd laser. The MH+ molecular ions were measured in reflector mode; the accuracy of monoisotopic mass peak measurement was within 30 ppm. Spectra were acquired by averaging of a minimum 1000 laser shots from "sweet spots" of matrix crystals. Spectra of fragmentation were obtained in LIFT mode, the accuracy of daughter ions measurement was within 1 Da range. Mass-spectra were processed with the use of FlexAnalysis 3.2 software (Bruker Daltonik, Germany) and analyzed manually.

In Vivo Sensitivity Test. Wild type *E. coli* B strain and its isogenic deletion mutants Δ*yejB* and Δ*sbmA* transformed with vector plasmid pBAD-His were used as tester strains. For protein target and immunity mechanism verification tests, *E. coli* B strain transformed with expression plasmids pBAD-AspRS, pBAD-ProRS, pBAD-MccF, and pBAD-MccE were used. Cells were grown in LB medium supplemented with 100 μg/mL ampicillin and 10 mM arabinose. The overnight culture was diluted 1000-fold with soft agar containing M9 medium supplemented with 1% glycerol, 0.01% yeast extract, 10 mM arabinose and 100 μg/mL ampicillin. 5-μL drops of 20 μM peptide−nucleotides solutions were deposited on the surface of the solidified agar and allowed to dry. Kanamycin (1 mg/mL) was used as a control growth inhibitor. Plates were incubated for 16 h at 30 °C to form a lawn.

tRNA Aminoacylation. *E. coli* BW25113 Δ*rimI* Δ*rimJ* Δ*rimL* were grown in 50 mL of LB medium at 37 °C until OD_{600} reached 0.8. Cells were collected by centrifugation, washed with 20 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 100 mM KCl, resuspended in 1 mL of the same buffer supplemented with 1 mM DTT, and disrupted by sonication. The S30 cell lysate was prepared as described earlier.[11] To process the peptide part, 25 μM peptidyl-nucleotides were preincubated with S30 cell lysate (3 mg/mL final protein concentration) for 2 h at 25 °C. Sixteen μL of the aminoacylation mixture (30 mM Tris-HCl, pH8.0, 30 mM KCl, 8 mM $MgCl_2$, 1 mM DTT, 3 mM ATP, 5 mg/mL bulk *E. coli* tRNA, 23 μM ^{14}C-Asp) were combined with 4 μL of processed peptidyl-nucleotide samples and incubated for 5 min at 25 °C. The reaction mixture was dried on Whatman 3MM paper filters. After washing with ice-cold 10% trichloroacetic acid, filters were washed with acetone and dried. The incorporation of radioactivity into insoluble fraction was determined in a scintillation counter.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b09853.

Figures S1−S3 and Table S1 (PDF)

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Notes

The authors declare no competing financial interest.

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