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The C-Terminal Part of Microcin B Is Crucial for DNA Gyrase Inhibition and Antibiotic Uptake by Sensitive Cells

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Microcin B (McB) is a ribosomally synthesized antibacterial peptide. It contains up to nine oxazole and thiazole heterocycles that are introduced posttranslationally and are required for activity. McB inhibits the DNA gyrase, a validated drug target. Previous structure-activity analyses indicated that two fused heterocycles located in the central part of McB are important for antibacterial action and gyrase inhibition. Here, we used site-specific mutagenesis of the McB precursor gene to assess the functional significance of the C-terminal part of McB that is located past the second fused heterocycle and contains two single heterocycles as well as an unmodified four-amino-acid C-terminal tail. We found that removal of unmodified C-terminal amino acids of McB, while having no effect on fused heterocycles, has a very strong negative effect on activity *in vivo* and *in vitro*. In fact, even nonconservative point substitutions in the last McB amino acid have a very strong effect by simultaneously decreasing uptake and ability to inhibit the gyrase. The results highlight the importance of unmodified McB amino acids for function and open the way for creation of recombinant McB derivatives with an altered or expanded spectrum of antibacterial action.

Microcin B (McB) is a founding member of an extended TOMM (thiazole/oxazole modified microcins) family of bacterial peptides (1). Members of the family have diverse functions that are thought to be determined by the heterocycles. McB is produced by some *Escherichia coli* strains harboring a plasmid-borne *mcbABCDEFG* operon (2, 3). The operon includes the *mcbA* gene, encoding a microcin B peptide precursor (4), genes *mcbBCD*, whose products form an enzyme complex that posttranslationally modifies McbA, genes *mcbEF*, whose products are responsible for transport of McB out of producing cells (5, 6), and the *mcbG* self-immunity gene (6).

The first 26 amino acids of the 69-amino-acid-long McbA propeptide McbA constitute a leader peptide that is recognized by the McbBCD enzyme complex (McB synthetase). McB synthetase modifies Gly-Cys or Gly-Ser dipeptides of McbA into thiazole or oxazole heterocycles, respectively. The McbA Gly-Ser-Cys and Gly-Cys-Ser McB tripeptides are converted into fused oxazole-thiazole (site A) and thiazole-oxazole (site B) heterocycles, respectively (7, 8). The heterocycles are formed in two steps (8). First, thiazoline and oxazoline intermediates are produced during a cyclodehydration reaction; second, dehydrogenation of those intermediates yields thiazole and oxazole heterocycles. Despite the fact that it had been convincingly shown as early as 1999 that the concerted action of McbB, McbC, and McbD is needed for McB maturation (9), for a long time the roles of individual enzymes in this process remained unclear. The mechanism of heterocycle formation was clarified during the analysis of Balh, a TOMM from *Bacillus* sp. Al Hakam (10). It was shown in this system that protein D, an McbD homolog, is actually responsible for catalyzing cyclodehydration in the synthase complex rather than just for substrate recognition as believed earlier (9). Protein D uses ATP to phosphorylate the carbonyl oxygen in the amino acid residue preceding a serine or cysteine residue being cyclized. Phosphorylation facilitates the removal of a water molecule to yield a cycle (10). Protein C, an McbB homolog, is a zinc binding protein (9) that cooperates with protein D to accelerate azoline (thiazoline and oxazoline) formation (10), through a mechanism that remains to be defined. McbC completes heterocycle synthesis. This protein is a flavin mononucleotide (FMN)-dependent dehydrogenase (9) that oxidizes thiazoline and oxazoline to the corresponding azoles (11) present in mature TOMMs.

The major form of McB (referred to below as Δ0) contains eight (four single and two fused) heterocycles. Most Δ0 McB extracted from the cells (referred to as an X type) contains an unusual depsipeptide bond between residues Cys^{51} and Ser^{52} (12). A minor portion of Δ0 McB (referred to as N type) carries a conventional amide bond at this position. The XΔ0 and NΔ0 forms of McB are equally active. The N-X isomerism involves Ser^{52}, which is part of a $Gly^{50}Cys^{51}Ser^{52}$ tripeptide. A minor fraction of McB contains an additional fused heterocycle (site C) formed from this tripeptide. This nine-ring form is referred to as Δ+1. McB maturation intermediates of both X and N types with reduced numbers of heterocycles—seven (Δ-1), six (Δ-2), and 5 (Δ-3)—are also produced in small amounts due to incomplete heterocyclization of C-terminal Gly-Cys or Gly-Ser dipeptides of McbA. The last C-terminal oxazole is formed from Ser^{65}. While the last serine of McbA, at position 67, is preceded by a glycine residue, it has not been observed to undergo cyclization (13).

After completion of heterocyclization, the McbA leader peptide is cleaved off to generate mature McB. The cleavage requires host *tldD* and *tldE* genes, which are thought to encode a protease (14). McB is exported into the periplasmic space of producing cells by a membrane-associated ATP-dependent transporter composed of McbE and McbF proteins. Cells producing McB are im-

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mune to the action of the antibiotic due to the combined action of McbE/F and McbG (6). McbG is a pentapeptide repeat protein; its homolog Qnr binds to DNA gyrase and protects it from quinolones (15), antibiotics that are structurally unrelated to McB.

McB targets the DNA gyrase (16). The mechanism of DNA gyrase inhibition by McB appears to be similar to that by quinolones. Both antibiotics stabilize a transient state that consists of DNA gyrase covalently bound to cleaved DNA, preventing DNA religation. Accumulation of double-stranded DNA breaks leads to cessation of DNA replication and cell death. Despite the similarities in the mechanisms of action, the regions of DNA gyrase with which McB and quinolones interact appear to be distinct. Mutations conferring resistance to quinolones mostly map to the GyrA subunit near the active site of the protein (17). Mutations providing resistance to McB map to position 751 of the GyrB subunit (16).

Here, we used site-specific mutagenesis of cloned *mcbA* gene to assess the functional significance of the McB region located past the site B fused heterocycle. This region contains two single oxazole heterocycles as well as an unmodified four-amino-acid C-terminal tail. We find that removal of unmodified C-terminal amino acids of McB, while having no effect on fused heterocycles, has a very strong negative effect on activity *in vivo* and *in vitro*. In fact, even nonconservative point substitutions in the last McB amino acid at position 69 strongly decrease the mutant antibiotic transport and its ability to inhibit the DNA gyrase. Our results both highlight the importance of unmodified McB amino acids for function and open the way for creation of recombinant McB derivatives with an altered or expanded spectrum of antibacterial action.

MATERIALS AND METHODS

Strains and plasmids. The *E. coli* strains used in this work were DH5α [F^- *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG* Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, *hsdR17*(r_K^- m_K^+), λ−]; BL21(DE3) [F^- *ompT gal dcm lon hsdS*$_B$(r_B^- m_B^-) λ(DE3 [*lacI lacUV5*-T7 gene *1 ind1 sam7 nin5*])]; ZK4*sbmA* (18; provided by Roberto Kolter); and CSH50 λ*sfiA*::*lacZ* Δ*tldD* Δ*tldE* (14; provided by Laurence Van Melderen). Plasmid pPY113 (19) harboring the entire *mcb* operon was also provided by Roberto Kolter.

Molecular cloning. The pPY113 plasmid was digested with BamHI and SmaI, and a 975-bp fragment containing *mcbA* and upstream sequences was cloned into the pUC18 vector to obtain the pUC18-*mcbA* plasmid.

pPY113 was linearized by ApaI, and 3′overhangs were removed with Klenow fragment (New England BioLabs). The plasmid was next digested with BamHI, and an ~5.6-kb fragment containing the entire *mcb* operon was recloned into pACYC184 to obtain pACYC184-*mcbABCDEFG*. A frameshift mutation was introduced into the fourth codon of *mcbA* in pACYC184-*mcbABCDEFG* using the QuikChange (Agilent) mutagenesis method and oligonucleotides McbAdisr_dir (5′-TCCTTATGGAATTAA GAAGCGAGTGAATTTG) and McbAdisr_rev (5′-CAAATTCACTCGC TTCTTAATTCCATAAGGA) (nonnatural bases are underlined) to yield the pACYC184-*mcbBCDEFG* plasmid.

To create truncated derivatives of *mcbA*, the pUC18-*mcbA* plasmid was amplified by outward PCR using an McbAter (5′-TGATACGTTGA ATTAACCGTTCAGGAGC) primer whose sequence contains the stop codon and the 3′ untranslated region (3′-UTR) of *mcbA* and appropriate primer annealing inside *mcbA* (sequences are available upon request). PCR fragments generated with PfuTurbo DNA polymerase (Stratagene) were treated with T4 polynucleotide kinase (New England BioLabs) and ligated, and, after transformation, desired recombinant plasmids were identified by DNA sequencing.

Purification of McB and its derivatives. Cultures of DH5α cells harboring plasmids encoding wild-type or mutant McB were grown overnight in liquid LB and used to inoculate a 0.8-liter volume of M63 minimal media supplemented with 5% of sodium succinate, 0.0005% thiamine chloride, 1 mM $MgSO_4$, 1 mM $FeSO_4$, and appropriate antibiotics. Cultures were grown for 24 h at 37°C. Cells were collected by centrifugation and boiled in 20 ml of 0.1 M acetic acid–1 mM EDTA for 15 min. The resulting suspensions were centrifuged at 10,000 × *g*, and clarified supernatants were applied onto C_{18} HyperSep cartridges (Thermo Scientific). The cartridges were extensively washed with 10% acetonitrile–0.1% trifluoroacetic acid (TFA). Microcin-containing fractions were eluted with 25% acetonitrile–0.1% TFA and vacuum dried. The resulting pellets were dissolved in dimethyl sulfoxide (DMSO) and applied onto an Phenomenex Jupiter C_{18} high-performance liquid chromatography (HPLC) column (Waters) in 10% DMSO–0.1% TFA. The column was equilibrated in 0.1% TFA. The bound material was eluted with a linear gradient of acetonitrile (from 0% to 50% acetonitrile in 30 min)–0.1% TFA. Absorbance of eluting material was monitored at 254 nm. Different forms of microcins were eluted between 12 and 16 min. Individual peaks were collected, vacuum dried, resuspended in DMSO, and stored at +4°C. The microcin concentration was determined according to the method described in reference 20.

MALDI-MS and MS-MS analyses. Mass spectra were recorded on an UltrafleXtreme matrix-assisted laser desorption ionization–tandem time of flight (MALDI-TOF-TOF) mass spectrometer (MS) (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 0.005%. Aliquots (1 μl) of HPLC fractions were mixed with 0.5 μl of 2,5-dihydroxybenzoic acid (Aldrich) solution (20 mg/ml in 30% acetonitrile–0.5% TFA) on a steel target, and droplets were left to dry at room temperature. Fragment ion spectra were generated by laser-induced dissociation in Lift mode; the accuracy of the mass peak measurement was 70 ppm for parent ions and 1 Da for daughter ions. The correspondence of the found masses to the peptides of microcin B and its derivatives and to tandem mass spectrometry (MS/MS) peptide fragments was revealed manually with the help of GPMAW 4.04 software (Lighthouse data).

Determination of antibiotic activity and SOS response. For determination of antibiotic activity of cells producing mutant microcins, 3-μl aliquots of suspensions of cultures of *E. coli* DH5α cells transformed with the McB plasmids tested were placed onto the surface of plates of M63 agar supplemented with 0.2% yeast extract, 2% glucose, 1 mM $MgSO_4$, 1 mM $FeSO_4$, and appropriate antibiotics and allowed to grow overnight at 37°C. On the following day, the plate was overlaid with 5 ml of 0.6% agar in distilled water mixed with 100 μl of overnight culture of McB-sensitive *E. coli* BL21(DE3) cells. Plates were incubated at a room temperature overnight, and the sizes of growth inhibition zones were recorded.

To measure antibiotic activities of pure microcins, 2-μl drops of microcin solutions of known concentrations (in DMSO) were spotted onto freshly prepared lawns of BL21(DE3) cells. Lawns were prepared by overlaying M63 or M9 (M9 salts supplemented with 0.4% glycerol, 0.2% Casamino Acids, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 0.002 mM $FeSO_4$, 0.0005% thiamine hydrochloride) agar plates with 5 ml of 0.6% agar mixed with 100 μl of overnight BL21(DE3) culture. Plates were incubated at room temperature overnight.

To test the SOS response, CSH50 cells transformed with appropriate McB plasmids were grown overnight in LB media, diluted into fresh M63 media supplemented with 0.2% yeast extract, 2% glucose, 1 mM $MgSO_4$, and 1 mM $FeSO_4$ and grown to an optical density at 600 nm (OD_{600}) of 0.6 to 0.8. Cells were harvested, and the β-galactosidase activity was measured according to a standard protocol (21).

***In vitro* DNA cleavage assay.** Relaxed DNA substrate was made from supercoiled pUC18 plasmid (prepared by alkaline lysis using a Qiagen QIAprep Spin Miniprep kit) by treatment with *E. coli* topoisomerase I (New England BioLabs) followed by Exonuclease T5 (Epicentre) treat-

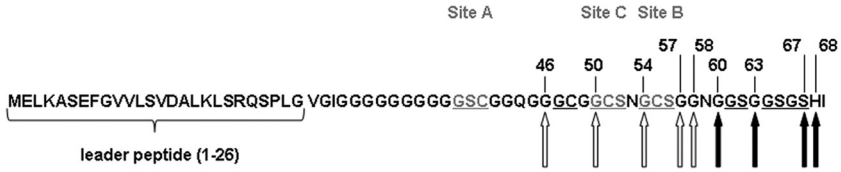

FIG 1 The primary structure of *E. coli* McbA and its mutants. The sequence of 69-amino-acid McbA is shown (single-letter code). The leader segment which interacts with the McbBCD synthase is indicated. The di- and tripeptides that are converted into heterocycles by the McbBCD synthase are underlined. The tripeptides (highlighted in gray) are converted into the site A, site B, and site C fused heterocycles. A fused heterocycle at site C is present only in the minor portion of wild-type McB. The vertical arrows below the McbA sequence indicate the endpoints of C-terminally deleted McbA mutants constructed in this work. White arrows indicate mutants for which no production of heterocycle-containing products was detected. Filled arrows indicate mutants for which production of heterocycle-containing material was observed.

ment to remove nicked DNA according to the manufacturer's instructions.

DNA cleavage assays were carried out using a method based on that described by Heddle et al. (22). Reaction mixtures contained 35 mM Tris-HCl (pH 7.5), 24 mM KCl, 4 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 1.75 mM ATP, 5 mM spermidine, 0.1 mg/ml bovine serum albumin, 6.5% (wt/vol) glycerol, 10 μg/ml yeast tRNA, 5 nM relaxed DNA substrate, 200 nM *E. coli* DNA gyrase, and various concentrations of mutant and wild-type microcins or ciprofloxacin. Ciprofloxacin (Sigma) was dissolved in water and further diluted into Tris-HCl (pH 7.5). After 90 min of incubation at 37°C, SDS (0.2%) and proteinase K (0.2 mg/ml) were added and incubation continued for 30 min. Reactions were quenched with an equal volume of chlorophorm:isoamyl alcohol (24:1) and a half-volume of gel loading buffer (0.1 M EDTA, 0.5 mg/ml bromphenol blue, 25% glycerol [wt/vol]). Reactions were mixed using a vortex device and centrifuged for 5 min on a microcentrifuge, and aqueous phases were loaded onto 1% agarose gels. The gel was stained in ethidium bromide solution (25 μg/ml) for 20 min.

Assay for DNA replication in permeabilized cells. An overnight culture of ZK4*sbmA* cells was diluted into 25 ml of LB and grown to reach an OD_{600} of 0.5. Cells were washed with 15 ml of 50 mM Tris-HCl (pH 7.4) and resuspended in 1 ml of the same buffer. Cells were permeabilized by freezing in liquid nitrogen followed by thawing in a 30°C water bath. Immediately after thawing, 14 μl of cell suspension was diluted with 55 μl of distilled water, mixed with 18 μl of DNA synthesis buffer (230 mM Tris-HCl [pH 7.5], 22 mM $MgCl_2$, 714 mM KCl, 4 mM ATP, 1 mM β-mercaptoethanol, and 200 mM [each] dNTP) (20). Wild-type McB or its mutants were added, and reaction mixtures were incubated for 10 min at 30°C. [α-^{32}P]dATP (5 μCi) was added, and 20-μl aliquots of reactions were withdrawn at various times after [α-^{32}P]dATP addition, quenched with 200 μl of cold 10% trichloroacetic acid, and loaded on nitrocellulose filters (0.45 μM pore size). The filters were washed 10 times with 200 μl of cold 0.01 N HCl and dried, and the amount of filter-bound radioactivity was determined.

RESULTS

Site-specific mutagenesis of the *mcbA* gene. To perform structure-activity analysis of McB, we constructed a series of nested deletions of cloned *mcbA* that encoded C-terminally truncated McbA peptides. Because of the large size of the pPY113 McB production plasmid and the extremely G/C-rich and repetitive sequence of the *mcbA* gene, a double-plasmid McB production system was created to facilitate mutagenesis. One pUC-based plasmid contained the *mcbA* gene or its mutational derivatives under the control of the P*mcb* promoter. Another compatible pACYC-based plasmid contained the entire *mcbABCDEFG* operon and its control region. The *mcbA* gene on the second plasmid was disrupted with a frameshift mutation at the beginning of the gene (codon 4). Cells harboring either of the plasmids alone did not produce McB (data not shown). However, cells harboring the second plasmid were as resistant to McB as control McB-producing cells harboring a wild-type *mcbABCDEFG* plasmid. Thus, disruption of the upstream *mcbA* gene has no significant effect on the level of activity of downstream gene products McbE, McbF, and McbG, which together are responsible for McB resistance (6). By extension, we reasoned that expression of genes in the middle of the operon, *mcbB*, *mcbC*, and *mcbD*, whose products are responsible for McB synthesis, is also not affected by mutation in *mcbA*. Indeed, when both plasmids were introduced in *E. coli* DH5α cells, robust production of McB was observed in a standard plate test. The amount of McB produced by cells harboring two plasmids was comparable to that observed with cells harboring a pACYC plasmid with the wild-type *mcb* operon.

Using site-specific mutagenesis of the pUC-based plasmid containing the *mcbA* gene only, nine derivatives were prepared that encoded McbA peptides containing 68, 67, 63, 60, 58, 57, 54, 50, and 46 N-terminal amino acids (the wild-type McbA is 69 amino acids long) (Fig. 1). In what follows, we refer to mutated *mcbA* genes and their products as $mcbA^{1-N}$ and McB^{1-N}, correspondingly, where N is the last sense codon of the gene or amino acid of the peptide. The first two deletions, $mcbA^{1-68}$ and $mcbA^{1-67}$, affected the four-amino-acid unmodified GSHI tail of McB. In $mcbA^{1-63}$, codons for a GS dipeptide that gets converted into the last oxazole cycle of mature McB were removed. The product of $mcbA^{1-60}$ lacks dipeptides that are converted into the ultimate and penultimate oxazole heterocycles of wild-type McB. Codons 54 to 56 of *mcbA* encode a tripeptide GCS that gets converted into the functionally important site B-fused heterocycle which is separated from the penultimate oxazole cycle by a four-amino-acid linker. Truncations at *mcbA* codons 58 and 57 occur in the linker but do not affect the site B tripeptide. Truncation at position 54 removes residues encoding the site B heterocycle. Truncation at position 50 removes the site B heterocycle, thiazole heterocycle, and an additional oxazole heterocycle. Finally, a construct truncated at position 46 encodes a peptide that was earlier used by Li et al. (8) for *in vitro* maturation studies using purified McbBCD McB synthase. It contains only the GSC tripeptide that gets converted into the site A fused heterocycle.

To determine if mutant *mcbA* genes encode peptides that can be converted into biologically active McB derivatives, cells containing mutant *mcbA* plasmids and a compatible plasmid that served as a source of the McbBCD synthase, the McbEF export pump, and the McbG self-immunity protein were deposited on lawns of McB-sensitive *E. coli* BL21(DE3) cells and growth inhibition zones around the patches of cells containing *mcb* plasmids were recorded after overnight growth. Cells harboring a wild-type

mcbA plasmid were used as a positive control. As expected, a robust growth inhibition zone around these cells was observed. A smaller growth inhibition zone was formed around cells harboring the $mcbA^{1-68}$ plasmid. The growth inhibition zone around cells harboring the $mcbA^{1-67}$ plasmid was 4 times smaller than that observed around control cells producing wild-type McB. No growth inhibition was observed around cells harboring other mutant plasmids (data not shown).

Production of McB mutants and their structures. The results presented above suggest that deletion of C-terminal residues of McbA has an unexpectedly strong effect on McB maturation, export from the producing cell, entry into the susceptible cell, or inhibition of the McB target, the DNA gyrase. A combination of these effects is also possible. To distinguish between these possibilities, we purified McB mutants from producing cells using a protocol developed for wild-type McB purification (20). During wild-type McB purification, a characteristic profile of one major HPLC peak corresponding to an X isoform of fully matured McB containing 8 heterocycles (12) as well as minor peaks corresponding to the N-type isoform of the 8-heterocycle compound (12) and maturation intermediates of both isoforms with 7 and 6 heterocycles are seen (Fig. 2A; the identity of each peak was established by MALDI-MS). During purification of McB^{1-68}, McB^{1-67}, McB^{1-63}, and McB^{1-60}, HPLC peaks eluting in approximately the same area of the acetonitrile gradient where wild-type McB elutes were observed. The amount of UV-absorbing material in these peaks during purification of the mutant microcins was comparable to that observed during wild-type McB purification. An example of McB^{1-60} (expected to contain 6 heterocycles when fully modified) is presented in Fig. 2B. In the particular example shown, in addition to peaks corresponding to the fully mature X- and N-type isoforms, a minor peak corresponding to a 7-heterocycle form of the N-type isoform (with a fused heterocycle at site C) is observed. In contrast, when purification from cultures expressing $mcbA^{1-58}$ or shorter versions of *mcbA* was undertaken, no HPLC peaks that could contain McB were observed (data not shown). Moreover, systematic MALDI-MS analysis of HPLC fractions collected during purification of McB^{1-58} and shorter versions of McB did not reveal any mass ions matching fully or partially modified truncated McbAs (data not shown). We therefore conclude either that maturation of McbA peptides that are shorter than 60 amino acids is prevented or, alternatively, that unmodified (or modified) mutant McbA peptides are very unstable and we therefore failed to detect the production of mutant microcins. In what follows, we concentrate our attention only on the four deletion variants of McBs that are produced.

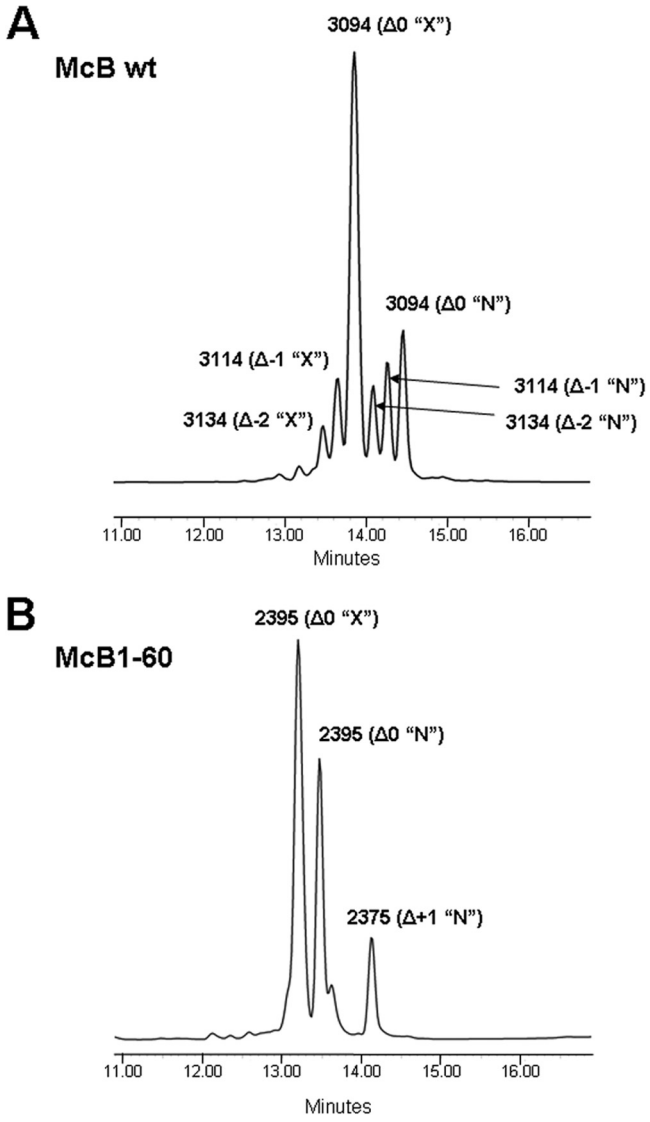

FIG 2 Purification of McB mutants. An HPLC chromatogram shows the final stage of purification from *E. coli* cells co-overexpressing *mcbBCDEFG* and wild-type *mcbA* (A) or the $mcbA^{1-60}$ mutant (B). Material from HPLC peaks was subjected to MALDI-MS, and *m/z* values were determined as shown for each peak. The peaks were further identified by MS-MS and are labeled according to the number of heterocycles.

The structures of individual forms of mutant microcins were determined by tandem MS analysis. The structures consistent with fragmentation analysis data and corresponding to the major X-type McB Δ0 species are presented in Fig. 3. In each case, the structures shown (and the corresponding N-type isoform) constituted the bulk of McB produced. In addition, small amounts of Δ+1 as well as Δ-1 and Δ-2 species were sometimes produced (see Fig. 2 for an example). The latter two forms, when produced, always lacked the ultimate (Δ-1) or the ultimate and penultimate (Δ-2) heterocycles, respectively (data not shown). The structures of McB^{1-68} and McB^{1-60} matched the ones expected from the sequence of the mutant McbA propeptides. In the McB^{1-67} and McB^{1-63} structures, the GS dipeptides at positions 64 and 65 and positions 61 and 62, correspondingly, that should have been converted into the ultimate oxazole heterocycle were unmodified. Interestingly, the last GS dipeptide at positions 66 and 67 of wild-type McB is also never modified. The result seems to suggest that the McB synthase requires a certain number of residues past the heterocycle precursor dipeptide for modification. The fact that McB^{1-68} contains a heterocycle formed from a GS dipeptide at positions 64 and 65, which in this mutant is followed by an unmodified tripeptide GSH, suggests that the minimal length of downstream sequence sufficient for modification is three amino acids. This length also appears to be a necessary one, since, as already mentioned, the last GS dipeptide at positions 66 and 67 of wild-type McB, which is followed by only two unmodified amino acids (HI), is never modified. To directly show that introduction of three downstream residues is sufficient for modification, we

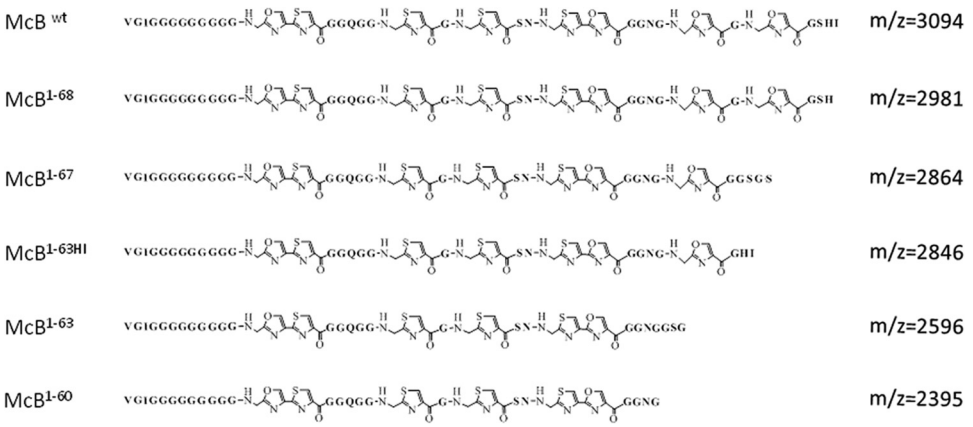

FIG 3 The structures of McB mutants. For each McB mutant for which HPLC peaks were detected during a standard purification procedure as used for wild-type McB, structures consistent with results of MS-MS analysis of material from major HPLC peaks are shown.

created an additional mutant, McbA$^{1-63}$HI, that contained two additional nonnatural amino acids after McbA residue 63. The corresponding microcin, McB$^{1-63}$HI, was purified as an equimolar mixture of forms containing 6 and 7 heterocycles. The latter form contained a heterocycle at positions 61 and 62 (Fig. 3). We therefore conclude that the presence of the third residue downstream of a modifiable dipeptide allows efficient modification to occur. Two residues are not sufficient.

***In vivo* activity of McB mutants.** Cells expressing many of the McB mutants studied here did not inhibit the growth of McB-sensitive tester cells, an effect that may have been due to various reasons, including low levels of production. We prepared sufficient amounts of Δ0 forms of McB mutants whose production could be detected. Solutions containing matching concentrations of these compounds were deposited on sensitive cell lawns to determine MIC values (defined here as the lowest concentration where a visible growth inhibition zone is detected). The results are presented in Table 1. As can be seen, removal of terminal McB amino acid leads to a strong, ca. 50-fold decrease in the activity level (MICs of 0.06 μM for wild-type McB and 3 μM for McB$^{1-68}$). Since McB$^{1-68}$ contains the same number of cycles as wild-type McB, the result suggests that the isoleucine residue at position 69 of intact McB contributes strongly to the biological activity. Shorter deletion variants of McB were very poorly active (MIC of 150 μM or more). Curiously, McB$^{1-63}$HI, which contains the same number of heterocycles as McB$^{1-67}$ but whose two last amino acids, HisIle, match terminal amino acids of wild-type McB, had a MIC of 30 μM, again supporting the idea that a terminal isoleucine stimulates the biological activity.

TABLE 1 Biological activity of McB C-terminal deletion mutants[a]

Microcin	Minimal concn (μM) producing growth inhibition zone
McB^{wt}	0.06
McB$^{1-68}$	3
McB$^{1-67}$	150
McB^{1-63HI}	30
McB$^{1-63}$	150
McB$^{1-60}$	300

[a] The indicated McB mutants were purified (most material was in the type XΔ0 form) and used to determine the ability to inhibit the growth of *E. coli* BL21(DE3) cells as described in Materials and Methods. Wild-type McB was used as a control.

***In vitro* activity of mutant microcins.** The data presented above clearly indicate that C-terminal deletions strongly affect the biological activity of McB; however, they do not allow one to distinguish if these effects are due to decreased ability of mutant McBs to penetrate the cell or are caused by altered interactions with the DNA gyrase (a combination of these effects is also possible). To assess the ability of microcin mutants to inhibit the DNA gyrase, their effects on DNA gyrase-catalyzed plasmid cleavage reactions were investigated *in vitro* (Fig. 4). In this reaction, the addition of wild-type McB to reaction mixtures containing relaxed covalently closed plasmid, DNA gyrase, and ATP causes accumulation of linear plasmid (labeled L) arising due to stabilization of enzyme complex with cleaved DNA in the presence of the drug. Increasing the concentration of wild-type McB increases the amount of the linear form, albeit the effect is not large (Fig. 4A). Ciprofloxacin, an unrelated gyrase inhibitor, also causes accumulation of linearized plasmid and is more efficient in this regard than McB (Fig. 4A), in agreement with earlier observations (22). Representative results for two mutant microcins, McB$^{1-68}$ and McB$^{1-60}$, are shown in Fig. 4B and C, respectively. As can be seen, both mutants were defective compared to the wild-type McB. Yet both mutants were able to cause some accumulation of linear DNA, indicating that they retained some residual ability to inhibit the gyrase. A similar result was recently reported by Collin et al. (23).

The relatively low efficiency of linearized plasmid accumulation even with wild-type McB makes it hard to draw quantitative conclusions about activity levels of McB mutants. As an independent assay for the ability of McB mutants to inhibit the gyrase (and cause accumulation of double-stranded breaks in DNA), we introduced wild-type or mutant McB-producing plasmids into *E. coli* CSH50 reporter cells (14), carrying a genomic fusion of the LexA-dependent *sfiA* promoter to the *lacZ* gene. These cells also lack the TldDE protease that is required for efficient McB export from the producing cell (14). When these cells undergo the SOS response caused by accumulation of double-stranded DNA breaks, the LexA-dependent inhibition of *sfiA*::*lacZ* fusion transcription is relieved, resulting in β-galactosidase production. The results of β-galactosidase activity measurements in cultures har-

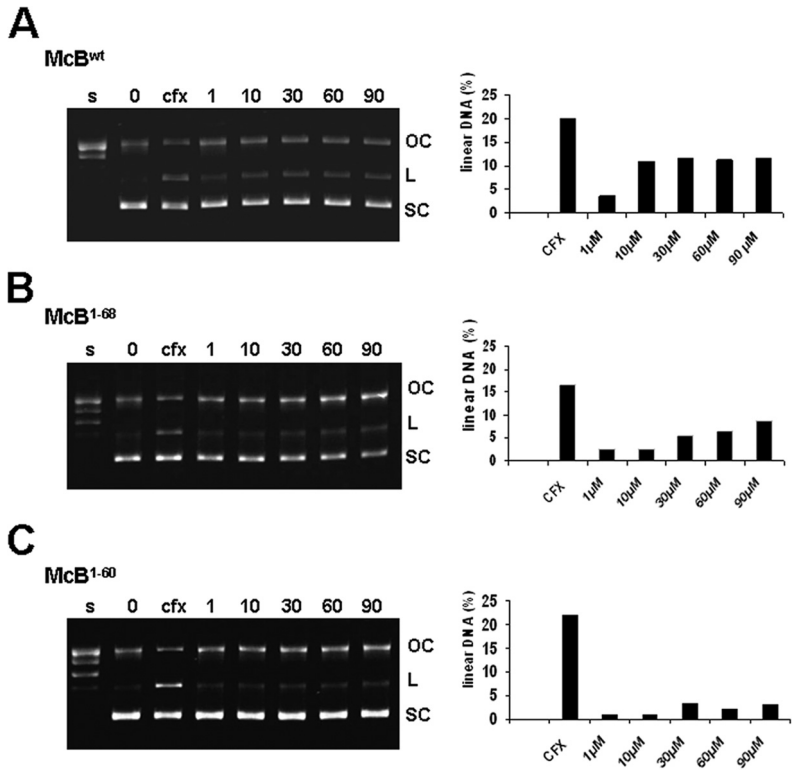

FIG 4 *In vitro* activity of mutant microcins. Plasmid pUC18 was incubated with *E. coli* gyrase in the absence or in the presence of increasing concentrations of wild-type McB (A), McB^{1-68} (B), and McB^{1-60} (C). The reaction products were resolved by agarose gel electrophoresis and revealed by ethidium bromide staining. "OC" stands for open circular form, "L" stands for linearized plasmid, and "SC" stands for supercoiled plasmid. s, reaction without enzyme, 0, reaction with no inhibitors; cfx, reaction with 1 μM ciprofloxacin as control; 1, 10, 30, 60, and 90, increasing concentrations (μM) of microcins. The gels shown on the left side were quantified by densitometry, and the percentage of linear plasmid for each lane was calculated and is presented in the right panel.

boring plasmids encoding various McbA mutants are presented in Fig. 5. As can be seen, cells producing McB^{1-68} demonstrated an elevated level of β-galactosidase, which, however, was much less than that obtained with wild-type McB producing culture. No

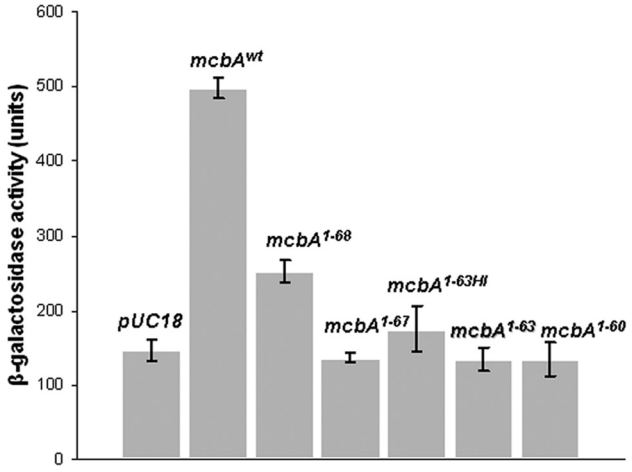

FIG 5 *In vivo* activity of mutant microcins. *E. coli* CSH50 tester cells containing an *sfiA*::*lacZ* fusion were transformed with plasmids co-overexpressing *mcbBCDEFG* and the indicated sets of *mcbA* genes (or the pUC18 vector control). Cell cultures were prepared, and levels of β-galactosidase activity in each culture were determined. The histograms present mean values and standard deviations obtained in three independent experiments.

β-galactosidase activity above the background (cultures containing a pUC18 vector instead of an *mcbA* expression plasmid) was detected in cultures producing McB^{1-67}, McB^{1-63}, and McB^{1-60}. Cells producing McB^{1-63HI} had a slightly elevated level of β-galactosidase, in agreement with a lower MIC value for this compound (Table 1). Therefore, it follows that even McB^{1-67}, which lacks just the last two C-terminal amino acids, HI (and has seven rather than eight heterocycles), is either inactive or very strongly defective in gyrase inhibition. In contrast, McB^{1-68}, which has eight heterocycles and lacks only the C-terminal isoleucine 69 of wild-type McB, appears to be partially active.

When normalized for background, the ability of intracellular McB^{1-68} to induce β-galactosidase synthesis is decreased 3-fold (Fig. 5). However, its ability to inhibit cell growth from the outside is compromised more (at least 50-fold) (Table 1). This suggests that the primary reason for the decreased biological activity of McB^{1-68} is its poor uptake. To test this conjecture, we determined the ability of *E. coli* ZK4 *sbmA* cells, which are resistant to McB, to synthesize DNA in the presence of wild-type or mutant McBs after freeze-thaw permeabilization. All McBs were tested at high 100 μM concentration. The results are presented in Fig. 6. As can be seen, at the concentration used, wild-type McB and McB^{1-68} were equally efficient in inhibiting DNA synthesis in permeabilized cells. Other mutants were inactive.

The terminal residue of McB is important for cell entry. The strongly decreased activity levels of McB^{1-68} point to the importance of C-terminal Ile^{69} for McB function (recall that McB^{1-68}

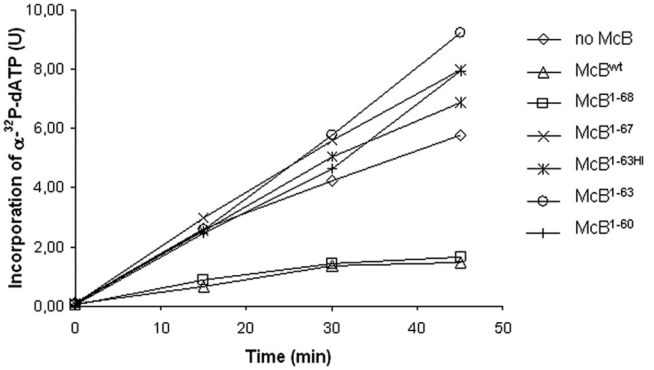

FIG 6 Inhibition of DNA synthesis in permeabilized cells by mutant microcins. McB-resistant *sbmA E. coli* cells were permeabilized and incubated in the presence of α-[^{32}P]dATP for various amounts of times in the absence or in the presence of 100 μM McB or its mutants. Incorporation of ^{32}P in acid-insoluble material (in arbitrary units) is shown. A representative result obtained in three independent experiments is shown.

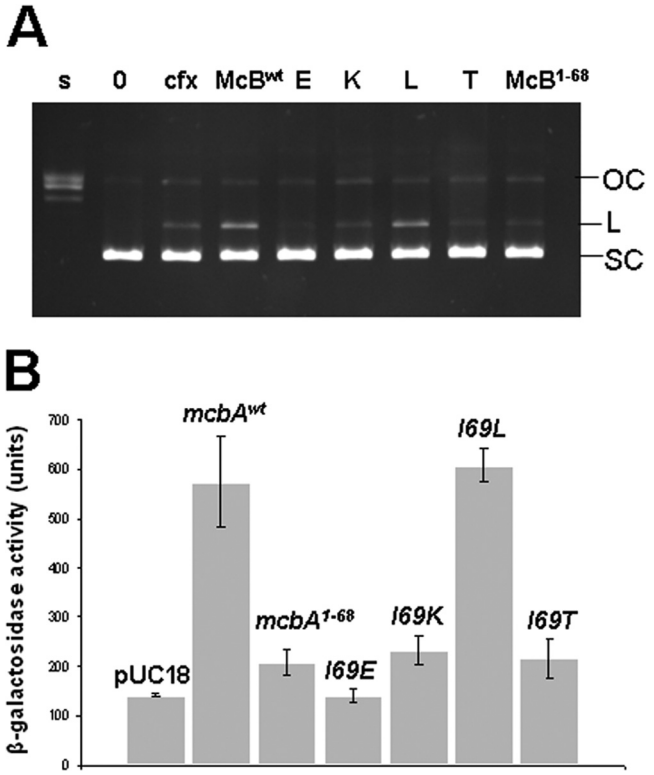

FIG 7 Analysis of McB mutants with substitution in the terminal amino acid. *In vitro* (A) and *in vivo* (B) activity of indicated McB mutants is shown (see, correspondingly, Fig. 4 and Fig. 5 legends for details). (A) Lanes E, K, L, and T, reaction mixtures were supplied with 60 μM concentrations of the I69E, I69K, I69L, and I69T mutants. Lanes McB^{wt} and McB$^{1-68}$, reaction mixtures were supplied with 60 μM concentrations of the corresponding McBs; lane cfx, 0.2 μM ciprofloxacin was added. (B) *I69K*, *I69E*, *I69L*, and *I69T*, *mcbA* alleles carrying designated mutations in the last codon.

contains the full complement of heterocycles). To determine whether the nature of amino acid at position 69 is also important, plasmids expressing *mcbA* variants coding for McbA peptides with a conservative I69L substitution, an I69T substitution introducing a polar residue, and I69E and I69K substitutions introducing opposite charges were constructed. All four mutant variants of McB were produced at a high yield and contained the same number of heterocycles as wild-type McB (data not shown). When the *in vivo* activity levels of mutant McBs were tested, I69L was indistinguishable from the wild type, while other mutants were defective (10-, 100-, and 500-fold drops in MIC values for I69T, I69K, and I69E, respectively; Table 2). It is noteworthy that I69K and I69E were even less active than McB$^{1-68}$. We next tested the mutants on lawns of sensitive cells overproducing SbmA, an inner membrane transporter responsible for McB uptake (24, 25). SbmA-overproducing cells were 10 times more sensitive to wild-type McB, indicating that the sensitivity of wild-type *E. coli* is limited by McB transport. The effect was specific to McB, since the sensitivity to ciprofloxacin, microcin C, an aminoacyl-tRNA synthetase inhibitor, or ampicillin was not affected by SbmA overproduction (Table 2). The sensitivity of SbmA-overproducing cells to I69L and I69T was also increased by a factor of 10 compared to control cells. In contrast, SbmA-overproducing cells were 50 times more sensitive to McB$^{1-68}$, I69K, and I69E than control cells with wild-type levels of SbmA. The result thus suggests that I69L and I69T are transported by SbmA normally whereas the transport of McB$^{1-68}$, I69K, and I69E is affected. However, defective transport by SbmA clearly cannot be the main reason of strongly decreased *in vivo* activity levels of these mutants. Indeed, *in vitro* activity measurements showed that, in agreement with the *in vivo* data, I69L was as active as the wild type, I69T, I69K, and McB$^{1-68}$ showed partial activity, while I69E was strongly defective (Fig. 7A). A similar conclusion was reached when plasmids overproducing these mutants were introduced in *E. coli* CSH50 tester cells. As can be seen from Fig. 7B, cells overproducing I69L underwent a strong SOS response, cells overproducing I69E showed background levels of β-galactosidase activity indicative of no SOS response, and cells overproducing I69T, I69K, and McB$^{1-68}$ exhibited a partial phenotype (elevated levels of β-galactosidase activity which were clearly below those seen in cells overproducing wild-type or I69L McB). We therefore conclude that the nature of side chain of amino acid at position 69 strongly affects the ability of McB to inhibit the DNA gyrase.

TABLE 2 Biological activity of McB mutants with substitutions at position 69[a]

Microcin	Minimal concn (μM) producing growth inhibition zone	
	BL21(DE3)	BL21(DE3) overexpressed SbmA
McB^{wt}	0.06	0.006
McB$^{1-68}$	3	0.06
McB I69L	0.06	0.006
McB I69T	0.6	0.06
McB I69K	6	0.12
McB I69E	30	0.6
Cfx	0.3	0.3
McC	10	10
Amp	200	200

[a] The indicated McB mutants and wild-type McB were purified (most material was in the type XΔ0 form) and used to determine the ability to inhibit the growth of *E. coli* BL21(DE3) cells with or without an *sbmA* expression plasmid under conditions of induction of the plasmid-borne gene as described in Materials and Methods. Ciprofloxacin (Cfx), microcin C (McC), and ampicillin (Amp) were used as controls.

DISCUSSION

The posttranslationally introduced heterocycles are generally believed to be responsible for the biological function of the TOMM family of peptides (1). In the case of McB, fused heterocycles in particular were for a long time considered to be the major determinants of activity. First, such heterocycles are found in some DNA binding drugs, for example, in anticancer agent bleomycin, and are important for their activity (26). Therefore, fused heterocycles could contribute to the binding to DNA gyrase-DNA complex, the likely target of McB. Second, the importance of fused heterocycles for McB activity is supported by the observation that the Δ+1 form of McB is more active than the standard Δ0 form (20). Third, mutations that prevent formation of site B fused heterocycle render McB inactive, while mutations interfering with site A fused heterocycle formation abolish McB production (20). Finally, a fragment of McB including residues from Gly^{46} to Ile^{69} and thus containing the site B fused heterocycle inhibited DNA gyrase *in vitro*, while a shorter fragment lacking the fused cycle was inactive (23). On the other hand, in the latter paper, data were presented on McB mutants lacking the ultimate and penultimate amino acids that showed that the ability to affect the gyrase is strongly affected by the deletions.

The heterocycles are introduced into McbA GS or GC dipeptides by the McB synthase in a directional manner, from the N terminus to the C terminus (13). One GS dipeptide, at positions 66 and 67, is never modified. Analysis of C-terminally truncated McB mutants provides an explanation to this observation. The McB synthase needs at least three amino acid residues past a modifiable residue to initiate the cyclization reaction. One can imagine that this minimal length requirement reflects the existence of an McB synthase site where a fragment of polypeptide chain downstream of modified residue must be bound.

The main impetus for this work was to obtain minimal active McB derivatives that could be used for further systematic structure-activity analyses. Our expectation was that removal of unmodified C-terminal residues of McB would have no effect on its function, as it should not affect the introduction of heterocycles which is initiated at the N-terminal end of McB. While deletions at the C terminus indeed did not have an effect on heterocycle formation, we found, contrary to our expectations, that unmodified C-terminal amino acids play an unexpectedly important role for *in vivo* and *in vitro* activity of McB. Removing the terminal isoleucine residue at position 69 does not affect the number of heterocycles but decreases antibiotic activity 50-fold. A similar drop of activity was noticed by Collin et al. (23), though in that work the composition of mutant McB with respect to heterocycle content was not determined. Both the transport of mutant antibiotic through an inner-membrane transporter, SbmA, and the ability to inhibit the intracellular target, the DNA gyrase, are affected. The nature of amino acid at position 69 is also critical for the *in vivo* and *in vitro* activity of McB. Introduction of a negatively charged residue at this position has no effect on heterocycle content but abolishes the ability to inhibit the gyrase. From these results, it therefore follows that unmodified residues may be at least as important as posttranslationally introduced heterocycles for McB function. Such residues may not be located just in the C-terminal portion of McB as our recent analysis of an McB analog produced by *Pseudomonas syringae* shows (27). There, we were able to show that a stretch of three unmodified amino acids at a location corresponding to *E. coli* McB site C is responsible for the ability of *P. syringae* McB to penetrate *P. aeruginosa* in an SbmA-independent manner. While no McB mutants with increased activity were obtained in the present work, one can imagine that introduction of nonnatural unmodified amino acids in the McB scaffold may afford new compounds with increased potency and/or an extended spectrum of activity by altering both intracellular transport and target interactions. The two-plasmid *mcb* expression system described here allows systematic searching for such McB derivatives through functional screening of extensive *mcbA* libraries. This work is currently ongoing in our laboratory.

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