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The Molecular Mechanism of Aminopropylation of Peptide-Nucleotide Antibiotic Microcin C

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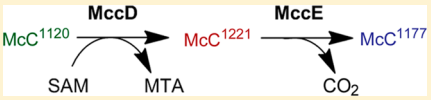
Supporting Information

ABSTRACT: Translation inhibitor microcin C (McC) is a heptapeptide with an aspartate α-carboxyl group linked to AMP via phosphoramidate bond. Modification of the McC phosphate by an aminopropyl moiety increases the biological activity by ~10-fold. Here, we determine the pathway of the aminopropylation reaction of McC. We show that the MccD enzyme uses *S*-adenosyl methionine to transfer 3-amino-3-carboxypropyl group onto a phosphate of an McC maturation intermediate consisting of adenylated heptapeptide. The carboxyl group is removed by the MccE enzyme, yielding mature McC. MccD is an inefficient enzyme that requires for its action the product of *Escherichia coli mtn* gene, a 5′-methylthioadenosine/*S*-adenosylhomocysteine nucleosidase, which hydrolyses 5′-methylthioadenosine, the product of MccD-catalyzed reaction, thus stimulating the amino-3-carboxypropylation reaction. Both MccD and MccE are capable of modifying McC-like compounds with divergent peptide moieties, opening way for preparation of more potent peptidyl-adenylates.



INTRODUCTION

Microcin C (McC) is a peptidyl-nucleotide antibiotic produced by *Escherichia coli* strains harboring a plasmid-borne *mcc* operon.[1,2] McC is active against *E. coli* strains as well as other enteric bacteria that lack such operon.[3] During the first step of McC biosynthesis, the C-terminal residue of the ribosomally synthesized MccA precursor heptapeptide fMRTGNAN is covalently linked via a phosphoramidate bond to adenosine by the ATP-dependent synthetase MccB.[4] The C-terminal asparagine residue of MccA is converted to a derivitized aspartate in the course of this modification.[4] During the second step of McC biosynthesis, the phosphate group of adenylated peptide is additionally decorated by an aminopropyl moiety (Figure 1). This step requires the products of the *mccD* and *mccE* genes.[5] Mature McC is actively uptaken by sensitive cells through inner-membrane transporter YejABEF.[6] Once inside the cell, McC undergoes proteolytic processing by cytoplasmic aminopeptidases.[7] As a result, the processed McC, which is a nonhydrolyzable C-terminally modified aspartyl-aminopropyl-adenylate, is released inside the cell. The processed McC inhibits the aspartyl-tRNA synthetase, causing cessation of protein synthesis and cell growth.[8]

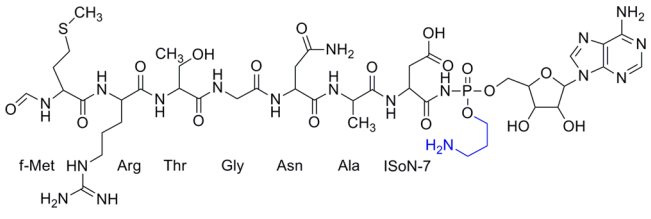


Figure 1. Structure of the mature microcin C (McC[1177]). Adenosine monophosphate part is attached to the formylated heptapeptide by the nonhydrolyzable phosphoramidate bond. 3-aminopropyl moiety, a characteristic attribute of the mature McC, is shown in blue.

The product of MccB-catalyzed adenylation of MccA, a peptidyl-adenylate with a molecular weight of 1119 Da, is biologically active, albeit its activity is ~10-fold less than that of the mature aminopropylated McC with a molecular weight of 1176 Da.[5] MccE, one of the two enzymes required for aminopropylation, consists of two domains. The N-terminal domain (NTD) is similar to pyridoxal 5-phosphate (PLP)-dependent decarboxylases, while the C-terminal domain (CTD) is similar to RimL-like GNAT acetyltransferases. The acetyltransferase activity of MccE-CTD is essential for self-immunity of the McC-producing cell[9,10] but appears to be dispensable for aminopropylation.[10] The fact that the two domains of MccE are encoded by separate genes in *mcc*-like operons from some bacteria other than *E. coli*[11,12] suggests that only the putative decarboxylase activity of MccE-NTD is involved in aminopropylation.

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Another enzyme required for McC aminopropylation is MccD. This protein belongs to a superfamily of SAM-dependent methyltransferases, and contains an *S*-adenosylmethionine (SAM) binding motif. Homologues of MccD are also present in *mcc*-like operons found in *Yersinia pseudotuberculosis*, *Synechococcus* sp.[13] and in *Pectobacterium wasabiae WPP163* (Gene ID: 8528966). SAM is a multipurpose molecule, which is commonly used in the cell as a methyl donor for DNA, RNA, and protein methylation reactions. SAM also serves as a 3-amino-3-carboxypropyl (ACP) donor and is involved in other types of group transfer reactions.[14] SAM-dependent ACP-transfer reactions are relatively rare and specialized.[15] ACP transfer to a nitrogen acceptor occurs in the biosynthesis of 3-(3-amino-3-carboxypropyl) uridine and 1-methyl-3-(3-amino-3-carboxypropyl) pseudouridine.[16,17] It was also demonstrated in the biosynthesis of 2-(3-amino-3-carboxypropyl)-isoxazolin-5-one, a neurotoxic amino acid from *Lathyrus odoratus*.[18] Examples of ACP transfer to an oxygen acceptor include the biosynthesis of a bacterial betaine lipid diacylglyceryl-*O*-4′-(*N*,*N*,*N*-trimethyl)homoserine[19] and the biosynthesis of isonocardicin, a β-lactam type antibiotic.[20] The ACP-transfer reactions presumably occur via a simple nucleophilic mechanism. Currently available data is insufficient for reliable identification of enzymes responsible for ACP-transfer reactions by bioinformatics means.[21,22]

Theoretically, the ACP group of SAM could be the source of aminopropyl moiety during McC maturation. In this scenario, one has to assume a two-step reaction with the initial ACP-transfer to the phosphate group of adenylated MccA by MccD with subsequent decarboxylation by MccE-NTD. An alternative route could be similar to the one described for polyamine biosynthesis, where SAM is first decarboxylated by MccE-NTD to yield decarboxy-SAM, followed by transfer of the 3-aminopropyl group[23] by MccD. In the present article we define the pathway of MccD/MccE-catalyzed aminopropylation of adenylated MccA and show that an additional enzyme encoded by the *E. coli* genome, Mtn (5′-methylthioadenosine/*S*-adenosylhomocysteine nucleosidase), is required for this modification. We further show that *E. coli* MccD/MccE pair can be used together with Mtn to efficiently modify heterologous peptidyl-adenylates leading to more potent antibacterials.

RESULTS AND DISCUSSION

***In vitro* Characterization of MccD/MccE-Mediated Reactions of McC Maturation.** To investigate the functional role of MccD and MccE in microcin C maturation pathway, we monitored the changes in molecular mass of McC maturation intermediate, adenylated MccA peptide MRTGNAD-AMP, m/z = 1120, hereafter referred to as McC^{1120}, in the presence of MccD and/or MccE using MALDI-TOF mass spectrometry (MS) (see Experimental Section for details). McC^{1120} was prepared from producing *E. coli* cells lacking the *mccD* and *mccE* genes.

Upon incubation of McC^{1120} with purified recombinant MccD, MccE, or both proteins in the presence of SAM, the likely ACP group donor, MALDI-MS analysis did not reveal any changes to McC^{1120}. This result may indicate that (i) our preparations of MccD and/or MccE proteins are inactive; (ii) MccD and/or MccE require an additional factor(s) to carry out the modification reaction; (iii) SAM is a wrong donor. The experiment was therefore repeated with extracts prepared from *E. coli* cells individually overexpressing *mccD* or *mccE* or co-overexpressing both genes from arabinose-inducible pBAD plasmid. Since the extracts of wild-type *E. coli* contain aminopeptidases PepA, PepB, and PepN each capable of rapidly processing McC^{1120},[7] and thus degrading the MccD/MccE reaction substrate, extracts were prepared from MccD- and/or MccE-overproducing *E. coli* cells lacking the *pepA*, *pepB*, and *pepN* genes. As expected, the incubation of McC^{1120} with the extracts of induced cells harboring an empty pBAD plasmid had no effect on the mass of McC^{1120} (Figure 2A). In contrast,

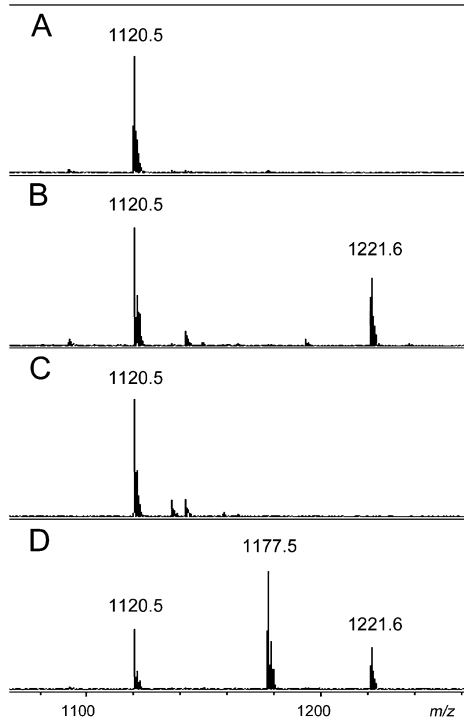

Figure 2. A two-step pathway of the McC maturation. MALDI-TOF MS analysis of products resulting from incubation of microcin C precursor McC^{1120} with cellular extract of *E. coli* BW28357 harboring the control pBAD plasmid (A), or plasmids overexpressing MccD (B), MccE (C), and both MccD and MccE (D). The mass peak 1177.5 corresponds to mature microcin C, while the mass peak 1221.6 has not been detected previously.

incubation of McC^{1120} with the extract prepared from cells overproducing MccD led to the appearance of a new mass peak with m/z = 1221 (Figure 2B). No peaks other than McC^{1120} were observed in reactions containing extracts from cells overproducing MccE alone (Figure 2C). In reaction containing the extract from cells co-overproducing both proteins an additional mass peak of 1177 was present (Figure 2D). The molecular weight of this species matches the 1176 Da value of mature McC (Figure 1). Similar results were obtained when purified recombinant MccD and/or MccE were combined with extracts of plasmid-free *E. coli pepA*, *pepB*, *pepN* cells in the presence of McC^{1120} (Figure S1). We conclude that recombinant MccD/E enzymes are functional and that *E. coli* extracts provide some missing component(s) for McC maturation. The maturation process itself appears to proceed in two separate steps: the first step involving the addition of a 101 Da group to McC^{1120} and the second involving the removal of 44 Da group to result in mature McC.

Characterization of the Products of MccD/MccE-Mediated Modification of McC by Tandem MS/MS. The m/z = 1221 mass peak as well as of the m/z = 1177 species

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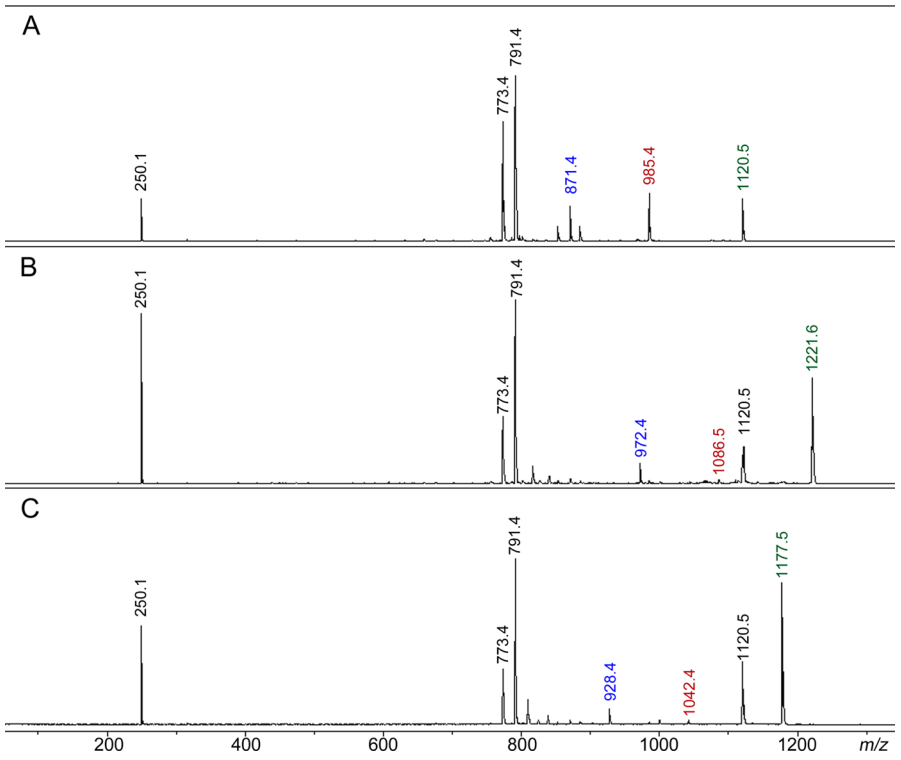
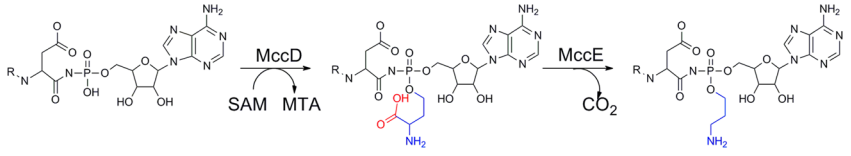


Figure 3. MS/MS analysis of McC^{1120} (A), McC^{1221} (B), and McC^{1177} (C). Peaks of the parental ion and McC fragments that lost adenine and adenosine moieties are marked in green, red, and blue, respectively. Mass shifts between the parental ions and the peak of the adenylated peptide fragment (m/z 1120.5) in panels B and C correspond, respectively, to the removal of the 3-amino-3-carboxypropyl and 3-aminopropyl groups from the phosphate of the corresponding parent ions. See text for details.

Scheme 1. Reaction Scheme for the Two-Step Microcin C Maturation Pathway[a]



[a]The six N-terminal residues of McC (MRTGNA) are denoted as R for clarity.

observed in reactions containing both MccD and MccE were analyzed by tandem MS/MS analysis (Figure 3). Control fragmentation spectrum of McC^{1120} revealed the presence of MccA peptide-containing fragments that lost adenine, adenosine, and AMP moieties (m/z of 985, 871, and 791, respectively, Figure 3A). In addition to the mass ion with m/z = 791 corresponding to formylated fMRTGNAD peptide of McC^{1120}, a mass peak with m/z = 773 corresponding to its dehydrated form was detected (Figure 3A). A mass peak with m/z = 250 matching adenosine was also observed (Figure 3A). Fragmentation of the 1221 m/z mass ion revealed the 791/773 m/z peptidyl fragments, the 250 m/z adenosine fragment, and the 1120 m/z fragment corresponding to a mass peak with a loss of 101 Da from the parent ion and matching adenylated peptide McC^{1120} (Figure 3B). Thus, the peptidyl and nucleoside moieties of McC^{1221} and McC^{1120} are identical. In addition, a new fragment (m/z = 972), corresponding to a parent ion with m/z = 1221 lacking adenosine was detected. This fragment is 101 Da heavier than the corresponding peptide fragment (m/z = 871) without adenosine observed during McC^{1120} fragmentation (Figure 3A). It therefore follows that the 101 Da group is attached to the phosphate moiety of McC^{1221}. The fragmentation pattern of the mass peak with m/z = 1177 from reactions containing MccD and MccE (Figure 3C) matched that of a mature McC prepared from the cells. It contained peaks with m/z = 791/773 corresponding to peptidyl fragments, and the mass peaks with m/z values of 928 and 1042 corresponding to fragments both carrying the 57 Da aminopropyl group at the phosphate, but lacking adenosine and adenine moieties, respectively.

The substrate and the products of MccD/MccE-catalyzed reactions were additionally analyzed by high-resolution mass spectrometry with an accuracy of measured monoisotopic masses within 1 ppm (Figure S2). The measured [MH$^+$] values were 1120.3987 for McC^{1120} ($C_{39}N_{17}O_{18}H_{63}S_1P_1$, calculated monoisotopic mass of 1120.3993), 1177.4563 for McC^{1177} (brutto formula $C_{42}N_{18}O_{18}H_{70}S_1P_1$, calculated monoisotopic mass 1177.4572), and 1221.4460 for McC^{1221} (expected formula $C_{43}N_{18}O_{20}H_{70}S_1P_1$, calculated mas 1221.4470). We conclude that that high-resolution mass spectrometric analysis supports the predicted structure of McC^{1221} (see Scheme 1).

Identification of SAM As a Substrate for MccD and an ACP-Donor for McC Modification. A mass shift of 101 Da observed in McC^{1221} fragmentation spectrum is consistent with MccD-dependent attachment of the 3-amino-3-carboxypropyl (ACP) group to the phosphate moiety of McC^{1120}. SAM is

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likely to be a donor of such a group.[14] To determine if SAM serves as an ACP donor, cell-free extract of MccD-overproducing *pepA*, *pepB*, *pepN E. coli* was passed through a gel-filtration column with a 6 kDa cutoff to remove the low-molecular weight compounds such as SAM, ATP, etc. Material eluting from the column, when combined with McC^{1120}, was unable to efficiently convert it to McC^{1221} (Figure 4A).

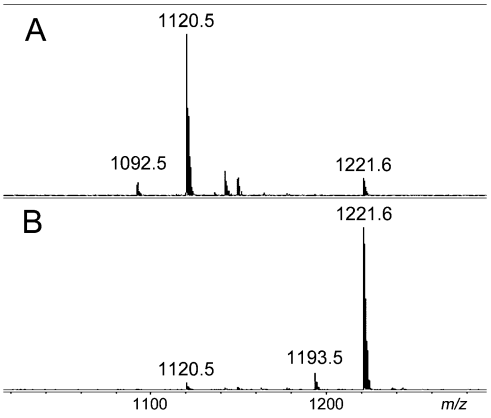

Figure 4. SAM is required for MccD-dependent conversion of McC^{1120}. (A) McC^{1120} was incubated with the high molecular weight fraction of cell extract of *E. coli* BW28357 overexpressing MccD, in the absence (A) or in the presence of 100 μM SAM (B).

However, when the column eluate was combined with McC^{1120} and SAM, a full conversion to McC^{1221} was observed (Figure 4B). It therefore follows that MccD indeed uses SAM as a substrate and as a donor of ACP group during modification of McC^{1120}. In all experiments described below the MccD-containing reactions were supplemented with SAM.

Proposed Pathway of McC Maturation. The McC maturation pathway that emerges from our data is summarized in Scheme 1. In the first step, MccD transfers the ACP group from SAM to McC^{1120} yielding McC^{1221}. The 5-methyl thioadenosine (MTA) should be the byproduct of this reaction. In the second step, McC^{1221} is decarboxylated by MccE, producing mature McC^{1177}. Since purified MccD in the presence of SAM is unable to convert McC^{1120} into McC^{1221} (above), it follows that *E. coli* extract provides a necessary component(s) for at least this step of the McC maturation pathway.

Identification of Mtn As an Enzyme Stimulating the ACP-Transfer Activity of MccD. We hypothesized that component(s) required for MccD-dependent McC modification step is a polypeptide. Accordingly, the whole-cell extract of *pepA*, *pepB*, *pepN E. coli* was fractionated through two bulk steps (polyethylenimine and ammonium sulfate precipitation) followed by hydrophobic interaction (HIC) and anion-exchange chromatography (see Experimental Section), and individual fractions were tested for their ability to promote the McC^{1120} to McC^{1221} conversion in the presence of SAM and pure MccD. Throughout fractionation, electrophoretic, and tryptic peptide MS fingerprint analyses were used to identify proteins specific for the active fractions. Analysis of an active fraction from the last purification step suggested that a single polypeptide with an apparent molecular weight of 25 kDa is responsible for stimulation of SAM-dependent MccD activity (Figure 5A,B). This protein was identified as 5′-methylthioadenosine/*S*-adenosylhomocysteine nucleosidase Mtn.

To prove that Mtn is indeed involved in McC maturation, two types of experiments were performed. First, an extract from cells lacking *mtn* (and *pepA*, *pepB*, and *pepN*) was prepared and tested for its ability to stimulate conversion of McC^{1120} into McC^{1221} by pure MccD. No conversion of McC^{1120} was observed in this extract, while control mtn^+ cell extract was highly active (Figure 5C). Second, a purified recombinant Mtn, when combined with MccD and SAM, stimulated full conversion of McC^{1120} into McC^{1221} in the absence of *E. coli* extract (Figure 5D). The results thus indicate that Mtn and

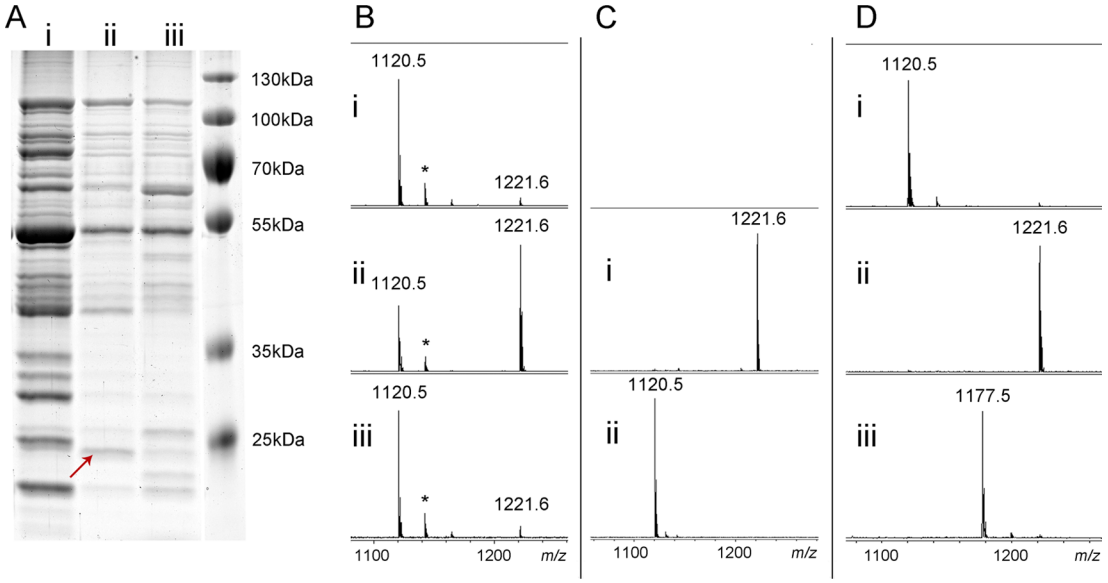

Figure 5. Identification of *E. coli* protein stimulating the MccD-catalyzed conversion of McC^{1120} to McC^{1221}. (A) SDS-PAGE electrophoregram of three consecutive fractions eluting from a MonoQ anion-exchange column and (B) MccD-stimulating activity in fractions i, ii, and iii detected by mass spectrometric analysis. Peaks labeled with an asterisk correspond to sodium salt of McC^{1120}. The protein band identified as Mtn is marked on panel A with an arrow. (C) Cell extracts of *E. coli* BW28357 (i) and *E. coli* BW28357 with deleted *mtn* (ii) were incubated with MccD and McC^{1120}. (D) McC^{1120} was incubated with 100 μM SAM and MccD alone (i) or in the presence of purified Mtn with (iii) or without (iii) MccE.

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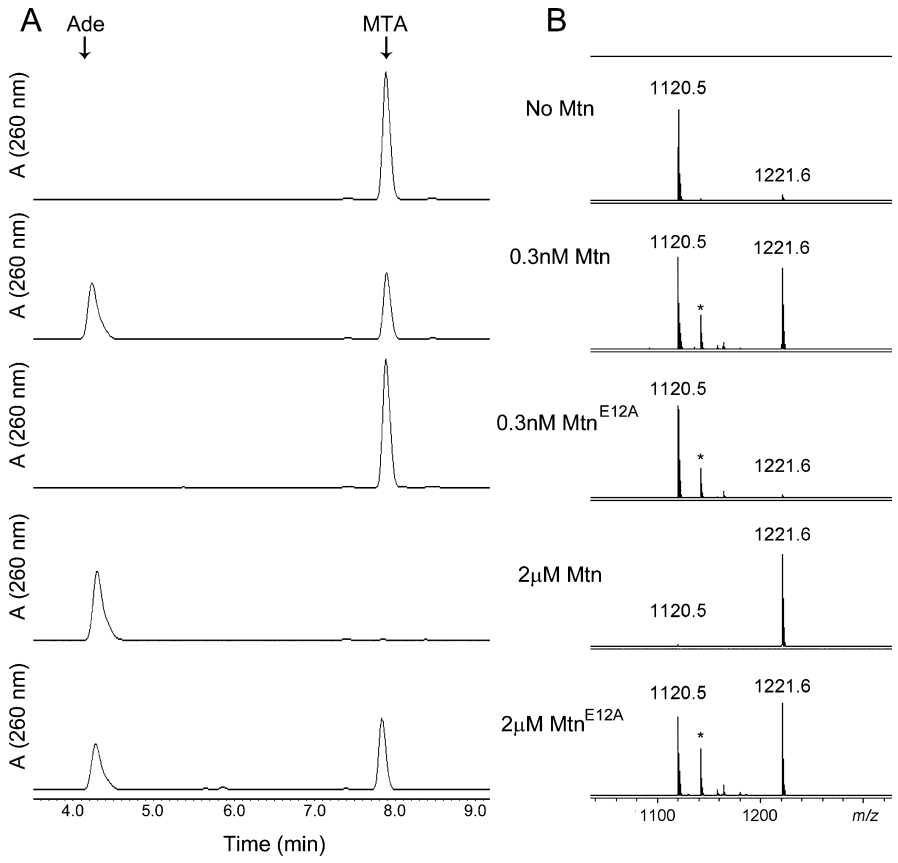

Figure 6. Efficiency of MccD-stimulating activity of Mtn depends on its catalytic activity. (A) Nucleosidase activity of the wild type Mtn and catalytically impaired Mtn^{E12A} mutant monitored by reverse phase HPLC. (B) MccD activity assayed in the presence of indicated concentrations of the wild-type Mtn or Mtn^{E12A}. The peak of sodium salt of McC^{1120} is marked with asterisk.

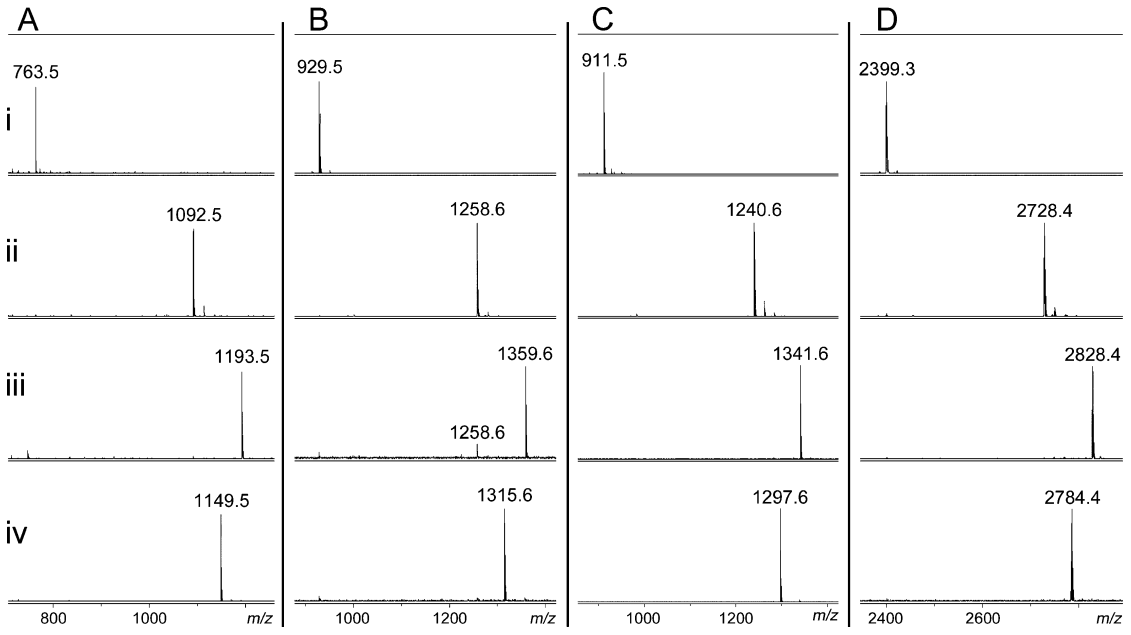

Figure 7. *In vitro* synthesis of aminopropylated microcin C-like compounds. Synthetic peptides encoded by the *E. coli mccA* gene (**A**) or *mccA* genes from the *mcc*-like operons of *L. johnsonii* (B), *H. pylori* (C), and a 20-amino acid C-terminal fragment of *Synechococcus* sp. CC9605 (D) were used for *in vitro* adenylation and aminopropylation reactions. The peptides were incubated with ATP and SAM alone (i), or together with cognate MccB proteins in the absence (ii), and in the presence of *E. coli* MccD and Mtn (iii) or MccD, Mtn and MccE (iv). The mass shift of 329 Da relative to the mass of the initial peptides (i) corresponds to the adenylated moiety (ii); further addition of 101 Da indicates carboxyaminopropylation of adenylated peptides (iii), whereas the loss of 44 Da signifies decarboxylation of the carboxyaminopropylated intermediate (iv).

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MccD are both necessary and sufficient for efficient conversion of McC^{1120} into McC^{1221} in the presence of SAM. The addition of MccE to a purified McC^{1221} was sufficient to convert it to a mature McC^{1177} (Figure 5D). Thus, unlike MccD, MccE does not require any additional protein factors for function.

Mtn may stimulate MccD through its MTA hydrolyzing activity by removing this byproduct of MccD-catalyzed reaction (Scheme 1). To test this possibility, a mutant Mtn carrying a substitution of catalytic Glu^{12} to Ala was prepared.[24] The MTA-hydrolyzing activity of Mtn^{E12A} was severely impaired (a ~5000-fold decrease compared to that of the wild-type Mtn; Figure 6A). Significantly, the ability of the mutant Mtn to promote the MccD-catalyzed modification of McC^{1120} was also decreased to approximately same degree (Figure 6B). The result thus establishes that the MTA-hydrolyzing activity of Mtn is essential for stimulation of MccD-catalyzed modification of McC^{1120}.

Modification of Heterologous Peptidyl-Adenylates by *E. coli* MccD/MccE Pair Together with Mtn Allows Production of More Potent Antibacterials. Recently, we reported the validation of several bioinformatically predicted McC-like compounds encoded by diverse bacteria.[12] Some of these compounds, for example from *Lactobacillus johnsonii* NCC 533 are encoded by a "minimal" *mcc*-like operon containing genes homologous to *mccA*, *mccB*, and *mccC*. Therefore, the corresponding heptapeptidyl-adenylates, when naturally produced, should not contain an aminopropyl modification. Other compounds, for example, from *Synechococcus* sp. CC9605, contain much longer MccA-like peptides and a full complement of *E. coli mcc* genes, however, the function of MccD and MccE enzymes homologues from these systems was not investigated. To determine if *E. coli* MccD/MccE enzyme pair can be used to obtain aminopropylated peptidyl-adenylates encoded by *mcc* operons of bacteria other than *E. coli*, *L. johnsonii* MccA peptide MHRIMKN (m/z = 929), *Helicobacter pylori* MccA peptide MKLSYRN (m/z = 911), and a 20 amino acid C-terminal fragment of *Synechococcus* sp. CC9605 MccA LQPKRLDKVAKNQLWADMMN (m/z = 2399) were incubated with cognate MccB enzymes in the presence of ATP to yield respective adenylates (Figure 7, second rows), yielding m/z values of expected peptidyl-adenylates. When the peptide substrates were combined with cognate MccB enzymes, ATP, SAM, *E. coli* MccD, and Mtn, the mass peaks corresponding to 3-amino-3-carboxypropylated species were observed in all cases (Figure 7, third rows). When reactions also contained *E. coli* MccE, quantitative conversion to mass peaks corresponding to aminopropylated species was observed for each compound (Figure 7, fourth rows).

In the case of *E. coli* McC, aminopropylation significantly increases its bioactivity.[5] To determine if aminopropylated forms of McC-like compounds are also more active, preparative amounts of *L. johnsonii* peptidyl-adenylate with and without aminopropyl decoration were prepared and tested for their ability to inhibit the growth of *E. coli* cells. The result, presented in Figure 8, shows that *L. johnsonii* peptidyl-adenylate modified by *E. coli* MccD/MccE was more active (at least 4-fold) than the unmodified compound that should be naturally produced by *L. johnsonii*.

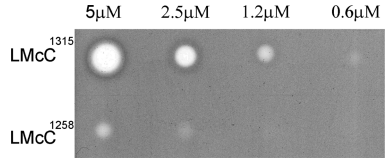

Figure 8. Aminopropylation increases toxicity of peptidyl-adenylate from *L. johnsonii in vivo*. 2-fold serial dilutions of *L. johnsonii* peptydyl-adenylate with ($LMcC^{1315}$) or without ($LMcC^{1258}$) aminopropyl decoration were placed over a growing lawn of *E. coli* cells. Growth inhibition zones are seen as clear circles on the turbid surface of cell lawn.

CONCLUDING REMARKS

In this work, we have defined a pathway of MccD/MccE-catalyzed aminopropylation and reconstituted an entire McC maturation process *in vitro* in a defined system. The McC aminopropylation proceeds in two sequential steps. In the first step, MccD uses SAM as a donor in a sulfur to oxygen ACP-transfer reaction to form McC^{1221}, the 3-amino-3-carboxypropylated intermediate of adenylated MccA, McC^{1120}. Reactions of ACP transfer are rare and typically proceed onto a hydroxycarbonyl group of the substrate.[19,20] In contrast, MccD catalyzes the ACP-transfer onto an oxygen of a phosphoamidate group. To the best of our knowledge, this is the first example of such SAM-dependent ACP transfer.

Reaction catalyzed by MccD alone is highly inefficient and appears to be a subject to product inhibition by MTA. This inhibition is relieved by the MTA-hydrolyzing activity of Mtn. MccE, which modifies another reaction product, McC^{1221}, is unable to stimulate MccD activity. The actual mechanism of MccD stimulation by Mtn requires further investigation. It would be interesting to determine if other known reactions of ACP transfer in bacteria are similarly stimulated by Mtn.

In the second step of the McC maturation pathway, a 3-amino-3-carboxypropylated McC is decarboxylated by MccE. This reaction proceeds efficiently *in vitro* and does not require additional *E. coli* proteins.

Our work shows that in the presence of MccA peptide, MccB, MccD, MccE, and Mtn enzymes, and ATP and SAM substrates, the entire *E. coli* McC maturation pathway can be carried out *in vitro* with high efficiency. Interestingly, when the *E. coli* MccA-MccB substrate-enzyme pair is substituted for cognate pairs encoded by *mcc*-like operons from other bacteria, aminopropylation reactions by *E. coli* enzymes lead to an efficient production of various peptidyl-nucleotides. Thus, the *E. coli* MccD/MccE enzymes are promiscuous and able to recognize peptidyl-adenylates, whose peptide moieties differ in sequence and length and efficiently convert them into aminopropylated forms. This opens a way for generation of novel compounds with more potent bioactivity than that of the natural peptidyl-nucleotides encoded by "minimal" operons of the *mccABC* structure described for many microbes.

EXPERIMENTAL SECTION

Materials, Media, Bacterial Strains, and McC^{1120} Purification. All chemicals were purchased from Sigma-Aldrich. Pfu polymerase and restriction enzymes were from Thermo Scientific. The *E. coli* strain DH5α was used as a host strain for all cloning. *E. coli* BL21(DE3) cells were used for recombinant protein expression. *E. coli* BW25113 and BW28357 (BW25113 lacking aminopeptidases PepA, PepB, and PepN) were used as parental strains for *mtn* deletion mutants construction, as described earlier.[25] Cells were grown in LB broth or on LB agar plates at 37 °C. When required, the antibiotics and inducer were used at 50 μg/mL for ampicillin and kanamycin, 20 μg/mL for chloramphenicol, 10 ng/mL for tetracycline, 1 mM for IPTG, and

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Table 1. Primers Used in the Study

primer	sequence	procedure
mccD_f	aatttaccATGgcgattaagcatgctgaagaattgacgga	cloning into pBAD/His and pet28b
mccE_f	aatttaccATGgcgcaaaaaataacaccatctaaa	
mccD_rs	aatttactcgtgTTAtttttgcgccatatttgcaatgtaa	cloning into pBAD/His
mccE_rs	aatttactcgtgTTAaccaattactttcgaataaatatttt	
mccD_r	aatttactcgtgtttttgcgccatatttgcaatgtaa	cloning into pet28b
mccE_r	aatttactcgtgaccaattactttcgaataaatatttt	
mtn_f	aatttaccATGgaaatcggcatcattggtgcaa	
mtn_r	aatttactcgtggccatgtgcaagtttctgcaccagt	
mtnE12_f	atcggcatcattggtgcaatggaagaagcggttacgctgctgcgtgacaaaatcgaa	mutagenesis
mtnE12_r	ttcgattttgtcacgcagcagcgtaaccgcttcttccattgcaccaatgatgccgat	

0.1% L-arabinose. McC^{1120} was purified from BW28357 harboring pUHAB plasmid lacking *mccD* and *mccE* genes, as described earlier.[5]

Molecular cloning. Primers used in the study are listed in Table 1.

The *mccD* and *mccE* genes and the *mccDE* gene pair were PCR-amplified using pUHAB plasmid[5] as a template and appropriate primer sets. PCR fragments were digested with *Nco*I and *Xho*I and ligated either into pet28b plasmid (Novagen) to create C-terminal fusions with His_6 tag sequence or pBAD/His vector (Invitrogen) for arabinose-dependent protein expression. The *mtn* gene was PCR amplified from BW25113 genomic DNA with appropriate primers and inserted into *Nde*I and *Xho*I sites of pet28b vector, creating N-terminal fusion with His_6 tag.

Site-directed mutagenesis of cloned *mtn* gene was performed by PCR-driven overlap extension[26] using the pet28-mtn plasmid as a template.

Protein Expression and Purification. Expression plasmids were transformed by electroporation into BL21(DE3) cells harboring the pG-KJE8 plasmid (TaKaRa), from which chaperones are expressed. His_6-tagged proteins were expressed and purified essentially as described by Roush et al., 2008[4] except that tetracycline and arabinose were added to the medium to induce the chaperones expression. Proteins were eluted from the Ni-NTA column in a buffer containing 20 mM Tris HCl, pH 7.5, 50 mM NaCl, and 300 mM imidazole, then diluted with an equal volume of 20 mM Tris HCl, pH 7.5, and applied onto a MonoQ column. Proteins were eluted in the 20 mM Tris HCl, pH 7.5 with a linear gradient of 50−500 mM NaCl. Fractions containing the target proteins that were ∼95% pure according to the Coomassie Brilliant Blue staining after SDS-PAGE were pooled, concentrated, and stored in the presence of 50% glycerol. Protein concentrations were measured with Quick Start Bradford Protein Assay kit (BioRad).

Cell Lysate Fractionation. BW28357 cells were harvested from 1 L of overnight culture, washed with ice-cold extraction buffer (50 mM Tris HCl, pH 7.5, 50 mM NaCl), resuspended in the same buffer containing 10 μg/mL lysozyme and complete protease inhibitor cocktail (Roche), and incubated on ice for 30 min. Cells were sonicated, and cell debris removed by centrifugation at 15,000 rcf for 20 min at 4 °C. The cleared lysate was precipitated with 0.5% polyethileneimine on ice for 20 min and centrifuged. The precipitate was extracted with 250 mM NaCl in 20 mM Tris HCl pH 7.5, and then the soluble protein fraction was precipitated with an equal volume of saturated $(NH_4)_2SO_4$. The precipitate was dissolved in loading buffer A for hydrophobic interaction chromatography (HIC) (1 M $(NH_4)_2SO_4$ in 20 mM Tris HCl, pH 7.5) and loaded onto the HiTrap phenyl-sepharose FF low sub column (GE Healthcare). The column was washed with 3 volumes of the HIC buffer A. The bound proteins were eluted with a 0−100% gradient of HIC buffer B (20 mM Tris HCl pH 7.5) in 10 min. Throughout purification, all fractions were analyzed by SDS PAGE and tested for their ability to stimulate MccD-catalyzed conversion of McC^{1120}. Fractions with highest activity were used for downstream steps. The highest levels of MccD-stimulating activity were detected in HIC fractions eluting in the presence of low salt concentrations in the buffer. These fractions were pooled, diluted with 1 volume of 20 mM Tris HCl, pH 7.5, and loaded onto MonoQ 5/50 GL anion exchange column (GE Healthcare) pre-equilibrated with the MQ buffer A (50 mM NaCl in 20 mM Tris HCl pH 7.5). Proteins were eluted using a 1 mL/min gradient of 50−500 mM NaCl in Tris HCl, pH 7.5 in 10 min.

Nucleosidase Activity of Mtn and Mtn^{E12A}. To determine the Mtn and Mtn^{E12A} activity, the MTA concentration was kept constant at 200 μM, while the concentration of enzymes was varied from 1 nM to 1 μM. The reactions were incubated at 28 °C for 20 min, then quenched by adding an equal volume of 0.5% trifluoroacetic acid (TFA) in water, and centrifuged to remove precipitated protein. The reaction products were separated by reverse phase HPLC (2.5 mL C18 column, Waters) at 1 mL/min in 0−30% gradient of acetonitrile (ACN) in 0.1% TFA in H_2O in 12 min.

MccD and MccE Activity Assays. 0.5 μM MccD was incubated with 10 μM McC^{1120} and 200 μM SAM in the reaction buffer (50 mM NaCl, 10 mM Tris HCl, pH 7.5) at 28 °C for 1 h. MccE and plp were added to the reaction at the final concentration 1 μM and 5 μM respectively, when specified. *E. coli* protein extract was prepared from the overnight culture by sonication in the reaction buffer supplemented with complete protease inhibitor cocktail (Roche) followed by centrifugation at 20,000 rcf for 20 min. To prepare the high molecular weight fraction of cellular proteins, the cleared lysate was passed through the Bio-Spin column with Bio-Gel P6 (Bio-Rad). The protein concentration was measured with Quick Start Bradford Protein Assay kit (BioRad) and adjusted to the final concentration of 1 mg/mL. The reaction was quenched with 10 volumes of 0.5% TFA and then subjected to MALDI-TOF MS analysis.

***In vitro* Microcin C Synthesis.** MccB proteins from *E. coli*, *L. johnsonii* NCC 533, *H. pylori* plasmid HPP12, and *Synechococcus* sp. CC9605 were expressed and purified as described earlier.[12] 50 μM cognate peptides were used for the *in vitro* adenylation with MccB enzyme. *E. coli* MccD, Mtn and MccE were added, where indicated. Reactions were carried out at 25 °C for 16 h in buffer containing 100 mM Tris pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, and 200 μM SAM. To test the toxicity of the synthesized compound, 5 μL of the serial dilution the reaction mixtures was deposited over the lawn of the sensitive *E. coli* BL21(DE3) cells.

Protein Identification. In-gel trypsin digestion was carried out essentially as described earlier.[27] The pieces (1−2 mm^3) of gel containing the proteins of interest were destained with 50 mM ammonium bicarbonate 40% acetonitrile, then dehydrated with 100 μL of 100% acetonitrile (ACN), and rehydrated with 5 μL of digestion solution containing 20 mM ammonium bicarbonate and 15 ng/μL sequencing grade trypsin (Promega, Madison, WI). Digestion was carried out at 37 °C for 5 h. Peptides were extracted with 10 μL of 0.5% TFA solution. To get the peptide mass fingerprint, 2 μL of extract subjected to MALDI-MS analysis. Protein identification was carried out by MS+MS/MS ion search with the use of Mascot software (Matrix Science) through the NCBI protein database. One missed cleavage, Met oxidation, and Cys-propionamide were permitted. Protein scores >88 were considered to be significant ($p < 0.05$).

MALDI-MS Analysis. Sample aliquots (1−2 μL) were mixed on a steel target with 0.5 μL of 20 mg/mL 2,5-dihydroxybenzoic acid in 0.5% TFA and 30% ACN water solution (Aldrich). Mass spectra were recorded on an UltrafleXtreme MALDI-TOF-TOF mass spectrometer (Bruker Daltonics) equipped with a neodymium laser. The $[MH]^+$

molecular ions were measured in reflector mode; the accuracy of mass peak measurement was within 0.1 Da. Fragment ion spectra were obtained in lift mode. The accuracy of fragment ion mass peak measurements was within 0.2 Da.

■ ASSOCIATED CONTENT

Supporting Information

Results of McC maturation in extracts of *E. coli* cells supplemented with purified recombinant Mcc protein (Figure S1) and high-resolution mass spectrometric analysis of McC maturation intermediated (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Guijarro, J. I.; González-Pastor, J. E.; Baleux, F.; San Millán, J. L.; Castilla, M. A.; Rico, M.; Moreno, F.; Delepierre, M. *J. Biol. Chem.* **1995**, *270*, 23520−23532.
(2) González-Pastor, J. E.; San Millán, J. L.; Castilla, M. A.; Moreno, F. *J. Bacteriol.* **1995**, *177*, 7131−7140.
(3) García-Bustos, J. F.; Pezzi, N.; Asensio, C. *Biochem. Biophys. Res. Commun.* **1984**, *119*, 779−785.
(4) Roush, R. F.; Nolan, E. M.; Löhr, F.; Walsh, C. T. *J. Am. Chem. Soc.* **2008**, *130*, 3603−3609.
(5) Metlitskaya, A.; Kazakov, T.; Vondenhoff, G. H.; Novikova, M.; Shashkov, A.; Zatsepin, T.; Semenova, E.; Zaitseva, N.; Ramensky, V.; Van Aerschot, A.; Severinov, K. *J. Bacteriol.* **2009**, *191*, 2380−2387.
(6) Novikova, M.; Metlitskaya, A.; Datsenko, K.; Kazakov, T.; Kazakov, A.; Wanner, B.; Severinov, K. *J. Bacteriol.* **2007**, *189*, 8361−8365.
(7) Kazakov, T.; Vondenhoff, G. H.; Datsenko, K. A.; Novikova, M.; Metlitskaya, A.; Wanner, B. L.; Severinov, K. *J. Bacteriol.* **2008**, *190*, 2607−2610.
(8) Metlitskaya, A.; Kazakov, T.; Kommer, A.; Pavlova, O.; Praetorius-Ibba, M.; Ibba, M.; Krasheninnikov, I.; Kolb, V.; Khmel, I.; Severinov, K. *J. Biol. Chem.* **2006**, *281*, 18033−18042.
(9) Novikova, M.; Kazakov, T.; Vondenhoff, G. H.; Semenova, E.; Rozenski, J.; Metlytskaya, A.; Zukher, I.; Tikhonov, A.; Van Aerschot, A.; Severinov, K. *J. Biol. Chem.* **2010**, *285*, 12662−12669.
(10) Agarwal, V.; Metlitskaya, A.; Severinov, K.; Nair, S. K. *J. Biol. Chem.* **2011**, *286*, 21295−21303.
(11) Paz-Yepes, J.; Brahamsha, B.; Palenik, B. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 12030−12035.
(12) Bantysh, O.; Serebryakova, M.; Makarova, K. S.; Dubiley, S.; Datsenko, K. A.; Severinov, K. *mBio* **2014**, *5*, e01059−14.
(13) Severinov, K.; Semenova, E.; Kazakov, A.; Kazakov, T.; Gelfand, M. S. *Mol. Microbiol.* **2007**, *65*, 1380−1394.
(14) Fontecave, M.; Atta, M.; Mulliez, E. *Trends Biochem. Sci.* **2004**, *29*, 243−249.
(15) Lin, H. *Bioorg. Chem.* **2011**, *39*, 161−170.
(16) Nishimura, S.; Taya, Y.; Kuchino, Y.; Oashi, Z. *Biochem. Biophys. Res. Commun.* **1974**, *57*, 702−708.
(17) Wurm, J. P.; Meyer, B.; Bahr, U.; Held, M.; Frolow, O.; Kötter, P.; Engels, J. W.; Heckel, A.; Karas, M.; Entian, K. D.; Wöhnert, J. *Nucleic Acids Res.* **2010**, *38*, 2387−2398.
(18) Callebaut, A.; Lambein, F.; Van Parijs, R. *Arch. Int. Physiol. Biochim.* **1977**, *85*, 157−158.
(19) Klug, R. M.; Benning, C. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98*, 5910−5915.
(20) Reeve, A. M.; Breazeale, S. D.; Townsend, C. A. *J. Biol. Chem.* **1998**, *273*, 30695−30703.
(21) Gunsior, M.; Breazeale, S. D.; Lind, A. J.; Ravel, J.; Janc, J. W.; Townsend, C. A. *Chem. Biol.* **2004**, *11*, 927−938.
(22) Riekhof, W. R.; Andre, C.; Benning, C. *Arch. Biochem. Biophys.* **2005**, *441*, 96−105.
(23) Ikeguchi, Y.; Bewley, M. C.; Pegg, A. E. *J. Biochem.* **2006**, *139*, 1−9.
(24) Lee, J. E.; Luong, W.; Huang, D. J.; Cornell, K. A.; Riscoe, M. K.; Howell, P. L. *Biochemistry* **2005**, *44*, 11049−11057.
(25) Datsenko, K. A.; Wanner, B. L. *Proc. Natl. Acad. Sci. U. S. A.* **2000**, *97*, 6640−6645.
(26) Heckman, K. L.; Pease, L. R. *Nat. Protoc.* **2007**, *2*, 924−932.
(27) Shevchenko, A.; Wilm, M.; Vorm, O.; Mann, M. *Anal. Chem.* **1996**, *68*, 850−858.