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Comments

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Aspartyl-tRNA Synthetase Is the Target of Peptide Nucleotide Antibiotic Microcin C*

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Microcin C is a ribosome-synthesized heptapeptide that contains a modified adenosine monophosphate covalently attached to the C-terminal aspartate. Microcin C is a potent inhibitor of bacterial cell growth. Based on the *in vivo* kinetics of inhibition of macromolecular synthesis, Microcin C targets translation, through a mechanism that remained undefined. Here, we show that Microcin C is a subject of specific degradation inside the sensitive cell. The product of degradation, a modified aspartyl-adenylate containing an *N*-acylphosphoramidate linkage, strongly inhibits translation by blocking the function of aspartyl-tRNA synthetase.

Microcins are a class of small (<10 kDa) ribosomally synthesized peptide antibiotics produced by *Enterobacteriaceae* (1). Whereas some microcins are active as unmodified peptides (2), others are produced as polypeptide precursors that are heavily modified by dedicated maturation enzymes (3). Interest is attached to such post-translationally modified microcins due to their highly unusual structures and the fact that they target important cellular processes that are attractive targets for antibacterial drug development.

Genes responsible for microcin production are usually plasmid-borne. Plasmids encoding microcin structural and maturation genes also encode determinants of immunity specific to the microcin produced. Based on cross-immunity, post-translationally modified microcins can be subdivided into the B, C, and J types. Microcin B (MccB)⁴ is a 43-residue peptide with 8 thiazole and oxazole rings that are synthesized by the MccBCD maturation enzyme complex from multiple serine and cysteine residues present in the MccB precursor (4). MccB is a potent inhibitor of DNA gyrase; it traps the enzyme at the stage of DNA strand passage (5). Microcin J, a 21-amino acid peptide, contains an unusual lactam bond between its N-terminal glycine and the δ -carboxyl group of an internal glutamate; it assumes

a highly unusual threaded-lasso structure (6–8). MccJ inhibits bacterial RNA polymerase by occluding a narrow channel that is used to traffic transcription substrates, NTPs, to the catalytic center of the enzyme (9, 10).

The structure of the subject of this study, Microcin C (McC) is shown in Fig. 1A. McC is a heptapeptide containing a modified adenosine monophosphate covalently attached to its C terminus through an *N*-acylphosphoramidate linkage (11, 12). The phosphoramidate group of the nucleotide part of McC is additionally modified by a propylamine group. Additionally, in mature McC, the peptide moiety, which is encoded by the *mccA* gene, is modified and the C-terminal asparagine residue specified by *mccA* is converted to an aspartate (18, 19), through an unknown mechanism. *In vivo*, McC appears to target translation (12). Guijarro *et al.* (12) also reported that large concentrations of McC, as well as of synthetic peptide of the same sequence but without the nucleotide modification, mildly inhibit translation *in vitro*. They therefore concluded that the peptide part of McC is responsible for translation inhibition, whereas the nucleotide part is involved in McC transport into the cell. Here, we define the molecular mechanism of McC action. Contrary to the suggestion of Guijarro *et al.* (12), we find that the peptide moiety of McC allows cell entry, whereas the nucleotide part is critical for translation inhibition. We show that inside the cell, McC is specifically degraded and that the product of degradation, a modified aspartyl-adenylate, strongly inhibits translation by preventing the synthesis of aminoacylated tRNA^{Asp} by aspartyl-tRNA synthetase.

EXPERIMENTAL PROCEDURES

Preparation of McC—The *Escherichia coli* strain TG1 harboring McC-producing plasmid pBM43 was grown for 18 h at 37 °C in M63 minimum medium (13) containing 0.2% glucose and 1 mg/liter of thiamine. Cells were removed by centrifugation and the cultured medium was loaded onto Sep-Pak C8 cartridge (Waters). The cartridge was washed with water followed by a 0.1% aqueous trifluoroacetic acid wash, and bound material was eluted stepwise with 5, 10, and 20% acetonitrile in 0.1% trifluoroacetic acid. The 10% acetonitrile fraction was concentrated by lyophilization, dissolved in water, and subjected to reverse phase-HPLC (1 ml/min) on a ReproSil-Pur 300 ODS-3 column (5 μ m, 250 \times 4 mm) using a 0–20% linear gradient of acetonitrile in 0.1% trifluoroacetic acid. The total gradient volume was 50 ml. Pure McC eluted as a single peak; it was lyophilized, dissolved in water, and stored at –20 °C. The yield of chromatographically and mass spectrometrically pure McC ranged from 5 to 10 mg/liter of cultured medium.

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⁴ The abbreviations used are: MccB, microcin B; MccJ, microcin J; McC, microcin C; HPLC, high performance liquid chromatography; DTT, dithiothreitol; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

Microcin C Targets Aspartyl-tRNA Synthetase

Preparation of Cell Extract for McC Processing—McC-sensitive *E. coli* B cells were grown at 37 °C to A_{600} 0.5 in 1 liter of M63 medium supplemented with 0.2% glucose and 0.1% yeast extract. Cells were collected by centrifugation, washed with 20 mM Tris-HCl, pH 7.5, resuspended in 3 ml of the same buffer, and disrupted by sonication on the Branson Ultrasonics sonifier with a microtip (5 × 10 s pulses, at maximal power setting). The lysate was centrifuged at 30,000 × *g* for 30 min. The supernatant (total protein concentration of 20 mg/ml) was divided into aliquots and stored at -70 °C until further use.

McC Processing—250 μg of McC was dissolved in 150 μl of 25 mM Tris-HCl, pH 8.0, 2 mM MgCl₂ and combined with 50 μl of cell extract prepared as described above. The reaction was incubated for 1 h at 37 °C with continuous shaking. The reaction was terminated by the addition of an equal volume of 100% acetonitrile, 0.1% trifluoroacetic acid. After a 30-min incubation at 4 °C, proteins were removed by centrifugation and the pellet was extracted three times with 200 μl of water. The extracts were pooled, lyophilized, redissolved in water, and fractionated on a ReproSil-Pur 300 ODS-3 column (5 μm, 250 × 4 mm). The column was first developed (0.5 ml/min) isocratically with 10 mM (NH₄)₂HPO₄, pH 7.6, followed by a linear (0–60%) gradient of acetonitrile in the same buffer. UV-absorbing fractions were collected, lyophilized, dissolved in water, and tested in an *in vitro* translation system and by mass spectrometry. Active, processed McC eluted at 11.4 min during the isocratic part of chromatography. Intact McC eluted at 27.2 min by 50% acetonitrile. Processed McC prepared in this way was chromatographically and mass spectrometrically pure. The material was lyophilized and dissolved in water. The concentration was determined using UV absorption at 260 nm (calculated extinction coefficient of processed McC 16,500 μg/mol).

Coupled *In Vitro* Transcription/Translation—Transcription and translation (coupled system) in *E. coli* S30 extract (Ref. 14, final total protein concentration, 2.5 mg/ml) was carried out at 30 °C in the presence of 100 mM Hepes KOH, pH 8.0, 2% PEG8000, 0.1 mM EDTA, 2 mM DTT, 0.5 g/liter of bulk *E. coli* tRNA, 0.1 g/liter of folinic acid, 0.1 mM luciferin, 20 mM acetyl phosphate, 20 mM phosphoenolpyruvate, 1 mM GTP, 1 mM UTP, 1 mM CTP, 1.4 mM ATP, 11.5 mM Mg(OAc)₂, 237 mM KOAc, 0.5 mM of each amino acid, 15 μM pT7luc plasmid (15), 0.04 g/liter of pyruvate kinase, and 2 units/μl of T7 RNA polymerase. The reaction mixture was placed into the cell of a Hidex Triathler luminometer and the appearance of luciferase activity in the course of translation was monitored.

***In Vitro* Translation**—Luciferase mRNA was prepared by *in vitro* transcription of the pT7luc plasmid with T7 RNA polymerase (22) and purified by phenol-chloroform treatment and precipitation with 3 M LiCl. Translation in *E. coli* S30 extract (Ref. 14, final total protein concentration, 3 mg/ml) was carried out at 30 °C in the presence of 26.1 mM Hepes KOH, pH 7.6, 4% PEG8000, 1.7 mM DTT, 0.175 g/liter of bulk *E. coli* tRNA, 0.03 g/liter of folinic acid, 0.1 mM luciferin, 80 mM creatine phosphate, 0.8 mM GTP, 1.2 mM ATP, 10 mM Mg(OAc)₂, 125 mM KOAc, 0.34 mM each amino acid, 74 nM luciferase mRNA, 0.25 g/liter of creatine kinase. The reaction progression was monitored as above.

Translation in wheat germ S30 extract (Ref. 16, final total protein concentration 3 mg/ml) was carried out at 30 °C in the presence of 26.1 mM Hepes KOH, pH 7.6, 2% glycerol, 1.6 mM DTT, 0.05 g/liter of bulk yeast tRNA, 0.1 mM luciferin, 16 mM creatine phosphate, 0.4 mM GTP, UTP, CTP, 1 mM ATP, 3 mM Mg(OAc)₂, 70 mM KOAc, 0.2 mM each amino acid, 0.45 mM spermidine, 74 nM luciferase mRNA, and 0.1 g/liter of creatine kinase. The reaction progression was monitored as above.

***In Vitro* Translation of Poly(U) RNA**—Poly(U) RNA translation in 3 mg/ml *E. coli* S30 extract (14) was carried out at 30 °C in the presence of

26.1 mM Hepes KOH, pH 7.6, 4% PEG8000, 1.7 mM DTT, 0.175 g/liter of bulk *E. coli* tRNA, 0.03 g/liter of folinic acid, 0.1 mM luciferin, 80 mM creatine phosphate, 0.8 mM GTP, 1.2 mM ATP, 10 mM Mg(OAc)₂, 125 mM KOAc, ~74 nM poly(U) RNA (molecular mass 800–1000 kDa, Sigma), 0.25 g/liter of creatine kinase. The reaction mixture contained [¹⁴C]Phe. The compound, which limits the reaction, was the poly(U) RNA.

Preparation of S100 Cell Extract from *E. coli*—*E. coli* MRE600 cells were grown to A_{600} 0.8 in LB medium. Cells were collected by centrifugation, and washed with 40 mM Tris-HCl, pH 7.6, 10 mM Mg(OAc)₂, 50 mM KOAc, 0.1 mM EDTA, 1 mM DTT. The cell pellet was resuspended in an equal volume of the same buffer and disrupted by a French press (pressure 1000 bar). The lysate was next centrifuged at 30,000 × *g* for 30 min. The supernatant was centrifuged at 100,000 × *g* for 2.5 h. The supernatant was loaded on the column with DE-52 cellulose, equilibrated by 40 mM Tris-HCl, pH 7.6, 10 mM Mg(OAc)₂, 50 mM KOAc, 0.1 mM EDTA, 1 mM DTT. The column was washed by the same buffer. The enzyme fraction was eluted by 40 mM Tris-HCl, pH 7.6, 10 mM Mg(OAc)₂, 250 mM KOAc, 0.1 mM EDTA, 1 mM DTT (total protein concentration was ~10 mg/ml).

***Francisella tularensis* AspRS**—The AspRS gene (*aspS*) was amplified by PCR, with an encoded C-terminal His₆ epitope, using genomic *F. tularensis* strain LVS DNA as template, the primers used were 5'-CATATGAGAACACATTATAGTTCA-3' and 5'-GGATCCTAATGGTGATGGTGATGGTGCGCGCCCCCTTCTTCTTTCTTAACA-3' and *Pfu* DNA polymerase (Stratagene). The PCR product was cloned into PCR-Blunt II-TOPO vector (Invitrogen), the sequence of the construct was confirmed, and the corresponding NdeI-BamHI-digested fragment ligated into NdeI-BamHI-digested pET11a.

Production of His₆-tagged AspRS was done by transforming BL21(DE3) with pET11a-FTAspRS and growing the resulting strain using the Overnight Express™ Autoinduction System 1 (Novagen) according to the manufacturer's instructions. Cell-free extract was prepared by re-suspending *E. coli* cells in lysis buffer (30 mM Tris, pH 8, 300 mM NaCl, 10 mM imidazole, 10% glycerol) and passage through a French pressure cell followed by centrifugation at 75,000 × *g*, at 4 °C for 45 min. The resulting supernatant was applied to a column containing BD TALON affinity resin (BD Bioscience), washed extensively with the lysis buffer, and AspRS-His₆ subsequently eluted using the lysis buffer supplemented with 300 mM imidazole. Fractions containing AspRS-His₆ (judged by Coomassie Brilliant Blue staining after SDS-PAGE to be >95% pure) were pooled, dialyzed extensively against storage buffer (50 mM Tris, pH 7.5, 5 mM 2-mercaptoethanol, 100 mM NaCl, 1 mM EDTA, 50% glycerol), and stored at -20 °C. The concentration of AspRS-His₆ was determined by active site titration as previously described (17), and the reactions were performed in duplicate for 5 and 10 min.

tRNA Aminoacylation Reaction—Aminoacylation reaction in *E. coli* S100 extract (final total protein concentration 0.1 mg/ml) was carried out at 30 °C in the presence of 30 mM Hepes KOH, pH 7.6, 1 mM DTT, 5 g/liter bulk of *E. coli* tRNA, 0.1 mM EDTA, 3 mM ATP, 8 mM Mg(OAc)₂, 30 mM KOAc, and 40 μM of the specified amino acid. The reaction products were either precipitated in cold 5% trichloroacetic acid and subjected to scintillation counting or purified by phenol-chloroform treatment/ethanol precipitation and added to *in vitro* transcription-translation reactions. At the conditions used, the aminoacylation reactions were limited by tRNA, because increase of this component only resulted in a proportional increase in trichloroacetic acid-insoluble radioactivity. Aminoacylation by purified *F. tularensis* AspRS was conducted at identical conditions with 40 nM active *F. tularensis* AspRS used instead of the S100 extract.

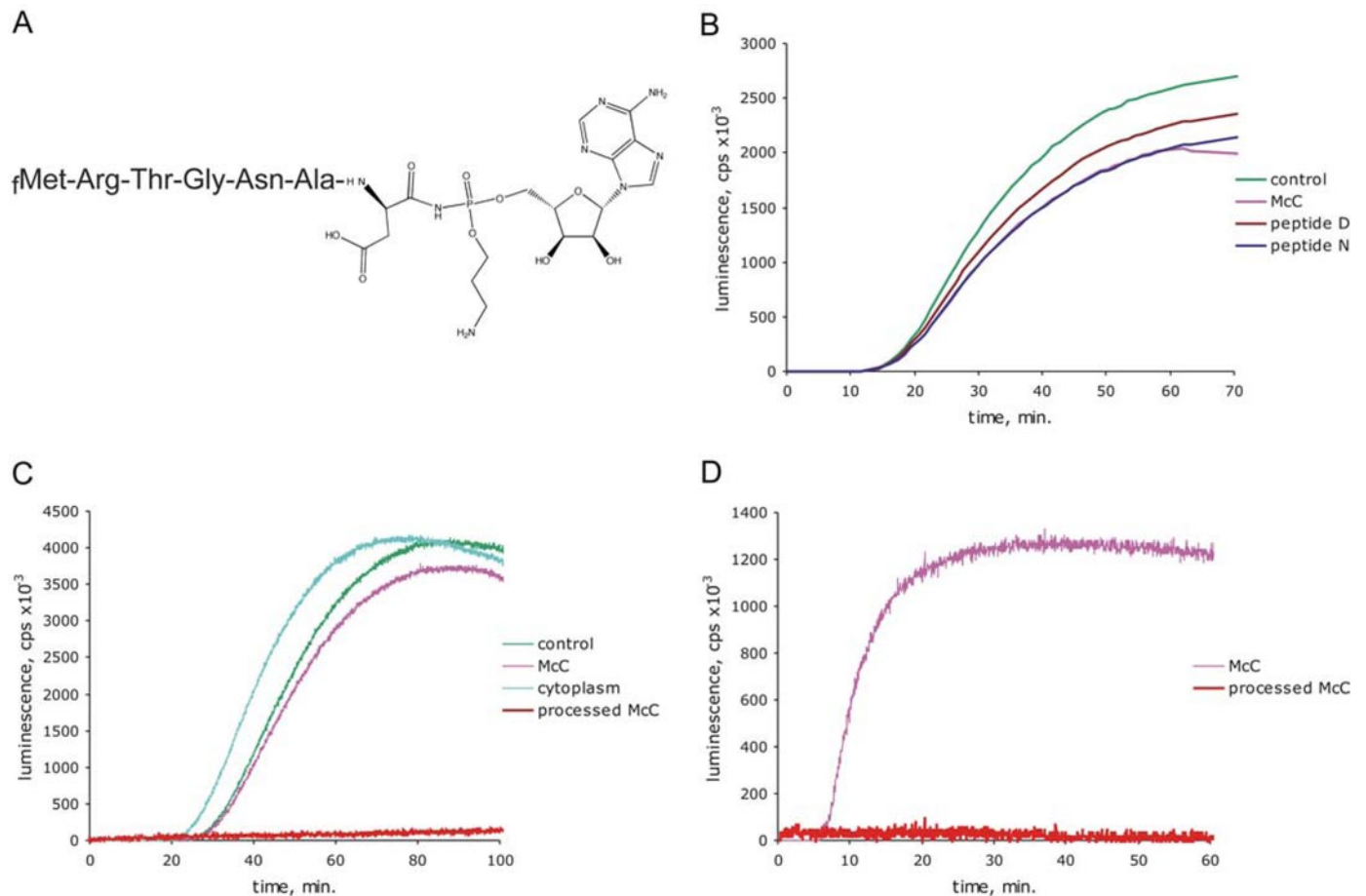
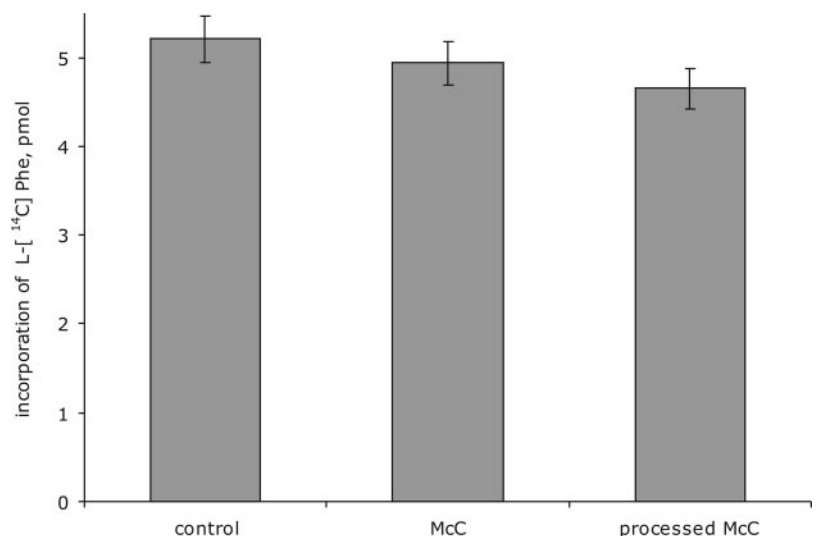


FIGURE 1. **Processed McC inhibits translation *in vitro*.** *A*, the structure of McC. *B*, *in vitro* transcription-translation of the luciferase gene in the presence of McC. The luciferase gene was used as a template for *in vitro* transcription-translation in *E. coli* S30 extract as described under "Experimental Procedures" in the presence of 10 μ M intact McC or MRTGNAD (peptide D) or MRTGNAN (peptide N) peptides. Water was added to the control reaction. A representative result from a series of three independent experiments is shown. *C*, *in vitro* transcription-translation of the luciferase gene in the presence of processed McC. The results of the *in vitro* transcription-translation of the luciferase gene in the presence of 10 μ M intact McC or the corresponding amount of McC that has been preincubated with *E. coli* extract (processed McC) are presented. Water (control) or *E. coli* extract preincubated without McC (cytoplasm) was added to control reactions. *D*, *in vitro* translation of luciferase RNA in the presence of processed McC. The luciferase RNA was translated *in vitro* using *E. coli* S30 extract as described under "Experimental Procedures" in the presence of 10 μ M intact McC or the corresponding amount of processed McC.

FIGURE 2. **Effect of McC on poly-Phe synthesis in a poly(U)-directed *in vitro* translation system.** *In vitro* translation of the poly(U) template was performed in the presence or absence of 10 μ M intact McC or the corresponding amount of processed McC.



The aminoacylation reaction in 1% wheat germ S30 extract was carried out at 30 °C in the presence of 30 mM HEPES-KOH, pH 7.6, 1 mM DTT, 5 g/liter of bulk yeast tRNA, 0.1 mM EDTA, 3 mM ATP, 8 mM Mg(OAc)₂, 30 mM KOAc, and 40 μ M of amino acid of choice. The

reaction products were precipitated in cold 5% trichloroacetic acid and subjected to scintillation counting.

ATP-PP_i Exchange Reaction—The reaction was carried out at 37 °C in 100 mM Na-HEPES, pH 7.2, 30 mM KCl, 10 mM MgCl₂, 2 mM NaF,

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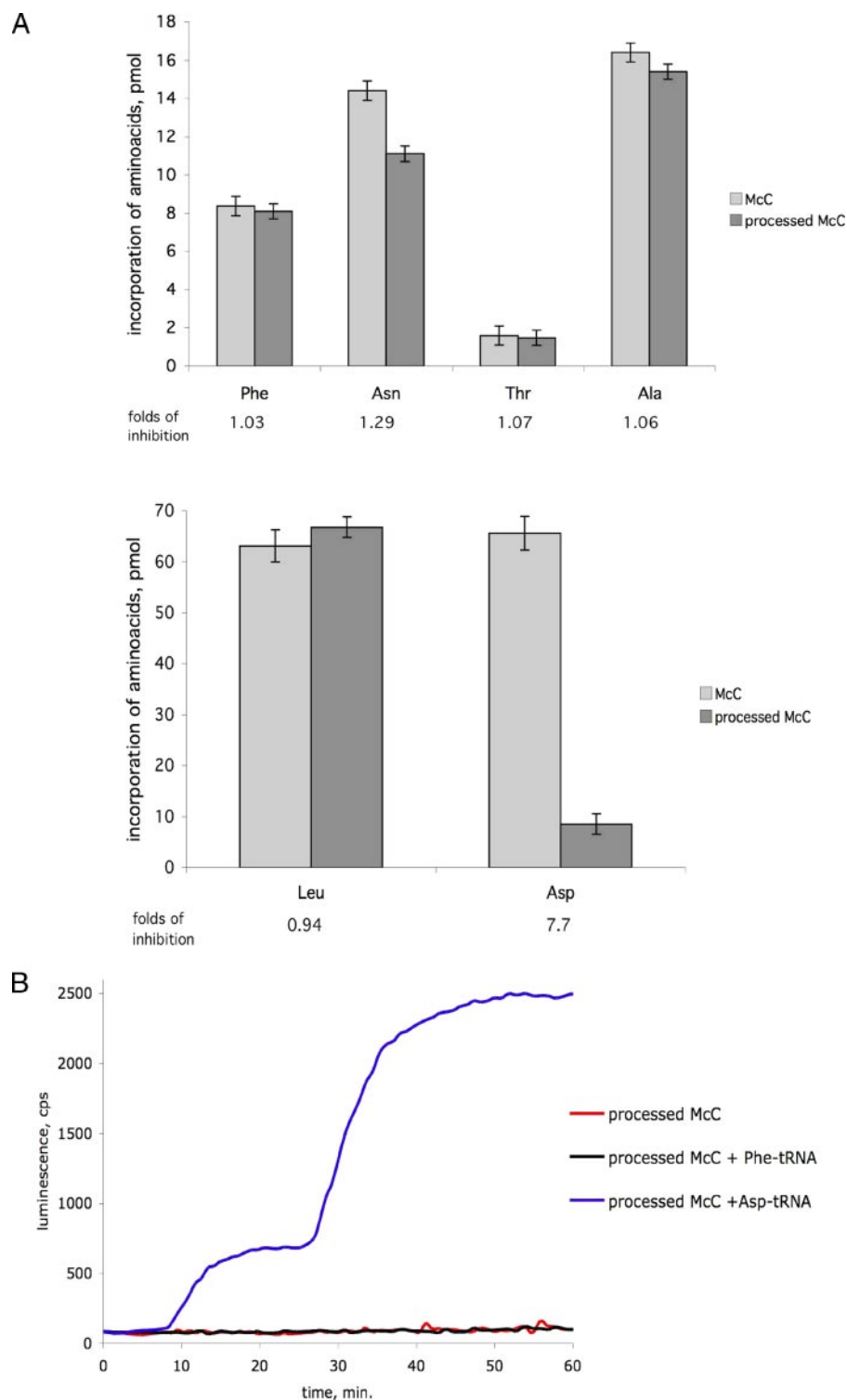


FIGURE 3. Processed McC inhibits aspartyl-tRNA synthetase. *A*, the indicated amino acids were used in *in vitro* tRNA aminoacylation reactions in the presence of 10 μM intact McC or the corresponding amount of processed McC. For each amino acid tested, aminoacylation observed in the presence of intact McC was identical to aminoacylation when water was added to the reaction instead of McC (data not shown). The bars show the mean values obtained in three independent experiments. *B*, the results of the luciferase gene transcription-translation in the presence of processed McC with the addition of aminoacylated tRNA^{Asp} or tRNA^{Phe} at 0 and 26-min time points are presented. *C*, the results of tRNA^{Asp} aminoacylation by 40 nM *F. tularensis* AspRS in the presence of increasing concentrations of processed McC are shown. *D*, [³²P]PP_i-ATP exchange by 30 nM *F. tularensis* AspRS in the presence of processed and intact McC. Circles, no McC; triangles, 200 nM intact McC; squares, 100 nM processed McC; diamonds, 300 nM processed McC.

2 mM ATP, 2 mM [³²P]PP_i (~1 cpm/pmol), 2 mM Asp, and 30 nM *F. tularensis* AspRS. After 1–10 min, 25 μl of the reaction were removed and added to 975 μl of solution containing 1% charcoal (NoritA), 5.6% perchloric acid, and 75 mM PP_i. The radiolabeled ATP bound to the charcoal was filtered through a 3MM Whatman filter disc under vacuum, washed 3 times with 5 ml of water and once with 5 ml of ethanol. The filters were dried and the radioactivity was counted by liquid scintillation counting (Ultima Gold, Packard Corp.).

RESULTS

McC Has No Effect on *in Vitro* Translation—It has previously been reported that the addition of 10 μM intact McC led to ~50% inhibition of a coupled *E. coli* *in vitro* transcription-translation system over 30 min (12). However, we failed to observe any effect of the addition of 10 μM highly pure, biologically active McC to the luciferase gene *in vitro* transcription-translation system after as much as a 60-min incubation (Fig. 1*B*). It has also been reported that the peptide MRTGNAD, corresponding to the peptide

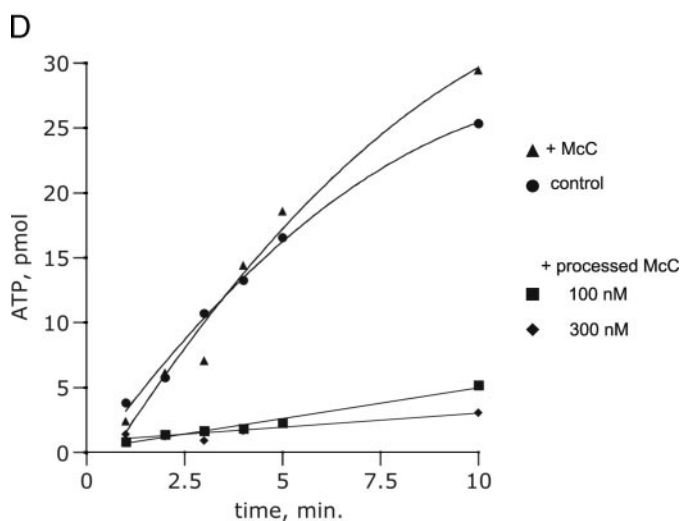
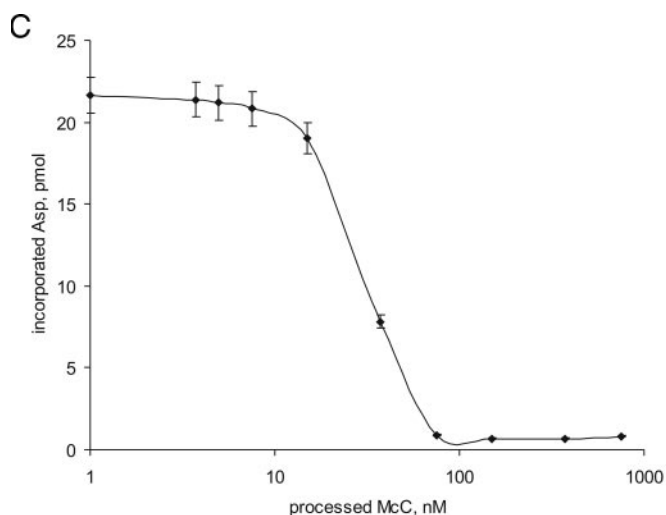


FIGURE 3—continued

moiety of mature McC inhibits *in vitro* translation as efficiently as intact McC (12). We synthesized the MRTGNAD peptide as well as the MRTGNAN peptide encoded by *mccA*, the structural gene for McC (18, 19), and confirmed the previous result (12) that such peptides have no effect on the growth of McC-sensitive cells (data not shown). The addition of these McC peptides (10 μ M) had no significant effect on the coupled *in vitro* transcription-translation system (Fig. 1B).

Incubation with Crude Cell Extract Inactivates Antibiotic Activity of McC but Makes It a Potent Inhibitor of Translation *In Vitro*—We hypothesized that the inability of pure McC to inhibit transcription-translation *in vitro* is due to a lack of processing that is required to convert it into active inhibitor. To test this hypothesis, McC was incubated with cell extract prepared from McC-sensitive *E. coli* cells as described under “Experimental Procedures.” The incubation resulted in inactivation of McC as judged by a dramatic decrease in its ability to produce growth inhibition zones on lawns of sensitive *E. coli* cells (data not shown). However, McC that was incubated with the cell extract (“processed McC”) strongly inhibited the coupled transcription-translation system (Fig. 1C), as well as the luciferase mRNA translation system (Fig. 1D). The addition of cell extract alone had no effect.

Whereas the observation above is consistent with an idea that McC is converted into an active form upon incubation with cell extract, a question arises why this conversion does not happen in the *in vitro* transcrip-

tion-translation reaction that contains the S30 extract of McC-sensitive MRE600 *E. coli* cells. The answer to this question appears to be that efficient processing of intact McC requires a high concentration of cell extract (or long incubation times) that exceed the cell extract concentration or incubation times used for *in vitro* transcription-translation experiments. In agreement with this idea, incubation of intact McC with the *in vitro* transcription-translation system for 70 min, the maximal time of *in vitro* transcription-translation in our experiments did not result in inactivation of McC as judged by its ability to inhibit the growth of sensitive cells (data not shown, recall that processed McC has no effect on cell growth, above). Thus, we conclude that intact McC is either processed in the cell extract or coupled with a factor(s) that converts it into an active, inhibitory form.

Active Form of McC Inhibits Aspartyl-tRNA Synthetase—What is the mechanism of translation inhibition by processed McC? A clue to this question is provided by the fact that no inhibition of poly(U)-primed translation is observed with either intact or processed McC (Fig. 2). Thus, it appears that the function of translation elongation factors and the elongation properties of the ribosome are not affected by processed McC. Therefore, the likely targets could be the process of translation initiation or the function of tRNAs other than tRNA^{Phe}. We tested the latter possibility by determining whether processed McC can inhibit the tRNA aminoacylation reaction. The results are presented in Fig. 3A. As can be seen, intact McC had no effect on tRNA aminoacylation by any of the 6 amino acids tested. In contrast, processed McC specifically inhibited tRNA aminoacylation by aspartate and, to a much lesser extent, of asparagine, but had no effect on tRNA aminoacylation by other amino acids tested. The results presented above suggest that the inhibition of aspartyl-tRNA synthetase (AspRS) may be responsible for the observed inhibition of *in vitro* translation by processed McC.

To test this idea, we supplemented the *in vitro* luciferase gene transcription-translation reaction inhibited by the addition of processed McC with aminoacylated tRNA^{Asp}; tRNA^{Phe} was used as a control. The results are presented in Fig. 3B. As can be seen, the addition of aminoacylated tRNA^{Asp} but not tRNA^{Phe} stimulated luciferase production. Thus, processed McC inhibits translation *in vitro* because it specifically inhibits the production of aminoacylated tRNA^{Asp}.

The final level of luciferase activity achieved after the addition of aminoacylated tRNA^{Asp} was much lower and the time during which the increase in the activity occurred was much shorter than seen in the absence of processed McC, suggesting that the exogenously added aminoacylated tRNA^{Asp} was quickly used up and no regeneration of aminoacylated tRNA^{Asp} occurred in the presence of processed McC. Indeed, the second addition of aminoacylated tRNA^{Asp} led to a further increase in luminescence. The second addition of aminoacylated tRNA^{Asp} led to a rapid increase in luciferase production, whereas a ~10-min lag was observed between the first addition of aminoacylated tRNA^{Asp} and the appearance of luciferase activity (a similar lag is observed in the absence of McC, Fig. 1, B and C). This behavior is expected, because for luciferase activity to appear, transcription of the luciferase gene must occur, translation of luciferase mRNA must be initiated, and the synthesis of the entire luciferase polypeptide must be completed. These processes take time and thus contribute to the observed lag in the appearance of luciferase activity. When the first pool of exogenously added aminoacylated tRNA^{Asp} is exhausted, translation elongation is stopped and ribosomes become stalled at Asp codons at various points of the luciferase mRNA. The second addition of aminoacylated tRNA^{Asp} to these “primed” ribosomes releases the translation block and leads to rapid increase in luciferase production.

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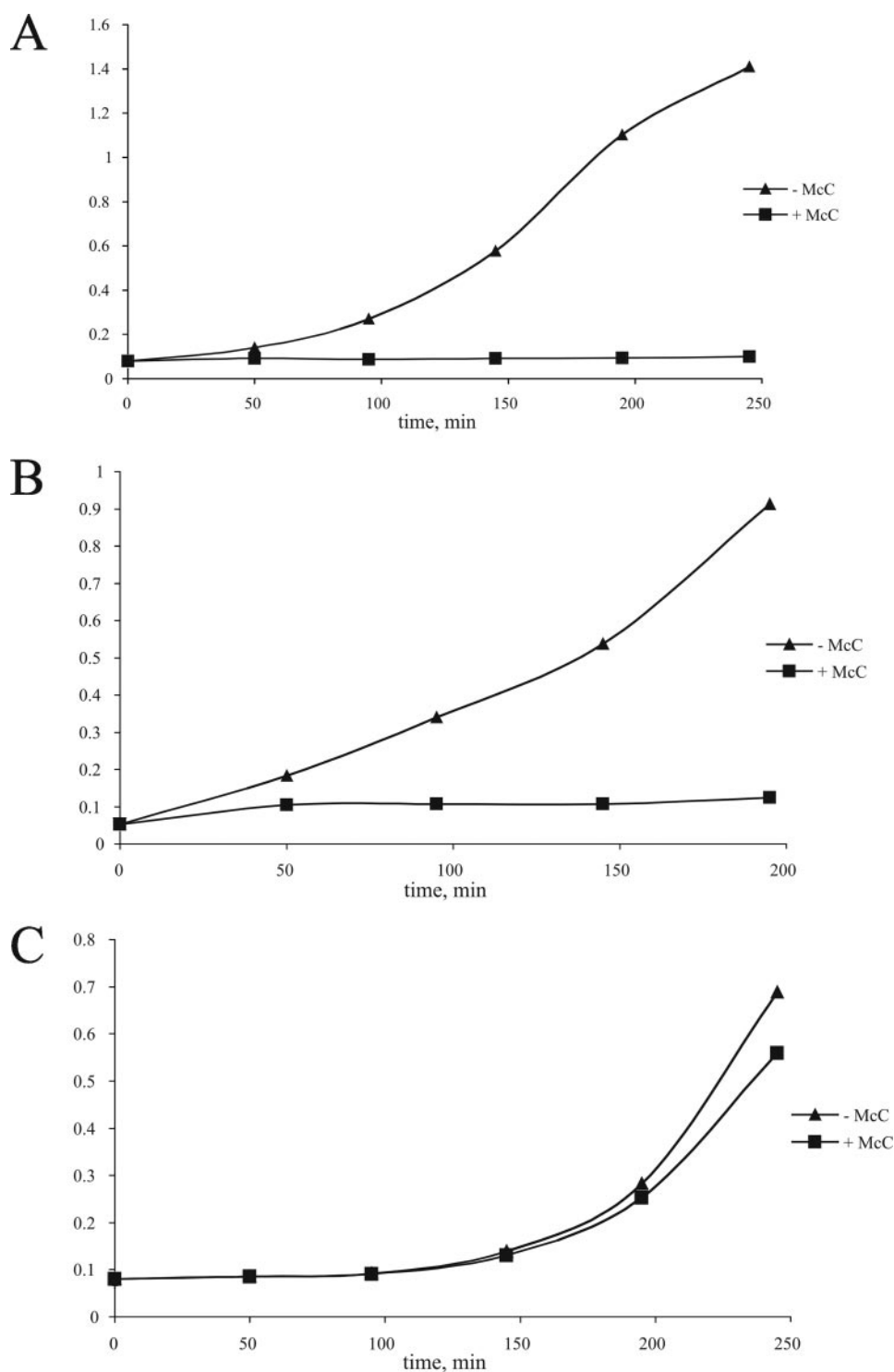


FIGURE 4. *E. coli* cells overproducing *D. radiodurans* AspRS become resistant to McC. The growth of *E. coli* cells harboring a pET vector plasmid (A), or plasmids expressing *D. radiodurans* ProRS (B), or AspRS (C) in the presence or absence of 1 $\mu\text{g/ml}$ (0.85 μM) McC is shown. The growth curves shown are representative of a series of independent experiments that were repeated three times.

To demonstrate that processed McC directly inhibits the function of AspRS in a pure system, we used a recombinant AspRS from *F. tularensis* that shares 58% amino acid identity to its *E. coli* homolog. We reasoned that because bacterial AspRSs are highly conserved, and intact McC is effective against a wide range of Gram-positive and Gram-negative bacteria (11), processed McC would be likely to inhibit the *F. tularensis* enzyme. This expectation was fulfilled. As can be seen from Fig. 3C, when processed McC was present in the reaction at concentrations below 20 nM, it had little effect on tRNA^{Asp} aminoacylation by *F. tularensis* AspRS. Higher concentrations of processed McC effec-

tively inhibited tRNA^{Asp} aminoacylation and no activity was detected above 100 nM processed McC. Because the concentration of active *F. tularensis* AspRS in the reaction was estimated at 40 nM, the result suggests that a stable stoichiometric complex between the inhibitor and the enzyme is readily formed.

To determine the effect of processed McC on aspartyl-adenylate synthesis, Asp-dependent [³²P]PP_i-ATP exchange reaction catalyzed by *F. tularensis* AspRS was monitored in the presence of processed or intact McC. The results, presented in Fig. 3D, clearly indicate that processed McC inhibits the pyrophosphate exchange, whereas intact McC

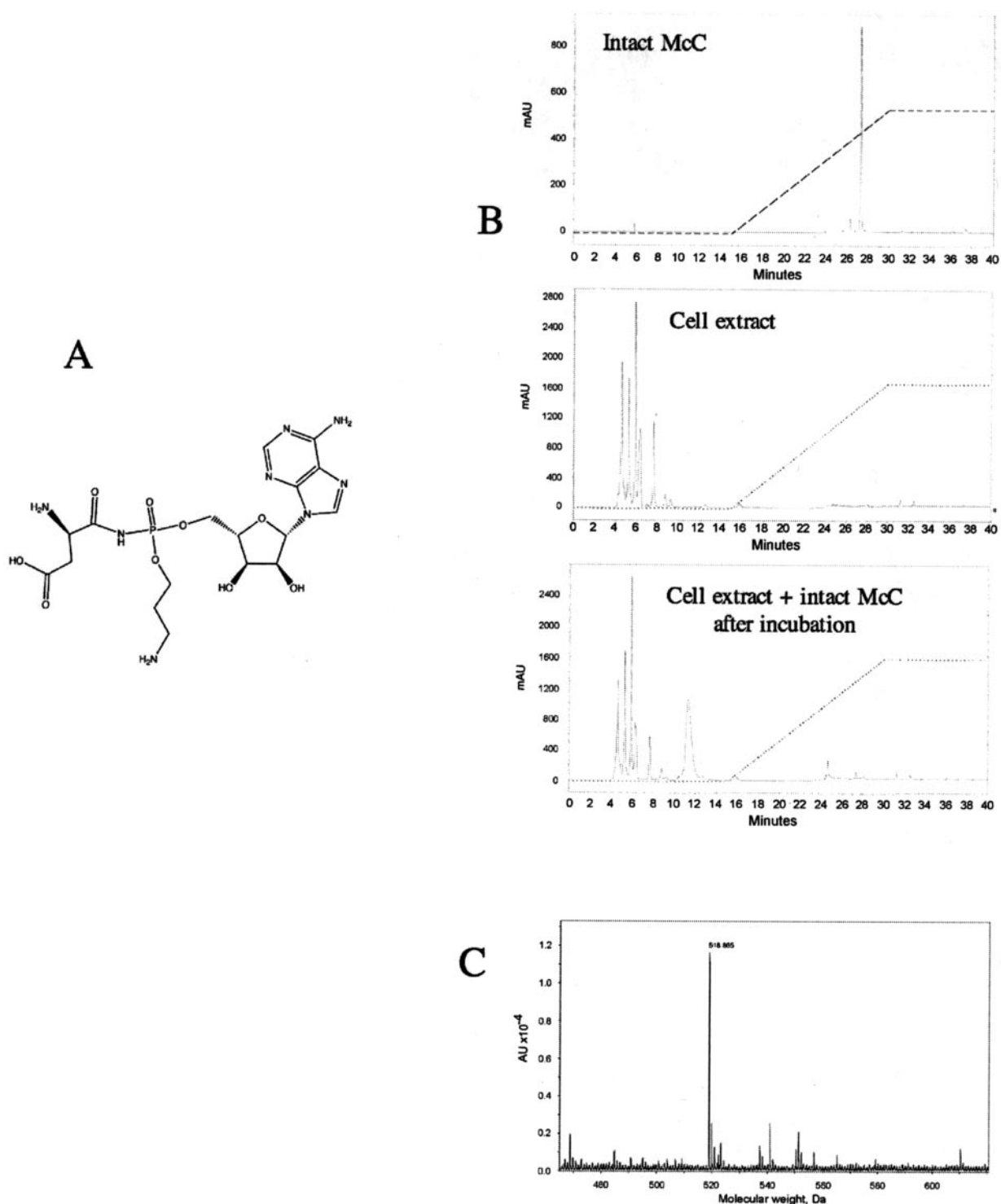


FIGURE 5. **Identification of processed McC.** *A*, the structure of the aspartyl adenylate analogue formed upon hydrolysis of the McC ultimate peptide bond. Molecular mass of the compound shown is 519 Da. *B*, HPLC separation of intact McC (*top panel*), cell extract (*middle panel*), and processed McC obtained by incubation of intact McC and cell extract (*bottom panel*). *C*, MALDI-TOF MS analysis of the indicated HPLC fraction that inhibited *in vitro* translation and aminoacylation of tRNA^{Asp} was performed on a Bruker Ultraflex mass spectrometer (reflector mode) using 2,5-dihydroxybenzoic acid as a matrix.

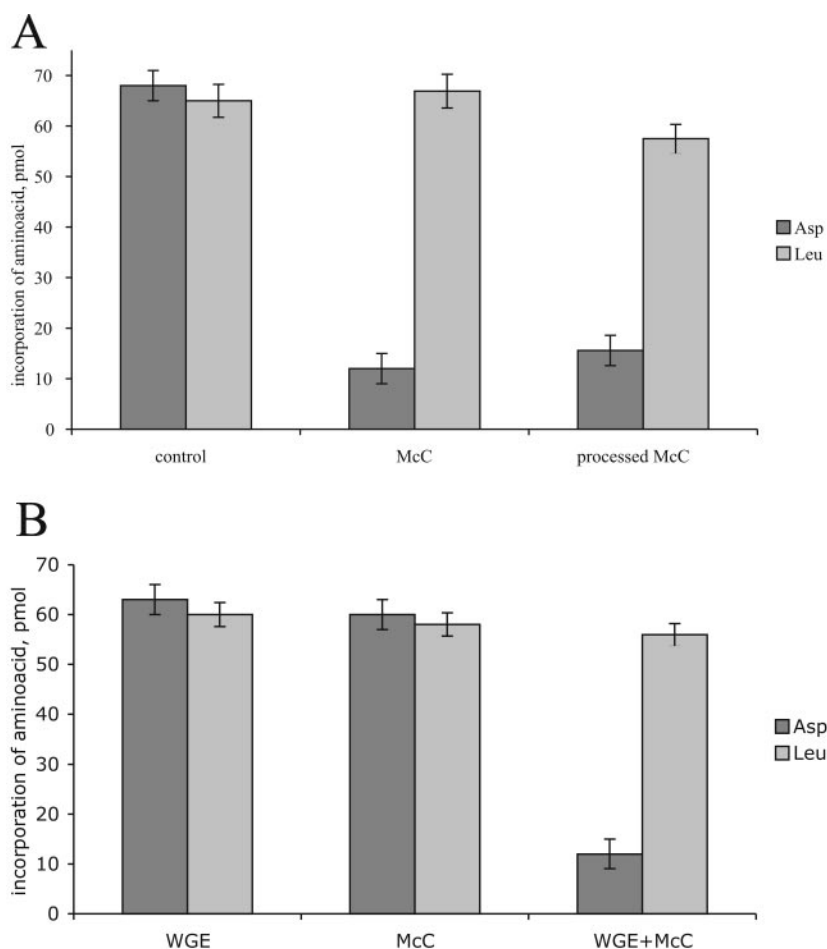
has no effect on the reaction. Thus, the inhibition of AspRS by processed McC occurs at the first step of the aminoacylation reaction, namely amino acid activation (Fig. 3D).

AspRS Is the in Vivo Target of McC—The results presented so far demonstrate that processed McC inhibits translation *in vitro* by targeting AspRS. We hypothesized that translation inhibition by McC *in vivo* occurs for the same reason and that therefore AspRS is the

physiological target of McC. If this were the case, cells overexpressing AspRS might become partially or completely resistant to McC. To test this prediction, *E. coli* BL21(DE3) cells were transformed with a pET vector-based plasmid overexpressing AspRS from *D. radiodurans* (20). As a control, we used cells overexpressing plasmid-borne *D. radiodurans* ProRS (21) or cells carrying the pET vector alone. The levels of McC resistance were determined by adding 1

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FIGURE 6. Effect of McC on tRNA aminoacylation in wheat germ extract. *A*, aminoacylation reactions in wheat germ extract were carried out with radioactive leucine and aspartate in the presence or absence of intact or processed McC as indicated. *B*, effect of McC preincubation with wheat germ extract (WGE) on tRNA aminoacylation in *E. coli* extract. WGE alone (bars at the left), intact McC (bars at the middle), or WGE and intact McC (bars at the right) were added to the *E. coli* aminoacylation reaction and incorporation of radioactive leucine or aspartate into trichloroacetic acid-insoluble fraction was followed.



$\mu\text{g/ml}$ ($0.85 \mu\text{M}$) of McC to cell cultures growing in liquid LB medium under conditions that induced expression of plasmid-borne genes. Cell growth was next monitored over time. Cultures that did not receive McC served as controls. The results are presented in Fig. 4. As can be seen, cells harboring the pET vector or overproducing PrRS ceased growth upon the addition of McC. In contrast, the McC had little effect on the growth of cells overproducing AspRS. The results are therefore consistent with the hypothesis that AspRS is the *in vivo* target of McC. It should be noted that the protection afforded by overexpression of *Deinococcus radiodurans* AspRS was partial, and cell growth was visibly inhibited in the presence of higher concentrations of McC (data not shown). The results suggest that *D. radiodurans* AspRS is also inhibited by McC, although the possibility that high concentrations of McC disrupt cell growth by affecting other targets cannot formally be excluded.

McC Processing Results in Hydrolysis of the Ultimate Peptide Bond—The C-terminal residue of intact McC is an aspartate covalently attached to modified AMP making it similar to the natural intermediate of the activation reaction catalyzed by AspRS, aspartyl adenylate. The mode of action of processed McC made us hypothesize that the processing event involves cleavage of the last McC peptide bond with the resultant generation of an aspartyl adenylate analogue (Fig. 5A) that poisons AspRS. To test this hypothesis, intact McC was incubated with cell extract and the reaction products were separated by reverse-phase HPLC as described under “Experimental Procedures.” Under the chromatographic conditions used, intact McC elutes at $\sim 50\%$ acetonitrile (Fig. 5B, top panel). Upon incubation with the cell extract, the peak of intact McC disappeared; instead, a peak that eluted during the isocratic

stage of the chromatography appeared (Fig. 5B, bottom panel). No such peak was present in the cell extract (Fig. 5B, middle panel). Spectrophotometric analysis indicated that the new peak contained a nucleotide, based on characteristic absorbance at 260 nm (data not shown). The material from this peak inhibited the cell-free transcription-translation system and the aminoacylation of tRNA^{Asp}, whereas other HPLC fractions did not (data not shown). When the active HPLC fraction was analyzed by MALDI-MS, a mass ion of 518.9 Da was detected (Fig. 5C). No such mass ion was detected in inactive fractions. In contrast, the minor peaks seen in the mass spectrogram presented in Fig. 5C were also seen in inactive fractions and must have come either from the chromatographic buffer or mass spectrometry matrix. The calculated mass of the hypothetical McC degradation product (Fig. 5A) is also 519. Thus, the results of MS analysis are consistent with an idea that processed McC is generated by hydrolysis of the ultimate peptide bond of McC, which results in generation of an aspartyl adenylate analogue that inhibits AspRS.

Processed McC Inhibits Aminoacylation by Eukaryotic AspRS—The inhibitory product of McC processing is very similar to the natural intermediate of the reaction catalyzed by AspRS and therefore likely inhibits the enzyme by binding in the active site. Aminoacyl-tRNA synthetases, including AspRS, are evolutionarily conserved enzymes and eukaryal proteins can often functionally complement bacterial enzymes *in vitro* and *in vivo* (see, for example, Moulinier *et al.*, Ref. 22). Therefore, one can expect that processed McC may also inhibit eukaryal AspRS. To test this prediction, we determined the effect of processed McC on *in vitro* translation in a wheat germ extract. In agreement with our hypothesis, processed McC inhibited translation

in this system (data not shown). Surprisingly, intact McC, which was used as a control, also inhibited translation in wheat germ extract (data not shown). Wheat AspRS appeared to be the target of inhibition because aminoacylation of tRNA^{Asp} but not tRNA^{Leu} was affected by both processed and intact McC (Fig. 6A). To explain this result, we postulate that intact McC is rapidly processed in wheat germ extract. Indeed, McC preincubated with wheat germ extract lost its bactericidal activity (data not shown) but inhibited aminoacylation of tRNA^{Asp} in the *E. coli* system (Fig. 6B), suggesting that McC processing activity is either evolutionarily conserved or, alternatively, is nonspecific.

DISCUSSION

Our results define the mechanism of translation inhibition by McC and open ways to designing specific inhibitors of aminoacyl-tRNA synthetases. The following sequence of events is consistent with our data. McC enters the sensitive cell through an unknown mechanism. During cell entry or once inside the cytoplasm, McC is processed by an unknown peptidase. The processing event produces a non-hydrolyzable analogue of aspartyl adenylate, with an *N*-acylphosphoramidate linkage and an amino propyl group at the phosphate. This compound specifically inhibits AspRS, which causes inhibition of translation and prevents further cell growth.

The *N*-acylphosphoramidate functionality is found in several natural products such as phosmidosine (23) and agrocin 84 (24). The synthesis of aminoacyl adenylate analogs having *N*-acylphosphoramidate linkage has also been reported (25).

The inhibition of AspRS by various analogues of aspartyl adenylate has been reported recently (26). AspRS inhibition by processed McC likely occurs at the level of substrate competition. Experiments with pure AspRS suggest that processed McC interacts with its target with high affinity and that the enzyme-inhibitor complex is very stable. Whereas further structural studies are now required to elucidate the exact mechanism of McC binding and inhibition, the highly specific inhibition of Asp-adenylate biosynthesis suggests that it binds the free enzyme thus effectively hindering subsequent Asp binding and activation.

The "Trojan horse" two-step mechanism of McC action (uptake of inactive compound by a sensitive cell, followed by conversion into an active, inhibitory compound within the cell) has been described for several unrelated systems whose active moiety is an amino acid coupled to a nucleotide. The antibiotic albomycin is taken inside the cell by the ferrichrome uptake system and, once inside the cell, is converted to an active form through the action of peptidase N, which cleaves the ultimate peptide bond of albomycin and releases the inhibitory aminoacyl-thioribosyl pyrimidine moiety (27). Another recently described inhibitor, agrocin 84, is imported inside the sensitive cell and, upon processing, generates a non-hydrolyzable analogue of leucyl adenylate, which inhibits LeuRS (24). Agrocin 84-producing cells are resistant to the drug due to an additional copy of the LeuRS gene carried on a plasmid that codes for agrocin 84 biosynthesis enzymes. The McC producing plasmid does not carry an AspRS gene. Previously, we showed that at least two genes, *mccC* and *mccE*, are responsible for immunity to McC. The product of *mccC* is related to multidrug-efflux transporters and must contribute to McC resistance by facilitating its efflux outside the producing cell. *MccE* is a bifunctional protein; one of its domains is related to diaminopimelate decarboxylases, whereas the other is very similar to ribosomal protein L7 serine acetyltransferase. It remains to be determined how the enzymatic activities of this protein contribute to McC resistance.

Because processed McC has no effect on cell growth, the N-terminal McC hexapeptide (or part thereof) is presumably essential for cell entry. Uptake systems that are known to participate in the transport of other microcins and colicins inside the cell (28) do not appear to be involved in McC transport because mutations affecting the TolC- and TolB-dependent pathways do not lead to McC resistance.⁵ An ongoing search for McC-resistant mutants should help to identify genes involved in McC transport.

Our results open two new avenues of research. From the inhibitory standpoint, development of processed McC analogues with amino acids other than Asp (either through chemical synthesis or genetic engineering of the *mccA* gene) may generate specific inhibitors of aminoacyl-tRNA synthetases other than AspRS. In addition, structure-activity studies of the McC peptide itself may permit the sequences required for cell entry to be defined. This could potentially allow the McC peptide to be used as a vehicle to transport other charged molecules into *E. coli* and possibly other bacteria.

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Aspartyl-tRNA Synthetase Is the Target of Peptide Nucleotide Antibiotic Microcin C

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