Enzymatic synthesis and functional characterization of bioactive microcin C-like compounds with altered peptide sequence and length

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Running title: Enzymatic synthesis of microcin C-like compounds

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ABSTRACT

Escherichia coli microcin C (McC) consists of a ribosomally-synthesized heptapeptide attached to a modified adenosine. McC is actively taken up by sensitive E. coli strains through the YejABEF transporter. Inside the cell McC is processed by aminopeptidases, which release nonhydrolyzable aminoacyl adenylate, an inhibitor of aspartyl-tRNA synthetase. McC is synthesized by the MccB enzyme, which terminally adenylates the MccA heptapeptide precursor MRTGNAN. Earlier, McC analogues with shortened peptide lengths were prepared by total chemical synthesis and were shown to have strongly reduced biological activity due to decreased uptake. Variants with longer peptide length were difficult to synthesize though. Here, we used recombinant MccB to prepare and characterize McC-like molecules with altered peptide moieties, including extended peptide lengths. We find that N-terminal extensions of E. coli MccA heptapeptide do not affect MccB-catalyzed adenylation and some extended peptide-length McC analogues show improved biological activity. When peptide length reaches 20 amino acids both YejABEF and SbmA can perform facilitated transport of toxic peptide-adenylates inside the cell. A C-terminal fusion of the MBP carrier protein with the MccA peptide is also recognized by MccB in vivo and in vitro allowing highly specific adenylation and/or radioactive labelling of cellular proteins.

IMPORTANCE

Enzymatic adenylation of chemically synthesized peptides allowed us to generate biologically active derivatives of peptide-nucleotide antibiotic microcin C with improved bioactivity and altered entry routes into target cells opening way for development of various antibacterial compounds not found in nature.
The antibiotic microcin C (McC) (Fig. 1) is produced by *Escherichia coli* strains harboring a plasmid-borne *mcc* operon. McC consists of a ribosomally-synthesized heptapeptide that is covalently linked through a phosphoramidate bond to adenosine; the phosphoramidate linker is esterified with an aminopropyl moiety (1). Inside a sensitive cell, the N-terminal formyl group of the McC peptide is removed by peptide deformylase, after which the peptide part is processed by aminopeptidases (2). As a result, processed McC - a modified non-hydrolysable aspartyl-adenylate - is released. Processed McC is a potent inhibitor of aspartyl-tRNA synthetase (AspRS), an essential enzyme (3).

McC penetrates the inner membrane of *E. coli* cells through the YejABEF transporter (4).

While intact McC inhibits the growth of sensitive *E. coli* cells at low micromolar concentrations (5, 6) processed McC does not affect cell growth, even at millimolar concentrations (3, 7). Thus, the peptide chain enables processed McC’ function through a Trojan-horse mechanism by promoting its active uptake via YejABEF. YejABEF is uniquely responsible for McC transport, since yej mutants are fully resistant to McC (4). The biological function of YejABEF (other than McC transport) is presently unknown. In *Salmonella*, it confers resistance to some antimicrobial peptides and allows proliferation inside activated macrophages, thus contributing to virulence (8). The latter property may be related to the fact that YejABEF interferes with peptide presentation on MHC Class I molecules (9).

The peptide part of McC is encoded by the *mccA* gene. At seven codons, this gene is considered to be the shortest natural gene known (10). The MccA peptide sequence is MRTGNAN. During McC maturation, MccA is C-terminally adenylated by the MccB synthetase (11). Bioinformatics searches identified MccB homologs in diverse bacteria and in some cases short nearby genes that could code for substrate peptides were also predicted (12, 13). Apart
from the C-terminal asparagine residue, most predicted MccA-like peptides have no sequence
similarity to each other or *E. coli* MccA. However, many predicted MccA-like peptides – from
*Helicobacter pylori*, *Bartonella*, *Lactobacillus*, and *Streptococcus* – have the same length as the
*E. coli* peptide. On the other hand, a 56 aminoacid long cyanobacterial MccA peptide and a 42
aminoacid long peptide from *Yersinia pseudotuberculosis* are subject to terminal adenylation by
their cognate MccB enzymes (13) suggesting that the length of MccB target peptides can exceed
the seven amino acids characteristic for most *mcc*-like operons.

*In vitro* adenylation of MRTGNAN peptide by recombinant *E. coli* MccB is very
efficient (11). The reaction proceeds in two steps. First, one ATP molecule is consumed to
convert the terminal asparagine into succinimide. This activated intermediate is coupled with the
second ATP molecule, resulting in biologically active peptide adenylate with terminal aspartate
(11). In the presence of the MccD and MccE enzyme pair an aminopropyl group is attached to
the product of MccB-catalyzed adenylation using SAM as a donor (14). The presence of
aminopropyl increases the biological activity several-fold, probably by increasing the avidity of
modified non-hydrolyzable aspartate-adenylate to the target enzyme (6).

Structure-activity analysis of the McC peptide was carried out by introduction of point
substitutions in the *mccA* gene (15). Using this approach codons 2-7 of *mccA* were each
systematically substituted for codons coding for 19 remaining standard amino acids and the
effects of these substitutions on the ability of cells harboring mutated *mcc* operons to produce
mutant microcins was determined. The role of the first methionine of the MccA peptide could
not be studied using this approach as this residue is required for translation initiation. The
analysis of the seventh codon of *mccA* was also not informative, since only peptides with a
terminal asparagine can be adenylated by the MccB enzyme (11). Despite these limitations, a
series of McC derivatives, including some with increased bioactivity, was obtained using this
approach, indicating that *E. coli* MccB is not strictly specific for its peptide substrates.

Total chemical synthesis of McC analogs yielded several active McC variants with non-natural residues at the seventh position of the peptide, targeting aminoacyl-tRNA synthetases other than AspRS targeted by natural McC (16). The chemical approach was also used to investigate peptide length requirements for facilitated *E. coli* McC transport. The results indicated that shortening of McC peptide by even one amino acid strongly decreased the biological activity by affecting YejABEF-facilitated transport (7). Further decrease of the peptide length abolished facilitated transport, resulting in bioactivity levels comparable to that of processed McC. McC variants with longer length wild-type MccA-based peptides proved impossible to obtain due to synthetic complications. Yet, a small amount of adenylated MTRGNAAG peptide, with an additional alanine highlighted in bold inserted after position 6 was prepared. This compound targeted GlyRS and was slightly more active compared to control chemically synthesized heptapeptide MRTGNAG adenylate (7). Overall, these results provided a lower bound for the peptide moiety of YejABEF substrates, which appears to coincide with the wild-type McC peptide length, and suggested that increasing the length of the transport peptide may lead to more potent McC-based antibacterials.

In this communication we use enzymatic synthesis of peptide adenylates by *E. coli* MccB to study McC structure-function. This approach is free from limitations of both the molecular genetics and chemical synthesis approaches and allowed us to prepare and characterize McC derivatives with substitutions of the N-terminal methionine and with extended peptide length. We show that the N-terminal amino acid of MccA plays a critical role in the binding to the MccB enzyme. We confirm that extension of McC peptide length up to a certain point increases bioactivity. The biological activity of longer peptide-adenylates can be further increased by aminopropylation. We finally show that MccA fusions to full-sized proteins such as 43 kDa
maltose-binding protein MBP are also subject to adenylation by MccB in vivo and in vitro, allowing efficient labeling of fusion proteins.
MATERIALS AND METHODS

DNA, molecular cloning

The *E. coli* mccB was cloned between the NcoI and BamHI of pET32b (pETMccB) vector or between the NcoI and Sall sites of MCS1 of vector pCOLADuet-1. The *E. coli* mhb fused with C-terminal sequence encoding GGGMRTGNAN (*E. coli* MccA with N-terminal tetraglycine linker) was next cloned between the Ndel and XhoI sites of MCS2 pCOLADuet-1 vector containing mccB (yielding pColMccB-MBP-MccA plasmid). A sequence encoding GGGMRTGNAN was also cloned between the EcoRI and HindIII sites of pMAL-c2X vector to generate pMAL-MBP-MccA.

Strains and strain construction

*E. coli* DH5-α was used for molecular cloning; for susceptibility tests we used *E. coli* BL21(DE3) and its ΔyejB, ΔsbmA and ΔyejBΔsbmA derivatives. The ΔyejB and ΔsbmA derivatives are described in (13). The ΔyejB ΔsbmA double mutant was constructed from ΔsbmA and ΔyejB single mutants (the latter marked by chloramphenicol resistance) using P1 transduction (17). *E. coli* 0256 and 0193 are clinical isolates obtained from St. Petersburg Research Institute of Children’s Infections of the Federal Medical and Biological Agency of Russia.

Protein expression and purification

*E. coli* BL21(DE3) cells were used as expression host. The BL21(DE3) cells transformed with appropriate expression plasmids were grown at 37°C in 200 ml LB-medium supplemented with 1% of glucose and necessary antibiotics until OD₆₀₀ reached 0.6. Cells were harvested by centrifugation, washed thrice with fresh LB medium and resuspended in 200 ml of fresh LB with antibiotics and 0.1-0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells harboring MccB protein plasmid were grown for 20 h at 18°C with vigorous agitation, cells harboring MBP
protein plasmid were grown for 4 h at 30°C. Cells were harvested and resuspended in 8 ml of appropriate loading buffer (MccB loading buffer: 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10 mM MgCl₂; MBP loading buffer: 20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, 1 mM sodium azide, 10 mM β-mercaptoethanol) and disrupted by sonication. The lysates were centrifuged at 30,000 × g for 30 min at 4°C. The supernatants were mixed with 200-300 µl of appropriate resin (MccB: His Bind Resin (Novagen), MBP: amylose resin (NEB)) equilibrated in the same buffer was added and proteins were allowed to bind for 2-4 h at 4°C with gentle agitation. The resin was allowed to settle by gravity, washed with 15 ml of wash buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10 mM MgCl₂) with (MccB) or without (MBP) 50 mM imidazole and bound proteins were eluted with 0.5 ml of elution buffer (MccB elution buffer: 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10 mM MgCl₂, 200 mM imidazole: MBP elution buffer: 20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, 1 mM sodium azide, 10 mM β-mercaptoethanol, 10 mM maltose). Five consecutive elutions were performed with each resin sample. Fractions were supplemented with glycerol up to 50% and stored at -20°C until further use. Proteins were at least 90% pure as judged by visual inspection of overloaded Coomassie-stained SDS gels.

His-tagged MccD, MccE, and Mtn proteins were prepared as described in (14).

**Synthesis of peptide substrates**

All peptides were synthesized by solid-phase synthesis by LLC “Syneuro”, Russia (at least 98% purity by HPLC and MS) or by GenScript USA Inc. (at least 85% purity).

**In vitro enzymatic assays**

Standard peptide adenylation reactions were performed in a total volume of 100 µl. 1x MccB buffer (75 mM Tris-HCl, pH 8.0, 5 mM MgCl₂) was supplemented with 5 µM MccB, 2.5 mM TCEP, 4 mM ATP. As substrates, 4 µM MBP-MccA, or 225 µM synthetic peptide was
used. Control reaction did not contain ATP. Reactions were incubated at 23°C for 20 h and were terminated by freezing at -20°C.

To set up competition assays, standard adenylation reactions were preincubated with 7.5 mM competitor peptide for 40 min and then were supplemented with 150 µM of MRTGNAN substrate peptide. Reactions were processed as described above and reaction products were separated by HPLC.

Aminopropylation of 40 µM HPLC-purified adenylated peptides was carried out with 2.7 μM MccD, 0.5 μM MccE and 0.8 μM Mtn in 20 mM Tris-HCl, pH 8.0 buffer supplemented with 50 mM NaCl and 500 μM S-Adenosyl-L-methionine (SAM) and 20 μM pyridoxal phosphate (PLP).

**In vitro radiolabeling**

Reactions were performed in the total volume of 50 µl containing 75 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 4 µM MccB, 4 mM DTT, 2.5 mM ATP and 0.75 µM [α-³²P]ATP (3000 Ci mmol⁻¹). The reactions were supplemented with either 4 µM MBP-MccA, or 200 µM of the MRTGNAN peptide. Reaction mixtures were incubated at 23°C for 24 h. 10 µl reaction aliquots were deposited on *E. coli* BL21(DE3) sensitive lawn to confirm peptide conversion into a toxic adenylated compound. The products were also resolved by 10% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue R-250 and radioactive proteins revealed by autoradiography.

**In vivo adenylation assays**

*E. coli* BL21(DE3) cells were transformed with pColMBP-MccA or pColMccB-MBP-MccA. Cells were grown at 37°C in 200 ml LB supplemented with 1% glucose and 50 µg ml⁻¹ kanamycin until OD₆₀₀ reached 0.6. Cells were harvested by centrifugation, washed thrice with fresh LB medium and resuspended in 200 ml of fresh LB with kanamycin and 0.3 mM IPTG.
Cells were grown for 5 h at 37°C with vigorous agitation, harvested, and MBP-MccA was purified using amylose resin. MBP-MccA protein fusion was incubated with protease Factor Xa in appropriate buffer for 3 h. Reaction mixtures were purified form MBP-protein using amylose resin and analyzed by MALDI-MS.

**Identification of adenylation reaction products**

The products of MccB reaction were identified by reverse-phase HPLC. Completed reactions were mixed with equal volume of acetonitrile supplemented with 0.1% TFA (trifluoroacetic acid) and incubated for 20 min on ice. Precipitated protein was removed by centrifugation at 30,000 × g for 10 min at +4°C. Supernatants were collected, dried, and resuspended in 100-200 µl of deionized water and applied on a 4.6×150 mm XSELECT HSS C-18 3.5 µm column (Waters) equilibrated with buffer A (0.1% TFA). The column was developed at 1 ml min⁻¹ using the following gradient: 0-10% buffer B (100% acetonitrile) for 5 min followed by 10-40% or 10-20% B in 40 min. Individual chromatographic peaks were analyzed by MALDI-MS.

**MALDI-MS and MS/MS analysis**

Mass spectra were recorded on Ultraflextreme MALDI-ToF/ToF mass spectrometer (Bruker Daltonik) equipped with an Nd laser (355 nm). The MH⁺ molecular ions were measured in reflector mode; the accuracy of monoisotopic mass peak measurement was 50 ppm. 1 µl aliquots of desalted adenylation reactions were mixed on a steel target with 0.5 µl of 2,5-dihydroxybenzoic acid (Aldrich) solution (20 mg ml⁻¹ in 30% MeCN, 0.5% TFA).

**In vivo sensitivity tests**

Wild-type or mutant (ΔyejB, ΔsbmA, or ΔyejBΔsbmA) cell cultures were grown in 10 ml of M63 broth without Fe²⁺ salts and supplemented with yeast extract at 37°C to an OD₆₀₀ of ~0.8. 1 ml of cell culture was added to 20 ml of melted top agar (0.65 g l⁻¹ of agar in M63 broth)
cooled to ~50°C. The mixture was poured on the surface of LB agar plates. After the agar solidified, 10-μl drops of completed adenylation reactions (above), aminopropylation reactions, or HPLC-purified adenylated peptides were placed on plate surface and allowed to dry. Plates were incubated for 4–6 h at 37°C and growth inhibition zones around the sites where samples were applied were visually detected.
RESULTS

Use of *in vitro* adenylation by MccB to prepare mutant McC variants

Several derivatives of MccA heptapeptide MRTGNAN were obtained and their ability to be adenylated by the recombinant *E. coli* MccB was studied *in vitro* using wild-type peptide as a control. Several peptides matched *mccA* mutants previously tested *in vivo* (15). These included MRTGNAD and MRTGNAQ with substitutions of the terminal residue and substitutions in the third (MRAGNAN, MRLGNAN, MRKGNAN, MRSGNAN, and MRCGNAN) and fourth (MRTWNAN) positions of the MccA peptide. For each reaction, the products were analyzed by MALDI-MS. As expected, the wild-type peptide was fully converted into adenylated form (Fig. 2). Both peptides with substituted C-terminal residues remained intact; neither the attachment of AMP nor succinimide intermediate of the adenylation reaction was detected (data not shown). The results agree with earlier data since cells harboring genetic constructs expressing the corresponding *mccA* mutant genes did not produce adenylated peptides (15). The MRKGNAN peptide was not modified in agreement with earlier *in vivo* data. The MRLGNAN and MRCGNAN peptides were adenylated, also in agreement with *in vivo* data (Fig. 2). MRAGNAN, which according to *in vivo* data was not modified, was poorly modified by the MccB enzyme *in vitro*, though small amounts of succinimide intermediate and trace amounts of adenylated product were detected (Fig. 2). MRSGNAN presents an interesting case, as the corresponding adenylate was not detected *in vivo*, but *in vitro* adenylation reaction proceeded to completion (Fig. 2). The MRTWNAN peptide with substitution at position 4 was modified in agreement with the published data.

We also tested a peptide containing non-natural D-enantiomer of threonine at position 3. The peptide, was not modified by MccB (Fig. 2).
To determine if the products of MccB-catalyzed reactions are biologically active, aliquots of completed reactions were deposited on lawns of McC-sensitive *E. coli* cells and formation of growth inhibition zones around deposited drops was monitored after overnight growth (Table 1).

As controls, biological activities of reaction aliquots were also tested on lawns of McC-resistant *yejB* mutant cells. For reactions containing wild-type MccA peptide, robust growth inhibition zones on wild-type but not mutant cell lawns were observed. No inhibition zones on either cell lawn were produced around deposited aliquots of reactions containing peptides with substituted terminal residues or with MRAGNAN or MRKGNAN, as expected, since these peptides were either not subject to adenylation or were very poorly modified (MRAGNAN). Reactions containing adenylated MRLGNAN, MRCGNAN and MRTWNAN inhibited bacterial growth, in agreement with earlier *in vivo* data (15). None of the active variants inhibited the growth of *yejB* mutant cells. The adenylate of MRSGNAN that was not produced *in vivo* was also biologically active. Overall, the results of this analysis show that there is a good correspondence between results obtained by the *in vivo* and the enzymatic *in vitro* approach. Yet, as suggested by the case of serine substitution at position 3, certain MccA peptide variants that are recognized and modified by MccB were missed during the *in vivo* screen possibly because of modified stability of mutant peptides in the cell. The *in vitro* approach is free of such limitations and thus allows more detailed structure-functional analysis of McC variants.

**N-terminal MccA residue is critical for the binding to MccB**

We next prepared several MccA peptide variants with substitutions of the N-terminal methionine. This residue is encoded by the starting codon of *mccA* and so its significance could not have been studied before using molecular genetic approach. Structural analysis of the MccB-MccA complex suggests that the side chain of MccA Met1 contributes to peptide binding to the enzyme (18). A total of 11 variants were tested (GRTGNAN, ARTGNAN, IRTGNAN, ...
VRTGNAN, LRTGNAN, FRTGNAN, NRTGNAN, QRTGNAN, DRTGNAN, ERTGNAN, and KRTGNAN) introducing side chains with different properties instead of Met. Among the peptides tested, LRTGNAN and IRTGNAN were partially adenylated, while FRTGNAN was adenylated to completion at our conditions (Fig. 3). The rest of the mutant peptides were not modified by the enzyme. This included, VRTGNAN, an unexpected result considering that LRTGNAN and IRTGNAN were good substrates for adenylation. Structural modeling using the existing MccB-MccA complex structure as a template (18) shows that the valine side chain is too small to make van der Waals contacts with the binding pocket in the protein (in contrast to leucine and isoleucine side chains, data not shown). All adenylated MccA peptides with substituted first amino acid were active against the wild-type *E. coli* but not the yejB mutant cells (Table 1).

Results obtained with N-terminally substituted MccA variants point to the importance of the first residue of MccA for the binding to MccB and/or proper presentation of the C-terminal residue of the bound peptide in the enzyme catalytic center. The following experiment was performed to determine if a bulky N-terminal hydrophobic residue of MccA is required for MccB binding. Wild-type MccB adenylation reactions were conducted in the presence of 50-fold excess of QRTGNAN or MRTGNAQ peptides (Fig. 4), neither of which is subject to adenylation. As control, we used the MRGTAAD peptide, which is also not adenylated and was previously shown to inhibit adenylation of wild-type MccB (11). Reaction products were resolved by HPLC and the size of an HPLC peak containing adenylated MccA was determined (Fig. 4A). As can be seen, MRGTAAD inhibited MccA adenylation, as expected. Both QRTGNAN and MRTGNAQ were much less effective inhibitors of MccA adenylation reaction (Fig. 4A), suggesting that they are equally compromised for binding to MccB.
The experiment was next modified such that competing peptides were added to purified adenylated MccA just prior to biological activity tests. Previously, it was shown that MccA and longer peptides containing the MccA sequence at their C-termini interfere with McC antibacterial activity by competing for entry through the YejABEF transporter (7). The MRTGNAQ peptide attenuated bioactivity of adenylated MccA as well as control wild-type MccA MRTGNAN (Fig. 4B), indicating that both peptides were recognized by YejABEF. In contrast, the addition of excess of QRTGNAQ had little or no effect on biological activity of adenylated MccA. This observation confirms the important role of Met¹ not just in the binding to the MccB enzyme but also in recognition by the YejABEF transporter. However, the MRTGNAD peptide, whose sequence is identical to the peptide part of McC, was also a poor competitor, indicating that the YejABEF specificity determinants are not limited by the nature of N-terminal amino acid.

Enzymatic synthesis and bioactivity of McC variants with longer peptide part

We next wondered if enzymatic synthesis can be used to obtain McC-like compounds with extended peptide lengths. We tested a series of wild-type MccA-based peptides extended by one, two, three, or six glycine residues at the N-terminus. Each peptide was readily adenylated by MccB (Fig. 5). The oligo-glycine tail by itself did not appear to increase the binding properties of MccA substrates since a (G)₆RTGNAQ peptide lacking a residue corresponding to MccA Met¹ was not adenylated (data not shown) further underscoring the significance of the methionine residue for binding to MccB.

Reactions containing elongated MccA adenylates inhibited the growth of wild-type but not of the yejB mutant E. coli (data not shown). To compare the levels of antimicrobial activities of longer-peptide McC-like compounds, each adenylate was purified, concentrations of each compound were matched, and their activities were semi-quantitatively determined by depositing...
drops containing serial dilutions of each compound on lawns of wild-type laboratory *E. coli* tester cells and two different *E. coli* clinical isolates. The results are shown in Table 2. One clinical strain, *E. coli* 0256, demonstrated low sensitivity to all compounds tested. In contrast, the other two strains were sensitive. Adenylates of extended MccA peptides were more active than wild-type MccA adenylate on the laboratory strain BL21(DE3). The activity of adenylated GMRTGNAN, (G)₂MRTGNAN, and (G)₃MRTGNAN was increased four-fold, while (G)₆MRTGNAN was twice as active as MccA-adenylate. A clinical isolate *E. coli* 0193 showed similar trends. Thus, increasing the length of the peptide moiety beyond the natural seven amino acids improves the bioactivity McC-like compounds.

The activity of the product of MccB-catalyzed adenylation of natural MccA is increased four-fold by aminopropyl decoration on the phosphate (14). Using the general conditions described in (14) we prepared aminopropylated variants of MccB adenylation products of peptides of various lengths (Fig. 5) and determined their bioactivity. The results are presented in Table 2. As can be seen, aminopropylated derivatives of longer-length peptide-adenylates were more active than corresponding compounds without this decoration. For *E. coli* 0256, which was practically resistant to compounds without aminopropyl, growth inhibition zones around modified 9, 10, and 13 aminoacid-long peptides were observed. For more susceptible BL21(DE3) and 0193 strains a 8 to 16-fold stimulatory effect of aminopropyl was observed. We conclude that increased peptide length and aminopropylation have an additive effect and together can increase the bioactivity of adenylated MccA heptapeptide by as much as 30-fold, changing the MIC value from 10 to 0.3 µM.

It could be argued that extending the MccA peptide with homogeneous contiguous glycines represent a special case that does not report on the MccB ability to recognize substrates with extra heterogeneous sequences containing bulky amino acids. We tested two MccA-based
peptides extended to a total length of 20 and 25 amino acids with a randomly chosen sequence. Both peptides were readily adenylated by MccB and resulting peptide adenylates inhibited growth of wild-type *E. coli* (Fig. 6). Interestingly, when activity of these peptide-adenylates was tested on MCC-resistant *yejB* mutant strain, clear growth inhibition zones (whose sizes were nevertheless much smaller than on wild-type cell lawns) were observed, suggesting that an additional transporter is involved in the uptake. When cells lacking SbmA, a transporter responsible for microcin B transport (19) were tested, growth inhibition zones around adenylated 20- and 25-aminoacid peptides were diminished marginally, while full resistance to McB was observed, as expected. However, when activity was tested on lawns of double mutant cells (*AyejBAsbmA*) lacking both transporters, no growth inhibition was detected with adenylated 20- and 25-aminoacid peptides, MCC, or McB. The double mutants cells were as sensitive to kanamycin, which was used as control, as single mutants or wild-type cells (Fig. 6). The result thus suggests that MCC analogues with longer peptide chains are transported inside the cells through a joint function of YejABEF and SbmA inner membrane transporters.

**Use of MccA/MccB for terminal protein labeling**

The results presented above clearly show that *E. coli* MccB can adenylate MccA-based peptides with substantial N-terminal extensions. This observation raised a question whether MccA can function as a C-terminal tag specifically recognized and adenylated by MccB. To answer this question, a plasmid expressing MBP C-terminally fused to MccA was created. A linker between MBP and MccA contained a site of recognition by factor Xa protease (Fig. 7A). Two types of experiments were performed. First the fusion protein was purified, combined with MccB in the presence or in the absence of ATP at conditions of MccA adenylation and the products were treated with factor Xa followed by mass-spectrometric analysis. The results are shown in Fig. 7B, left. As can be seen, a mass-peak with m/z = 1467.5 was seen in reactions
This mass-peak corresponds to expected peptide ISEFGGGGMRTGNAN that shall be generated upon factor Xa cleavage (Fig. 7A). In reactions containing ATP this mass-peak was not present. Instead, a mass-peak with m/z = 1796.8, matching adenylated ISEFGGGGMRTGNAD was observed. The *in vitro* adenylation experiment was repeated using [α-32P]ATP and reaction products were separated by SDS PAGE. The gel was stained and then subjected to autoradiography. As can be seen, in reactions containing the MBP-MccA fusion protein, MccB, and [α-32P]ATP, the MBP-MccA became radioactively labeled. No labeling was observed in lanes where one of reaction components was missing (Fig. 7C).

To show that labeling of proteins tagged by the MccA peptide tag can also occur *in vivo*, *E. coli* cells co-expressing MBP-MccA and MccB were obtained. Upon induction, MccB and MBP-MccA co-overproducing cell cultures continued growth for several hours, however, stopped growing afterwards. No such effect was seen in cultures co-overexpressing MccB and MBP. The cessation of growth of cultures co-overproducing MccB and MBP-MccA could have been due to degradation of adenylated MBP-MccA fusion which should eventually lead to accumulation of processed McC. Indeed, when extracts of cells co-overproducing MccB and MBP-MccA were loaded on an amylose resin column followed by factor Xa treatment of affinity purified material, a mass-peak with m/z = 1796.8 matching adenylated ISEFGGGGMRTGNAD terminal peptide of MBP-MccA was detected (Fig. 7B, right). Only a mass-peak with m/z = 1487.5 corresponding to unmodified ISEFGGGGMRTGNAN was present in cells expressing MBP-MccA alone. We conclude that the MccA peptide can serve as a terminal tag, which is adenylated by MccB either *in vivo* or *in vitro* with high specificity and efficiency.
DISCUSSION

In this work, we extend the structure-activity analysis of microcin C by preparing a panel of modified Mc in an in vitro adenylation reaction catalyzed by the McC synthase MccB.

Earlier, structure-activity relationships of McC were studied by introducing mutations in cloned mccA gene or by chemical synthesis of McC analogs. Introducing substitutions in the mccA gene did not allow the role of MccA peptide Met1 to be assessed. The chemical approach has proven to be impossible or very complicated when attempts to synthesize McC-like compounds with longer peptide parts were made. Further, since a sulfonamide rather than a phosphoamide bond was used to connect adenosine to the peptide during chemical synthesis, decoration with aminopropyl or other modifications, which could increase bioactivity, became impossible. The enzymatic synthetic approach is free from these limitations. The MccB enzyme is highly active in vitro and large amounts (tens of milligrams) of peptide adenylates can be routinely prepared using chemically synthesized peptides as adenylation substrates. Using this strategy we show that the N-terminal amino acid of MccA plays a critical role in adenylation by the MccB enzyme. Only peptides with bulky hydrophobic residues (which includes the natural methionine) are accepted by the enzyme.

McC variants with substituted N-terminal methionine residue are taken up through the YejABEF inner membrane transporter. While the physiological function of YejABEF is not known, it has been proposed that in Salmonella it is involved in the uptake of peptides containing N-terminal formyl-methionine, contributing to avoidance of host immune response (9, 20). Our data show that mutant MccA peptide with substitution of the N-terminal methionine for glutamine does not compete with McC, confirming the importance of terminal Met residue for YejABEF recognition. However, just like in the case of MccB binding, the situation appears to be more complex, since MRTGNAD also poorly competes with McC, indicating the presence of
N-terminal methionine cannot be the only determinant of recognition by YejABEF. While MRTGNAD is unable to compete with McC for entry through YejABEF, its sequence is identical to the peptide moiety of McC. This observation suggests that YejABEF also recognizes the nucleotide part of McC and may thus be functionally similar to its close relative NppABCD from *Pseudomonas aeruginosa*, which contributes to resistance to peptidyl-nucleoside antibiotics (21).

The wild-type McC processing pathway requires deformylation by methionine deformylase followed by the action of methionine aminopeptidase (MAP) and degradation by non-specific aminopeptidases A, N, or B of the resulting hexapeptide-adenylate (2). Compounds with substituted Met must be processed without the involvement of methionine deformylase or MAP. The bioactivity of some McC-like compounds produced by bacteria other than *E. coli* has been shown to be limited by the rate of proteolytic processing (13). Combining peptide-stability/degradation rate analysis with MccB-catalyzed *in vitro* adenylation could yield adenylation-competent peptides with faster processing, which may result in peptide-adenylates with increased bioactivity.

Enzymatic synthesis allowed us to probe longer peptides as adenylation substrates and determine bioactivity of resulting adenylates. Using a series of N-terminally extended MccA variants we show that extension of McC peptide length by a single amino acid increases bioactivity. Further increase has no additional effect, followed by eventual decline in activity.

The transport of longer peptide-adenylates remains YejABEF dependent up to a certain point. However, McC variants with peptides lengths of 20 and 25 amino acids are transported jointly by YejABEF and SbmA. Earlier, we showed that a peptide adenylate prepared by adenylation of 25 aminoacid long MccA-like peptide from *Synechococcus sp.* by cognate MccB enzyme enters *E. coli* cells exclusively through SbmA (13). The difference between entry pathways of *E. coli* and
Synechococcus sp. peptides based adenylates indicates that both YejABEF and SbmA exhibit sequence or structural specificity towards the peptides they transport. The fact that certain peptide adenylates are taken up by two transport systems is potentially very attractive as it could allow to limit the appearance of spontaneously resistant mutants.

The biological activity of longer peptide-adenylates can be further increased by aminopropylation, providing an additional way of obtaining compounds with higher activity levels. Since multiple MccA-MccB pairs have been validated in in vitro adenylation reactions, the opportunities for obtaining modified peptide-adenylates with increased activity levels explored here using the E. coli system are also fully open for other McC-like compounds some of which are produced by (and presumably target) important pathogens.

We finally show that MccA fusions to full-sized proteins such as MBP are also subject to adenylation by MccB in vivo and in vitro. This finding opens several interesting possibilities. First, C-terminal MccA tag offers an opportunity to selectively label proteins in a way that is complementary and orthogonal to popular methods that rely on phosphorylation by a kinase (22). Second, an perhaps more interestingly, introducing an adenylated tag into a protein in vivo should allow one to study protein stability, for upon degradation of a tagged protein processed toxic McC should accumulate inside the cell.

ACKNOWLEDGEMENTS

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REFERENCES


**FIGURE LEGENDS**

**Figure 1. The structure of microcin C.**

The chemical structure of the part of the molecule corresponding to toxic processed McC (modified aspartyl-adenylate) released inside the cell is shown. The transport part – the first 6 residues of the MccA peptide are indicated in a single amino acid code. The N-terminal methionine is formylated.

**Figure 2. In vitro adenylation of E. coli MccA peptide mutants by MccB.** Chemically synthesized peptides corresponding to 7-amino-acid-long wild-type MccA MRTGNAN, mutants bearing indicated single amino acid substitutions (underlined), or an MRTGNAN peptide containing a D-stereoisomer of threonine at position 3 were combined with recombinant MccB in the absence (top) and the presence (bottom) of ATP. Reaction products were analyzed by MALDI-MS. The m/z values of mass-peaks corresponding to starting peptides and adenylation products are indicated. Adenylation by MccB adds 329 Daltons to substrate peptide. Asterisks indicate peaks corresponding to adenylation reaction intermediates containing a terminal succinimide.

**Figure 3. In vitro adenylation of E. coli MccA peptide mutants with substitution of N-terminal methionine residue.** Reactions were set up and analyzed as described in Fig. 2 legend. Results are shown only for peptides that were modified by MccB. Other tested peptides bearing N-terminal substitutions are listed in Table 1.

**Figure 4. Effect of MccA variants that are not subject to adenylation on wild-type MccA adenylation and on bioactivity of adenylated MccA.** (A) Adenylation reaction of wild-
type MccA was conducted in the presence of 50-fold excess of QRTGNAN, MRTGNAQ, or MRTGNAD. Reaction products were separated by reverse-phase HPLC and adenylation of wild-type MccA was determined by monitoring absorbance at 260 nm. The presence of adenylated MccA in the peak was confirmed by MALDI-MS. (B) The product of MccA adenylation was purified, combined with indicated concentrations of MccA peptide variants and 10-μl reaction aliquots were deposited onto cell lawns formed by *E. coli* cells. The results of overnight growth at 37 °C are shown. The plate shown is representative of one of three independent experiments.

**Figure 5. In vitro adenylation of extended length MccA peptide variants.** Chemically synthesized peptides corresponding to wild-type MccA MRTGNAN and indicated N-terminally extended variants were combined with recombinant MccB in the absence (top) and the presence (middle) of ATP. Reactions shown at the bottom were incubated in the presence of ATP and recombinant MccD, MccE, and Mtn enzymes and SAM substrate and PLP cofactor at conditions promoting aminopropylation of peptidyl adenylates. The addition of aminopropyl adds 57 Daltons to adenylated peptides.

**Figure 6. In vitro adenylated extended-length MccA variants are active and enter cells through both YejABEF and SbmA.** (A) Wild-type MccA or 20 and 25 aminoacid long peptides containing C-terminal MccA sequences were adenylated by MccB *in vitro* and products analyzed by MALDI-MS. (B) Adenylation products were purified and 10-μl reaction aliquots were deposited onto cell lawns formed by wild-type *E. coli* or indicated mutants along with several control antibiotics. The results of overnight growth at 37 °C are shown. The plate shown is representative of one of three independent experiments.
Figure 7. MccA can be used as a C-terminal tag for adenylation of proteins \textit{in vivo} and \textit{in vitro}. (A) The structure of the fusion protein containing MBP fused to MccA through a linker containing factor Xa cleavage site is schematically shown. Factor Xa cleavage results in generation of an ISEFGGGMRTGNAN peptide with Mw=1466.7. (B) Left: the MBP-MccA fusion protein was purified, combined with MccB in the presence or in the absence of ATP at conditions favoring adenylation. Reactions were treated with factor Xa and subjected to MALDI-MS. Right: MBP-MccA protein was expressed in cells with or without MccB, purified, subjected to factor Xa treatment and products analyzed by MALDI-MS. (C) Purified MBP-MccA was incubated with MccB and in the presence or in the absence of $[\alpha^{32P}]$ATP. Reaction products were resolved by SDS-PAGE and stained with Coomassie (left, bands corresponding to MBP-MccA and MccB are indicated). The leftmost lane contains molecular weight markers. An autogradiograph of the gel is shown on the right.
Table 1. In vitro adenylation and in vivo activity of MccA heptapeptide point mutants.

<table>
<thead>
<tr>
<th>Peptide sequence</th>
<th>Adenylation by MccB in vitro</th>
<th>In vivo activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Wild-type cells</td>
</tr>
<tr>
<td>MRTGNAN</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MRTGNAD</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MRTGNAQ</td>
<td>-</td>
<td>-</td>
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<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>MRLGNAN</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MRSGNAN</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MRCGNAN</td>
<td>+/-</td>
<td>+</td>
</tr>
<tr>
<td>MRKGNAN</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MR (D- Thr) GNAN</td>
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<td>-</td>
</tr>
<tr>
<td>MRTWGNAN</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GRTGNAN</td>
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</tr>
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<td>+</td>
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<tr>
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<td>-</td>
</tr>
<tr>
<td>ERTGNAN</td>
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</table>
The table presents the results of *in vitro* adenylation by MccB of each peptide listed in the first column as detected by MALDI-MS of reactions conducted at standard conditions (see, for example, Fig. 2 and the Material and Methods section) and the results of bioactivity testing of 10 µl aliquots of completed adenylation reactions on lawns of wild-type or *yejB* mutant *E.coli* cell lawns. “+” indicates that clear growth inhibition zone was detected, “-” – no growth inhibition zone observed.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>-</th>
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</tr>
</thead>
<tbody>
<tr>
<td>KRTGNAN</td>
<td>-</td>
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Table 2. Bioactivity of elongated MccA peptide adenylates.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Minimal inhibitory concentration (MIC), μM&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
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<tr>
<td></td>
<td>E. coli</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td></td>
</tr>
<tr>
<td>MRTGNAD-AMP</td>
<td>10</td>
</tr>
<tr>
<td>MRTGNAD-AMP (ap)</td>
<td>2.5</td>
</tr>
<tr>
<td>GMRTGNAD-AMP</td>
<td>2.5</td>
</tr>
<tr>
<td>GMRTGNAD-AMP (ap)</td>
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<tr>
<td>GGMRTGNAD-AMP (ap)</td>
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<tr>
<td>GGGGRTGNAD-AMP</td>
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<tr>
<td>GGGGRTGNAD-AMP (ap)</td>
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</tr>
<tr>
<td>GGGGGGRTGNAD-AMP</td>
<td>5</td>
</tr>
<tr>
<td>GGGGGGRTGNAD-AMP (ap)</td>
<td>1.3</td>
</tr>
</tbody>
</table>

<sup>a</sup>Bioactivity was measured with serial two-fold dilutions of each compound in the diffusion test on the lawn of the indicated E. coli cells. MIC values presented show a minimal concentration at which the inhibition zone was visible around a 10 μl compound aliquot.
A

\[ \text{Xa Factor} \rightarrow \text{MBP} \]

\[ \text{LGIEGRIFGGGMRTGNAN} \]

\[ \text{Mw 1466.7} \]

B

\textbf{in vitro labeling}

\begin{align*}
\text{- ATP} & : 1467.7 \\
\text{+ ATP} & : 1467.7, 1796.8
\end{align*}

\textbf{in vivo labeling}

\begin{align*}
\text{- MccB} & : 1467.7, 1796.8 \\
\text{+ MccB} & : 1467.7
\end{align*}

C

\textbf{- ATP + ATP}

- MBP-MccA
- MccB

\textbf{- ATP + ATP}

- MBP-MccA