JOURNAL OF BACTERIOLOGY, Mar. 2007, p. 2114–2118 Vol. 189, No. 5
0021-9193/07/$08.00+0 doi:10.1128/JB.01609-06

Amino Acid Residues Required for Maturation, Cell Uptake, and Processing of Translation Inhibitor Microcin C▽

Teymur Kazakov,[1,2] Anastasia Metlitskaya,[3] and Konstantin Severinov[1,3,4]*

Waksman Institute, Piscataway, New Jersey 08854[1]; Institute of Protein Research, Russian Academy of Sciences, Puschino, 142292 Russia[2]; Institute of Molecular Genetics, Russian Academy of Sciences, Moscow, 123182 Russia[3]; and Department of Molecular Biology and Biochemistry, Rutgers, The State University, Piscataway, New Jersey 08854[4]

Received 16 October 2006/Accepted 29 November 2006

Microcin C (McC), a peptide-nucleotide Trojan horse antibiotic, targets aspartyl-tRNA synthetase. We present the results of a systematic mutational study of the 7-amino-acid ribosomally synthesized peptide moiety of McC. Our results define amino acid positions important for McC maturation and cell uptake and processing and open the way for creation of more potent McC-based inhibitors.

The microcins are a class of small (<10 kDa) antibiotics produced from ribosomally synthesized peptide precursors (1). Genes responsible for microcin production are usually plasmid borne (2). Plasmids carrying microcin structural genes also provide immunity to the microcin produced. Some microcins are heavily modified by dedicated maturation enzymes that are also encoded by genes carried on plasmids that harbor microcin structural and immunity genes (9).

Posttranslationally modified microcins have highly unusual structures; they also inhibit important cellular processes, such as replication, transcription, and translation (3). The subject of this study, microcin C (McC) (Fig. 1A), is a heptapeptide containing a modified AMP covalently attached to its C terminus through an *N*-acyl phosphoramidate linkage (6, 8). The nucleotide part of McC is additionally modified by a propylamine group attached to the phosphate. The peptide moiety of McC is encoded by *mccA*, which is 21 bp long and is one of the shortest genes known (5). The last codon of the *mccA* gene, AAC, codes for asparagine. However, mature McC contains an aspartate at this position. Apparently, conversion of the asparagine specified by *mccA* to aspartate occurs during posttranslational maturation of the MccA peptide.

The mechanism of McC function has recently been determined (7). McC is specifically processed inside a sensitive cell, and the product of this processing, modified aspartyl-adenylate, strongly inhibits translation by preventing the synthesis of aminoacylated $tRNA^{Asp}$ by aspartyl-tRNA synthetase. Unprocessed McC has no effect on the aminoacylation reaction. On the other hand, processed McC, while active against aspartyl-tRNA synthetase in vitro, has no effect on McC-sensitive cells. Thus, Mcc belongs to the so-called Trojan horse type of inhibitors (10); the peptide moiety allows McC to enter sensitive cells, where an unidentified peptidase(s) performs suicidal McC processing that liberates the inhibitory aminoacyl nucleotide part of the drug. The fact that the peptide part of McC is encoded by a gene makes it amenable to structure-activity analysis by standard genetic engineering techniques. While the initiating codon of *mccA* cannot be mutated without affecting MccA translation, mutations in the remaining six codons can be used to study the effects of amino acid substitutions on McC maturation, cell uptake, processing inside the cytoplasm, and inhibition of the target enzyme. Here, we report the results of a study in which a panel of 114 *mccA* point mutants (6 *mccA* codons × 19 non-wild-type amino acid positions per codon) was created and analyzed.

The *mccA* mutations were created by standard site-specific mutagenesis of the previously described pUHAB plasmid containing the entire cluster of *mcc* genes (4). Each mutation was confirmed by DNA sequencing. Every *mccA* point mutation plasmid was transformed into *Escherichia coli* DH5a cells, and equal amounts of transformants were spotted on M63 minimal salt agar plates supplemented with 0.2% yeast extract (7). DH5a cells producing wild-type McC and cells harboring a vector plasmid without *mcc* genes were used as controls. After a 12-h incubation at 37°C, spots of cells harboring plasmids with individual *mccA* mutations were overlaid with M63 soft agar containing McC-sensitive *E. coli* B cells. After overnight growth at 37°C, the appearance of growth inhibition zones around spots of cells carrying *mcc* genes was recorded. Clear cell-free growth inhibition zones were easily discernible on the turbid background of an *E. coli* B cell lawn. The results of this analysis are shown schematically in Fig. 1B. Robust growth inhibition zones were observed around DH5a cells harboring 28 *mccA* point mutations. The sizes of the inhibition zones were equal to or larger than (see below) the sizes of the growth inhibition zones produced by cells harboring wild-type pUHAB. In all 28 cases, matrix-assisted laser desorption ionization mass spectrometric analysis of cultured media revealed the presence of expected mass ions corresponding to mutant microcins (data not shown).

Mass spectrometric analysis of cultured media was also conducted for cells that did not produce inhibition zones. Mass ions corresponding to four mutants, R2Y, R2H, A6M, and A6F, were detected. Since there were no growth inhibition zones around cells producing these mutant microcins, the mutants were likely to be functionally defective or insufficient amounts were produced or both. Mass spectrometric analysis of cultured media from cells harboring the remaining mutant *mccA* plasmids did not reveal the presence of mass ions cor-

* Corresponding author. Mailing address: Waksman Institute, 190 Frelinghuysen Road, Piscataway, NJ 08854. Phone: (732) 445-6095. Fax: (732) 445-5735. E-mail: severik@waksman.rutgers.edu.

▽ Published ahead of print on 8 December 2006.

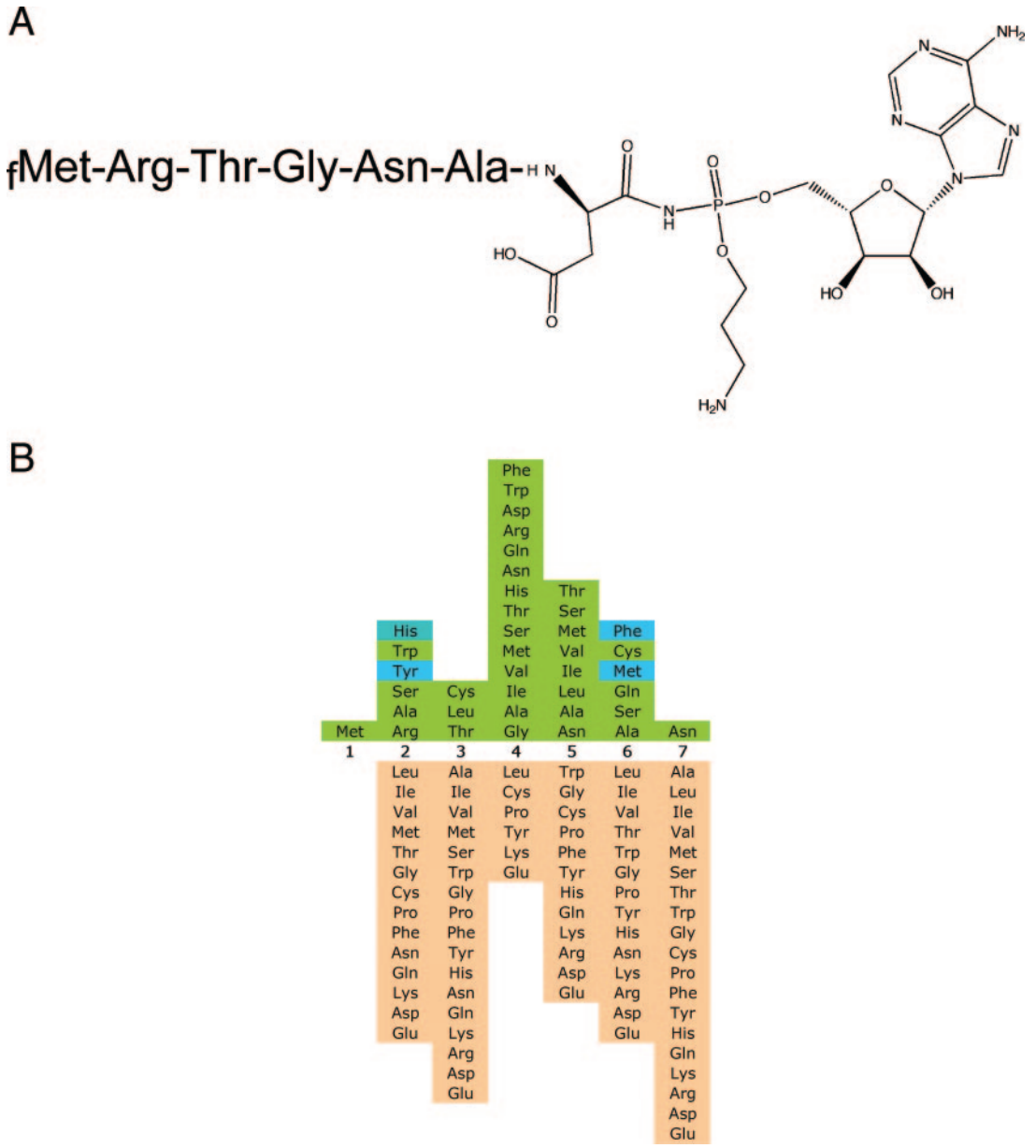

FIG. 1. Summary of the structure-activity analysis of a panel of 114 mutants with *mccA* point mutations. (A) Structure of McC. (B) Point substitutions obtained in this study are shown above (mature McC produced) or below (no mature McC produced) the MccA peptide sequence. Cells producing McC with the substitutions indicated by an olive background resulted in growth inhibition zones that were comparable to the growth inhibition zones produced by cells producing wild-type McC. Cells producing McC with substitutions indicated by a cyan background did not produce growth inhibition zones on lawns of McC-sensitive cells.

responding to the mutant microcins (or in fact any mass ions different from those found in control mass spectra obtained with culture medium of cells harboring the vector plasmid). Apparently, the corresponding substitutions in *mccA* completely eliminated maturation of the MccA peptide into McC. An alternative possibility, that the absence of microcin in the medium was caused by accumulation of mutant microcins in the cells, is unlikely, for one would expect at least some such cells to be very sick due to poisoning of their aspartyl-tRNA synthetase by accumulated processed McC. However, cells that did not produce McC mutants were healthy (data not shown). Moreover, mass spectrometric analysis of cytoplasmic extracts of such cells did not reveal mass ions corresponding to mutant microcins (data not shown).

The diameters of the growth inhibition zones around cells producing the N5A, N5L, and G4V mutants were consistently twofold larger than the diameters of the growth inhibition zones around cells producing wild-type McC. This effect may have been due to altered antibacterial activities of the mutant microcins; alternatively, the amounts of mutant microcins produced may have differed from the amounts of McC produced by cells harboring wild-type *mcc* genes. To determine which of

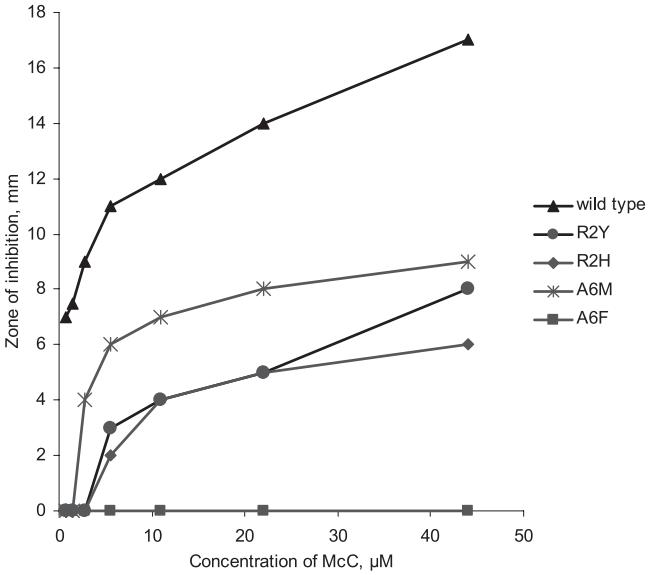

FIG. 2. Antibacterial activities of McC mutants with activities lower than the wild-type activity. Drops of solutions containing different concentrations of wild-type or mutant microcins were spotted on lawns of McC-sensitive *E. coli* B cells. The plates were incubated overnight, and the diameters of growth inhibition zones were determined.

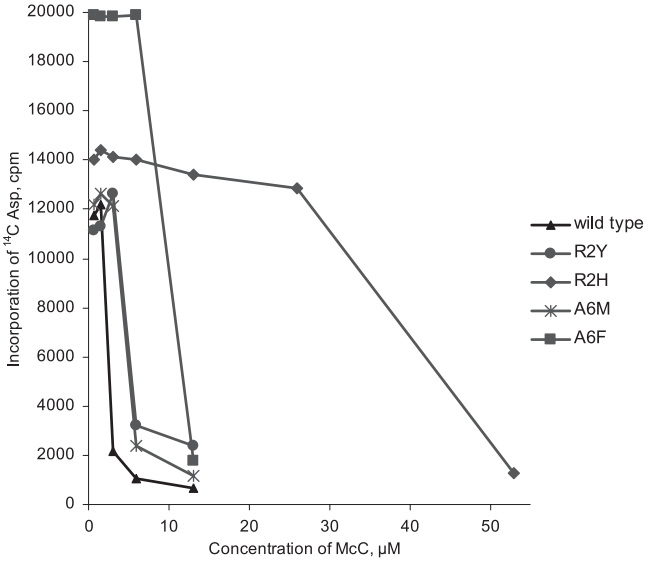

FIG. 3. Inhibition of the $tRNA^{Asp}$ aminoacylation reaction by McC mutants with activities lower than the wild-type activity. Different amounts of purified wild-type or mutant microcins were incubated with *E. coli* B cell extracts for 60 min, and this was followed by the $tRNA^{Asp}$ aminoacylation reaction.

these possibilities was true, mutant microcins were purified (7) and equal volumes of solutions containing various concentrations of wild-type or mutant microcins were deposited on sensitive cell lawns. The cells were allowed to grow, and the diameters of growth inhibition zones were determined. Each of the apparently more active mutants produced zones that were the same size as the zones produced by wild-type McC. Thus, reproducible twofold-larger growth inhibition zones observed for cells producing these microcins suggest that production levels increased at least fourfold (since the zone diameter is determined by diffusion of the drug). The reasons for this effect were not investigated further but may include increased synthesis of the MccA peptide due to altered codon composition of the mutant *mccA* genes or increased efficiency of maturation of mutant MccA or both.

As indicated above, no growth inhibition zones were detected around cells producing the R2Y, R2H, A6F, and A6M mutants. Mutant microcins were purified, and their abilities to produce growth inhibition zones on lawns of sensitive bacteria were examined (Fig. 2). As Fig. 2 shows, the diameters of the zones produced by the R2Y, R2H, and A6M mutants were at least twofold less than the diameters of the zones produced by identical amounts of wild-type McC. The A6F mutant produced no zones (small, turbid zones were apparent when solutions containing high [200 μM] concentrations of mutant microcin were added to sensitive cell lawns [data not shown]).

The partially active mutants could be defective in cell uptake or processing inside the cell or both. To distinguish between these possibilities, the abilities of purified mutant microcins to inhibit $tRNA^{Asp}$ aminoacylation in cell extracts (7) were investigated. Prior to the addition of a radioactively labeled reaction substrate, ^{14}C-labeled aspartic acid, microcins were preincubated with cell extracts for 60 min. Preliminary experiments showed that wild-type McC was completely processed under these conditions (see Fig. 4). The results (Fig. 3) showed that R2Y and A6M McC were only slightly less active than wild-type McC in inhibition of the $tRNA^{Asp}$ aminoacylation reaction (the differences were less than twofold). Approximately fourfold-higher concentrations of the A6F mutant than of the wild-type McC were required to obtain 50% inhibition of the $tRNA^{Asp}$ aminoacylation reaction. Considering that the A6F mutant was severely defective in growth inhibition zone formation (compared to the R2Y and A6M mutants), the results suggest that the A6F substitution strongly interferes with cell uptake. The amount of the R2H mutant needed to obtain 50% inhibition of the $tRNA^{Asp}$ aminoacylation reaction was ~80 times larger than the amount of the wild-type McC needed. Since the structure (and the potency) of processed McC was not affected by substitutions in the second position of MccA, the R2H substitution likely affected the rate of McC processing. To confirm that this was indeed the case, wild-type or mutant R2H McC was incubated with cell extracts for different amounts of time (to allow processing), and this was followed by the $tRNA^{Asp}$ aminoacylation reaction (note that the time needed to complete the $tRNA^{Asp}$ aminoacylation reaction is less than 30 s [our unpublished observations] and thus is negligible compared to the processing time). The results (Fig. 4) show that wild-type McC completely inhibited the aminoacylation reaction after 30 min of processing. In contrast, the mutant McC required a 140-min preincubation with cell extract to block $tRNA^{Asp}$ aminoacylation. This effect was not due to nonspecific inactivation of aspartyl-tRNA synthetase in the extract during preincubation since robust aminoacylation of $tRNA^{Asp}$ was observed in extracts incubated for 140 min without McC (Fig. 4). Thus, the R2H substitution decreased the rate of processing at least fourfold. Since in the growth inhibition assay the R2H mutant had the same activity as the R2Y

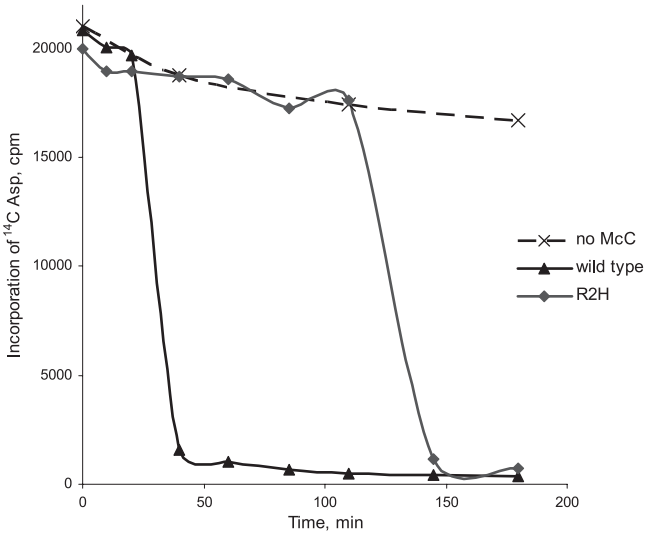

FIG. 4. McC R2H mutant processing rate is lower than the wild-type McC processing rate. Wild-type or R2H McC (13 μM) was incubated with *E. coli* B cell extracts for different amounts of time, and this was followed by the $tRNA^{Asp}$ aminoacylation reaction.

and A6M mutants, it is possible that the R2H substitution actually increased cell uptake. However, an alternative possibility, that even the low processing rate was sufficient for effective inhibition of sensitive cell growth, cannot be excluded.

Analysis of the distribution of active and partially active McC mutants with mutations along the length of the MccA peptide revealed several noteworthy features. First, active mutants were obtained for each of the *mccA* codons subjected to mutagenesis, with the notable exception of the last codon. The latter result is definitely a setback since alteration of the last McC amino acid was expected to generate mutants with altered specificity (i.e., mutants targeting aminoacyl-tRNA synthetases other than aspartyl-tRNA synthetase). Particularly surprising is the fact that the N7D mutation, which introduced an aspartate found in mature McC instead of encoded asparagines, was not produced. The results show that the amide group of the Asn^7 side chain is essential for McC maturation and are consistent with the idea that this amide group is incorporated into the modified nucleotide moiety of mature McC to become part of either the phosphoamidate linkage or the propylamine group.

The fourth position of McC appears to be most permissive; only six amino acid substitutions in this position were not tolerated. The reasons why a particular substitution is or is not tolerated in this position or in other positions of MccA are difficult to determine based on known physicochemical properties of amino acids. For example, Lys and Glu are not tolerated in the fourth position, while their chemical counterparts, Arg and Asp, respectively, are acceptable. For the fifth position, the functional substitutions include nonaromatic hydrophobic amino acids (Val, Ile, Leu, Ala, and Met) and polar amino acids (Ser and Thr). However, Gln, which is most similar to Asn found in the wild-type sequence, is not tolerated. The situation is similar for other positions; for example, substitution of wild-type Arg^2 for Lys is not permitted, while Trp and Tyr (but not Phe) are acceptable. Position 6 is particularly interesting since the penultimate peptide bond between Ala^6 and Asp^7 in mature McC must be hydrolyzed to release processed McC. It appears that the peptidase(s) responsible for cleavage is not very specific with respect to the amino acid N terminal of the scissile bond (in addition to wild-type Ala, Phe, Cys, Met, Gln, and Ser are tolerated) or, alternatively, that the cleavage is specified by the C-terminal Asp residue. On the other hand, analysis of the processing-defective R2H mutant revealed that McC residues that are far from the processing site can significantly affect the rate of McC digestion by the peptidase.

From a practical standpoint, functional substitution of natural McC residues for Cys (T3C and A6C) may allow preparation of McC derivatives containing, for example, cross-linkable probes at these positions that can be used to study McC uptake and processing. Likewise, functional R2W and G4W mutants can be used in experiments in which the interaction of McC with cellular machinery is measured by fluorescent methods.

In summary, our results revealed that of 114 point substitutions in the MccA peptide, 28 did not significantly alter the biological activity of McC and therefore are unlikely to have a strong effect on McC maturation, cell export, cell intake, processing, and inhibitory functions. Four additional substitutions did not affect McC maturation but appeared to interfere with uptake or processing. Thus, approximately 30% of the point substitutions in the MccA peptide resulted in functional or partially functional mature microcin. This is an unusually high number for such a small peptide, indicating that the informational content of the MccA peptide is relatively low (and, consequently, the sequence requirement for McC maturation, uptake, and processing is relatively relaxed). It is therefore likely that functional McC mutants having double or even triple substitutions in the peptide moiety are possible.

Functional substitutions were obtained in every MccA position mutated except the last (seventh) position. There appears to be no simple chemical rule that explains why a particular substitution in MccA is or is not permissive. The asparagine residue encoded by the seventh codon of *mccA* is apparently strictly required for maturation of McC, a peptide-nucleotide. Enzymological studies of McC maturation process are needed to determine if mature McC derivatives with substitutions at the seventh position can be created. Such derivatives are of considerable interest as they should target aminoacyl-tRNA synthetases other than aspartyl-tRNA synthetase. Total chemical synthesis provides an alternative approach for preparation of McC derivatives with altered specificity. A list of acceptable substitutions in the MccA peptide generated in our work should facilitate chemical synthesis of microcins with altered specificity by allowing workers to select functional peptide sequences that can be efficiently conjugated with the nucleotide moiety of McC.

This work was supported by a Burroughs Wellcome Career Award and by NIH grant R01GM64530 to K.S. and by grant 06-04-48865 from the Russian Foundation for Basic Research to A.M.

REFERENCES

1. **Baquero, F., and F. Moreno.** 1984. The microcins. FEMS Microbiol. Lett. **23:**117–124.
2. **Baquero, F., D. Bouanchaud, M. C. Martinez-Perez, and C. Fernandez.** 1978. Microcin plasmids: a group of extrachromosomal elements coding for

low-molecular-weight antibiotics in *Escherichia coli*. J. Bacteriol. **135:**342–347.

3. **Destoumieux-Garzon, D., J. Peduzzi, and S. Rebuffat.** 2002. Focus on modified microcins: structural features and mechanisms of action. Biochimie **84:**511–519.
4. **Fomenko, D. E., A. Z. Metlitskaya, J. Peduzzi, C. Goulard, G. Katrukha, L. V. Gening, S. Rebuffat, and I. A. Khmel.** 2003. Microcin C51 plasmid genes: possible source of horizontal gene transfer. Antimicrob. Agents Chemother. **47:**2868–2874.
5. **Gonzalez-Pastor, J. E., J. L. San Millan, and F. Moreno.** 1994. The smallest known gene. Nature **369:**281.
6. **Guijarro, J. I., J. E. Gonzalez-Pastor, F. Baleux, J. L. San Millan, M. A. Castilla, M. Rico, F. Moreno, and M. Delepierre.** 1995. Chemical structure and translation inhibition studies of the antibiotic microcin C7. J. Biol. Chem. **270:**23520–23532.
7. **Metlitskaya, A., T. Kazakov, A. Kommer, O. Pavlova, I. Krashenninikov, V. Kolb, I. Khmel, and K. Severinov.** 2006. Aspartyl-tRNA synthetase is the target of peptidenucleotide antibiotic Microcin C. J. Biol. Chem. **281:**18033–18042.
8. **Metlitskaya, A. Z., G. S. Katrukha, A. S. Shashkov, D. A. Zaitsev, T. A. Egorov, and I. A. Khmel.** 1995. Structure of microcin C51, a new antibiotic with a broad spectrum of activity. FEBS Lett. **357:**235–238.
9. **Moreno, F., J. E. Gonzalez-Pastor, M. R. Baquero, and D. Bravo.** 2002. The regulation of microcin B, C and J operons. Biochimie **84:**521–529.
10. **Reader, J. S., P. T. Ordoukhanian, J. G. Kim, V. de Crecy-Lagard, I. Hwang, S. Farrand, and P. Schimmel.** 2005. Major biocontrol of plant tumors targets tRNA synthetase. Science **309:**1533.