Molecular Microbiology (2007) **65**(6), 1380–1394
doi:10.1111/j.1365-2958.2007.05874.x
First published online 17 August 2007

MicroReview

Low-molecular-weight post-translationally modified microcins

Konstantin Severinov,[1,2,3]* Ekaterina Semenova,[1] Alexey Kazakov,[4] Teymur Kazakov[1,2,5] and Mikhail S. Gelfand[4,6]**

[1]*Waksman Institute for Microbiology,*
[2]*Department of Molecular Biology and Biochemistry, Rutgers, the State University of New Jersey, Piscataway, NJ 08854, USA.*
[3]*Institute of Molecular Genetics, Russian Academy of Sciences, Moscow, 123182, Russia.*
[4]*Institute for Information Transmission Problems, Russian Academy of Sciences, Moscow, 127994, Russia.*
[5]*Institute of Protein Research, Russian Academy of Sciences, Puschino, 142292, Russia.*
[6]*Faculty of Bioengineering and Bioinformatics, Lomonosov Moscow State University, Moscow, 119991, Russia.*

Summary

Microcins are a class of ribosomally synthesized antibacterial peptides produced by *Enterobacteriaceae* and active against closely related bacterial species. While some microcins are active as unmodified peptides, others are heavily modified by dedicated maturation enzymes. Low-molecular-weight microcins from the post-translationally modified group target essential molecular machines inside the cells. In this review, available structural and functional data about three such microcins – microcin J25, microcin B17 and microcin C7-C51 – are discussed. While all three low-molecular-weight post-translationally modified microcins are produced by *Escherichia coli*, inferences based on sequence and structural similarities with peptides encoded or produced by phylogenetically diverse bacteria are made whenever possible to put these compounds into a larger perspective.

Accepted 4 July, 2007. For correspondence. *E-mail severik@waksman.rutgers.edu; Tel. (+7) 732 445 6095; Fax (+7) 732 445 5735; **E-mail: gelfand@iitp.ru; Tel. +7-495-6504225; Fax +7-495-6500679.

Introduction

Microcins are a diverse class of small ribosomally synthesized peptide antibiotics produced by *Enterobacteriaceae* and active against closely related bacterial species (Baquero *et al*., 1978). This review concerns with microcin J25 (MccJ25), microcin B17 (MccB17), and microcins C7 and C51, two chemically identical compounds encoded by slightly different genetic systems and referred to as MccC7-MccC51 in this review. The three microcins are unrelated to each other but share the following characteristics: (i) low molecular weights (2106 Da for MccJ25, 3093 for MccB17, and 1178 Da for MccC7-MccC51); (ii) highly unusual structures due to extensive post-translational modifications; and (iii) they target essential molecular machines in the cytoplasm of sensitive cells. Genes responsible for production of low-molecular-weight post-translationally modified microcins (*mcj*, *mcb* and *mcc* coding respectively for MccJ25, MccB17 and MccC7-MccC51) are carried on plasmids and are tightly clustered. In addition to a structural gene coding for a microcin precursor (promicrocin), each cluster contains genes whose products carry out post-translational modifications necessary for microcin production, as well as genes whose products actively pump microcin from the producing cell and thus provide resistance to both internally produced and externally added microcin. Each export protein or export protein complex is specific to the microcin produced and, therefore, cells producing a particular microcin remain sensitive to other microcins (in fact the nomenclature used to refer to different microcins, J, B, C, etc., refers to results of determination of cross-immunity of producing cells to different microcins). Microcin-producing cells of the same immunity type have been isolated multiple times in different, geographically remote laboratories. Numbers behind letters indicating the immunity type refer to particular isolates; for example, cells producing MccC7 and MccC51 were isolated in Spain and Russia respectively (González-Pastor *et al*., 1995; Fomenko *et al*., 2003).

Each microcin is taken up by sensitive cells through specialized transport systems that will not be discussed here. Microcin synthesis is sharply activated when cells

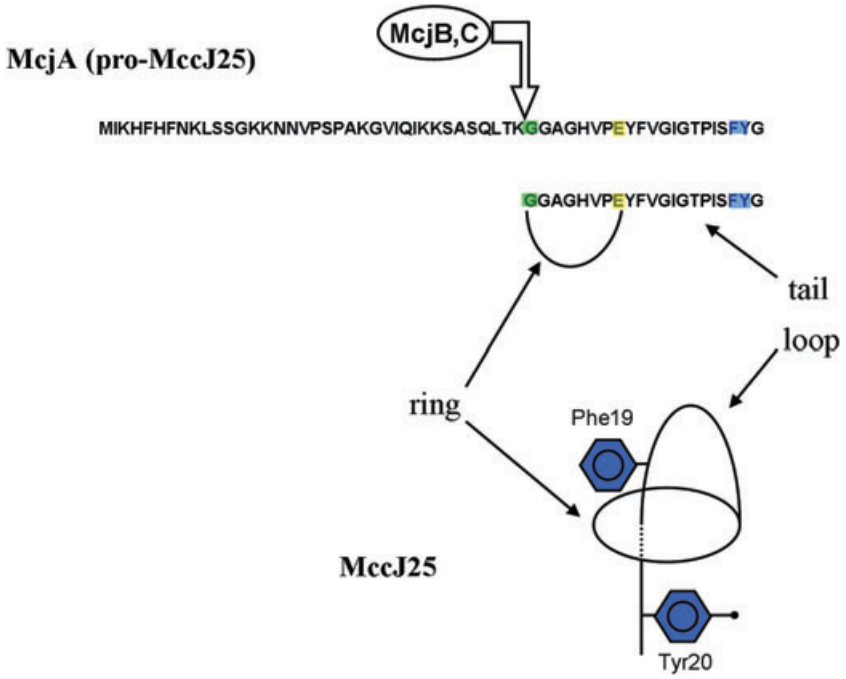


Fig. 1. Maturation and structure of MccJ25, a threaded-lasso peptide. The amino acid sequence of pro-MccJ25 is shown at the top. The covalent structure of mature MccJ25 is schematically presented below. The products of *mcj* genes involved in the maturation process and pro-MccJ25 residues important for MccJ25 structure formation are coloured to match the alignment of Fig. 2B. Mature MccJ25 assumes a threaded-lasso structure, as shown at the right bottom part of the figure. Phe[19] and Tyr[20] form a lock that keeps the MccJ25 tail inside the ring.

reach stationary phase or by phosphate, carbon or nitrogen starvation (MccB17, Hernández-Chico *et al*., 1986; Connel *et al*., 1987), carbon or phosphate starvation (MccJ25, Chiuchiolo *et al*., 2001), or carbon starvation (MccC51, Fomenko *et al.* 2001). Presumably, expression of microcins genes allows producing cells to survive by killing microcin-sensitive cells and thus making more resources available for themselves. Microcins might also act as signalling molecules. The sharp activation of microcin genes transcription at stationary phase is due to the use of σ^{70} promoters activated by OmpR and IHF (MccB17, Hernández-Chico *et al*., 1986; Connell *et al*., 1987; Bohannon *et al*., 1991) or the combined action of ppGpp, Lrp and IHF (MccJ25, Chiuchiolo *et al*., 2001). Activated transcription of *mcc* genes is σ^{S}-dependent and requires CRP (Fomenko *et al*., 2001). The synthesis of both MccB17 and MccC7 is attenuated by MprA, a DNA binding regulator of the MarR family (del Castillo *et al*., 1990). Transcription of the *mcb* (Moreno *et al*., 2002) and *mcc* genes is also negatively regulated by H-NS and Lrp (Fomenko *et al*., 2001).

It is intuitively clear that the amount of microcin precursors synthesized should exceed that of maturation/immunity gene products by a wide margin. The mechanisms that ensure this regulation are not fully understood. In the case of MccJ25, the precursor gene and the operon containing maturation/immunity genes are transcribed from divergent promoters, and so differential promoter strengths might produce the optimal ratio of precursor and biosynthesis transcripts. In the case of MccB17 and MccC7-MccC51, genes encoding the precursor and biosynthesis enzymes are transcribed from the same promoter, with precursor genes being promoter-proximal. Thus, regulation through transcription termination might operate in this case. Indeed, sequence analysis reveals putative terminators between the MccC7-MccC51 structural and maturation genes. In the case of MccB17, there exists an antisense transcript that originates from a promoter located inside the *mcbD* gene and that might downregulate expression of MccB17 biosynthesis genes (Genilloud *et al*., 1989).

While microcin synthesis is inducible, microcin immunity genes are constitutively expressed. Constitutive expression is achieved through σ^{S}-independent transcription of the MccC7 immunity genes or separate transcription of MccB17 immunity genes from a minor promoter (Fig. 4A, Genilloud *et al*., 1989).

Microcin J25

Microcin J25, a 21-amino-acid peptide, is produced from 58-amino-acid-long pro-MccJ25, a product of the *mcjA* gene (Solbiati *et al*., 1999). Pro-MccJ25 consists of a 37-amino-acid N-terminal leader and the C-terminal part that becomes MccJ25. MccJ25 is highly resistant to proteolysis and even withstands autoclaving without the loss of activity (Blond *et al*., 1999). The structure of MccJ25 explains this exceptional stability (Fig. 1). The amino group of MccJ25 Gly[1] is linked to the side-chain carboxyl of Glu[8] by a lactam bond, creating a ring. The C-terminal tail of MccJ25 forms a two-β-strand loop, the end of which passes through the ring to form a threaded-lasso structure

(Bayro *et al*., 2003; Rosengren *et al*., 2003; Wilson *et al*., 2003). The side-chains of Phe^{19} and Tyr^{20} at the end of the tail straddle the ring and make the movement of the tail inside the ring impossible. Extensive digestion of MccJ25 with thermolysin results in cleavage of the polypeptide chain between amino acids 10 and 11 (Rosengren *et al*., 2004). Hydrolysis of MccJ25 in 12 M HCl results in a derivative lacking amino acids 13–17 in the loop (Rosengren *et al*., 2004). Both of these compounds contain two separate polypeptide chains that remain stably associated with each other due to the lock formed by Phe^{19} and Tyr^{20}.

The *mcjB* and *mcjC* genes are required for production of MccJ25 from pro-MccJ25. Although no *in vitro* maturation system for MccJ25 exists, some insights into the process can be obtained by sequence analysis. McjC is distantly related to the widely distributed and highly conserved family of class B asparagine synthetases (AS-B) and β-lactam synthetases (β-LS) (sequence identities in the range of 20–23%, BLAST*e*-values approximately 0.0001). AS-B converts aspartic acid to asparagine (Larsen *et al*., 1999), while β-LS catalyses the formation of β-lactam ring in clavulanic acid (Miller *et al*., 2001; 2002). Both enzymes use ATP and Mg^{2+} as cofactors. AS-Bs are two-domain proteins. The N-terminal domain contains a glutamine binding site where hydrolysis of glutamine to glutamic acid and ammonia takes place. The C-terminal domain catalyses the adenylation of aspartic acid and its subsequent reaction with ammonia to form asparagine. The two domains are connected by a tunnel that allows ammonia generated by the action of the N-terminal domain to reach the active site of C-terminal domain synthetase (Larsen *et al*., 1999). A Cys residue essential for catalysis by AS-Bs is not present in McjC, suggesting that McjC does not have a glutaminase function. In this respect, McjC is similar to β-LS, and so it is likely responsible for the formation of the β-lactam bond between the amino group of MccJ25 Gly^1 (generated upon proteolytic cleavage of pro-MccJ25) and the side-chain of Glu^8. McjB is similar to transglutaminases, enzymes that establish covalent cross-links between proteins through an acyl-transfer reaction between the γ-carboxamide group of glutamine in one protein and ε-amino group of lysine in another protein, although a variety of primary amines can serve as acyl acceptors. The enzymes of this superfamily have three conserved sequence motifs that centre around conserved cysteine, histidine and aspartate residues that form a catalytic triad in the structurally characterized transglutaminase, the human blood coagulation factor XIII (Yee *et al*., 1994). These residues are conserved in McjB. Many microbial homologues of transglutaminases are proteases that can catalyse the reverse reaction of peptide bond hydrolysis (Makarova *et al*., 1999). Thus, McjB might hydrolyse the peptide bond between the last lysine of the McjA leader and a glycine that becomes Gly^1 of mature MccJ25. McjB might also fold pro-MccJ25 to form the ring around the tail part of MccJ25 to favour β-lactam formation in the reaction catalysed by McjC.

Microcin J25-producing cells are resistant to internally produced and externally added MccJ25 due to the action of McjD, an ABC transporter (Solbiati *et al*., 1996). Most spontaneous *Escherichia coli* MccJ25-resistance mutations affect genes encoding membrane proteins responsible for MccJ25 uptake, for example the FhuA transporter (Destoumieux-Garzón *et al*., 2005), but a rare MccJ25-resistant *E. coli* carrying a mutation in the *rpoC* gene, encoding the RNA polymerase (RNAP) β′ subunit, was also identified (Delgado *et al*., 2001), suggesting that RNAP might be the cellular target of MccJ25. In agreement with this idea, the *in vitro* activity of *E. coli* RNAP is inhibited in the presence of micromolar concentrations of MccJ25 (Delgado *et al*., 2001). Conversely, RNAP from MccJ25-resistant *rpoC* mutant cells is resistant to MccJ25 *in vitro*, thus proving that RNAP is the cellular target of the drug (Yuzenkova *et al*., 2002).

Targeted mutagenesis of the *E. coli rpoC* gene resulted in isolation of numerous additional MccJ25-resistant mutants (Yuzenkova *et al*., 2002; Mukhopadhyay *et al*., 2004). In the structural model of *Thermus aquaticus* RNAP, residues corresponding to *E. coli* amino acids that, when substituted, lead to MccJ25 resistance are located around the circumference of the secondary channel, a narrow opening that leads from the RNAP surface to the catalytic centre (Zhang *et al*., 1999). Although *E. coli rpoC* mutations that lead to MccJ25 resistance affect residues that are evolutionary conserved, the inhibitory activity of MccJ25 is limited to RNAPs from proteobacteria (Yuzenkova *et al*., 2002). This suggests that evolutionarily variable regions of RNAP might also contribute to MccJ25 binding to the RNAP target. Indeed, a functionally dispensable β′ subunit loop present in RNAPs from proteobacteria, but absent from homologues from Gram-positive bacteria, contributes to MccJ25 sensitivity of the former group of enzymes (Yuzenkova *et al*., 2002).

Based on structural considerations, the secondary channel is thought to direct NTP substrates towards the RNAP active site (reviewed in Borukhov *et al*., 2005). The tight clustering of MccJ25-resistance mutations in the secondary channel suggests a novel mechanism of RNAP inhibition: occlusion of the secondary channel by bound MccJ25 and prevention of NTP entry to the enzyme catalytic centre. As expected for such a mode of inhibition, MccJ25 binding instantaneously stops RNAP on its tracks (Adelman *et al*., 2004). When MccJ25 dissociates, transcription resumes at a normal rate until another MccJ25 molecule binds to the transcription complex.

Structural modelling suggests that MccJ25 can fit inside the secondary channel of *T. aquaticus* RNAP (with an obvious caveat that this enzyme is resistant to MccJ25) and fully block it (Adelman *et al*., 2004; Mukhopadhyay *et al*., 2004). A mode of transcription inhibition through binding in, and total occlusion of, the RNAP secondary channel invites very specific predictions about the effects that MccJ25 should have on transcription arrest and factor-dependent transcript cleavage. In the arrested conformation of the transcription elongation complex, the 3′ end-proximal portion of the nascent RNA is thought to be threaded through the secondary channel; transcript cleavage factors Gre induce endonucleolytic transcript cleavage activity of the RNAP catalytic centre by reaching into the catalytic centre through the secondary channel (Borukhov *et al*., 2005). By binding in the secondary channel, MccJ25 should prevent formation of arrested transcription complexes (by preventing the threading of the transcript through the channel) and inhibit Gre factor-dependent cleavage (by preventing factor access to the catalytic centre). Such behaviour is indeed observed experimentally (Adelman *et al*., 2004), providing further support for a 'cork-in-the-bottle' model of MccJ25 binding in the secondary channel that prevents all traffic through the channel.

A systematic structure-activity analysis of MccJ25 has not been carried out. The previously mentioned MccJ25 derivatives obtained by thermolysin cleavage or acid hydrolysis inhibit RNAP *in vitro* but do not exhibit antibiotic activity (Semenova *et al*., 2005). Thus, the loop of the tail is important for cell uptake but is not involved in interactions with RNAP. Several substitutions in the ring also affect the antibiotic activity without preventing RNAP inhibition *in vitro* (Cristóbal *et al*., 2006).

The structure of the MccJ25 ring and tail lock is virtually superimposable on the structure of RP71955, a threaded-lasso peptide produced by *Streptomyces* (Frechet *et al*., 1994; Semenova *et al*., 2005). RP71995 is identical to aborycin, an antibiotic that inhibits the growth of Gram-positive, but not Gram-negative, bacteria through an unidentified mechanism (Potterat *et al*., 1994). Several other pharmacologically active threaded-lasso peptides are produced by various bacteria (Wyss *et al*., 1991; Chokekijchai *et al*., 1995; Katahira *et al*., 1995; 1996; Esumi *et al*., 2002; Iwatsuki *et al*., 2006). These peptides were isolated for their ability to inhibit processes as diverse as the HIV-1 infection, the function of endothelin 1, and the function of smooth muscle myosin kinase. These properties, although useful, must be fortuitous and cannot reflect the true biological function of these compounds. The structural similarity to MccJ25 suggests that threaded-lasso peptides might be functionally analogous to it as well. Preliminary data indicate that some of the threaded-lasso peptides do inhibit transcription by bacterial RNAPs (E. Semenova and K. Severinov, unpubl. obs.).

Genes coding for threaded-lasso peptides other than MccJ25 have not been identified. The small size of pro-MccJ25, MccJ25 and other known threaded-lasso peptides makes straightforward similarity searches for homologous genes useless. Clustering of prokaryotic genes has been successfully used for functional annotations (Overbeek *et al*., 1999; Osterman and Overbeek, 2003). We therefore hypothesized that a presence of adjacent *mcjB* and *mcjC* homologues identified using BLAST searches may point to possible MccJ25-like biosynthetic loci, which should contain adjacent *mcjA*-like genes. Such «guilt-by-association» searches reveal, first, that clusters of adjacent homologues of *mcjB* and *mcjC* with or without nearby ABC transporter genes are quite common in a variety of bacteria from such diverse groups as α-, β- and γ-proteobacteria and actinobacteria (Fig. 2A). Further searches upstream of putative maturation enzyme genes reveal short open reading frames (ORFs) that could encode threaded-lasso peptide precursors. Multiple alignment of the products of such candidate *mcjA* genes together with known threaded-lasso peptides demonstrates conservation of residues that could be involved in ring formation: a glycine (in some cases, serine, alanine or, rarely, cysteine) separated by six or seven amino acids from glutamate or aspartate (Fig. 2B). In most cases, the C-terminal region of these peptides contains one or several aromatic amino acids that can form a lock to keep the tail inside the ring. Notably, the candidate pro-MccJ25 sequences are similar within major branches of the McjC (Fig. 2C) and McjB (data not shown) phylogenetic trees. While the branches are formed by genes from related species, the overall branching pattern of the tree does not reflect the species taxonomy, indicating that *mcj* homologues are horizontally transferred.

The genomes of *Sphingopyxis alaskensis* RB2256, *Caulobacter* sp. K31, *Frankia* sp. and *Streptomyces avermitilis* contain several candidate *mcj* loci and, in several cases, multiple *mcjA* genes can be identified (Fig. 2A). Thus, these bacteria might produce several lariat peptides. Establishment of the functions of candidate *mcj* loci in different bacteria and determination of activities of lariat peptides they likely encode remains the subject of future investigations.

Microcin B17

Microcin B17 is synthesized from 69-amino-acid-long pro-MccB17, a product of the *mcbA* gene (Fig. 3A). MccB17 contains oxazole and thiazole rings that are post-translationally synthesized by the McbBCD enzyme in subsequent reactions of cyclization, dehydration and

dehydrogenation from GlySer (oxazole) and GlyCys (thiazole) dipeptides present in pro-MccB17 (Li *et al*., 1996). Modification of pro-MccB17 tripeptides $Gly^{39}Ser^{40}Cys^{41}$ and $Gly^{54}Cys^{55}Ser^{56}$ results in the formation of two fused 4,2-bis-heterocycles (oxazole–thiazole and thiazole–oxazole heterocycle respectively, Fig. 3A). Completion of the McbBCD action results in formation of pro-MccB17. The N-terminal 26-amino-acid-long leader peptide of modified pro-MccB17 is removed, resulting in mature MccB17. The products of the *pmbA* (also called *tldE*) (Rodríguez-Sáinz *et al*., 1990) and *tldD* genes (Allali *et al*., 2002) are required for pro-MccB17 cleavage. The products of these genes are evolutionary conserved in *Eubacteria* and *Archaea*; however, their cellular function and exact role in MccB17 maturation is not firmly established. They may either act as proteases and/or promote the interaction of MccB17 with the export pump composed of the *mcbE* and *mcbF* gene products (Garrido *et al*., 1988). Be that as it may, the cleavage of the leader peptide does not depend on the presence of post-translational modifications by McbBCD (Madison *et al*., 1997).

The McbB sequence does not have obvious similarity to any sequences in public databases. It contains pairs of cysteines characteristic of zinc binding motifs. Biochemical analysis indicates that: (i) McbB is indeed a zinc metalloprotein, and (ii) zinc is necessary for the enzymatic activity of the McbBCD synthetase (Zamble *et al*., 2000). McbC has a conserved 'mcbC-like oxidoreductase' domain (Schneider *et al*., 2003) that is also present in several FMN-dependent enzymes, such as EpoB (involved in the epothilone biosynthesis), TubD (tubulysin biosynthesis), MtsD (myxothiazol biosynthesis), IndC (indigoidine biosynthesis) and TfxB (trifolitoxin biosynthesis). These enzymes oxidize the products of cyclization of thioesters. Purified McbC also contains a non-covalently bound flavin (Milne *et al*., 1999). During formation of the MccB17 cycles, the McbC subunit of the McbBCD synthetase functions as a dehydrogenase with oxygen serving as an acceptor of electrons (Milne *et al*., 1999). The N-terminal portion of McbD contains motifs reminiscent of the Walker B box in ATP utilizing enzymes and of motifs found in small G protein GTPases. Substitutions of conserved aspartate residues in these motifs reduce MccB17 production *in vivo* and heterocycle formation *in vitro* (Milne *et al*., 1998).

Microcin B17 is the only microcin for which an *in vitro* maturation system has been established and considerable effort has been put into trying to understand how the maturation occurs. McbA fragments lacking the leader peptide are not modified by McbBCD either *in vitro* or *in vivo* (Madison *et al*., 1997). On the other hand, the leader peptide added *in trans* inhibits McbBCD function (Li *et al*., 1996), indicating that the leader peptide (i) is recognized by McbBCD and (ii) needs to be covalently connected to the rest of McbA for modification to occur. The leader peptide forms an amphipathic helix, in which residues forming a hydrophobic patch on one side are essential for interaction with McbBCD (Roy *et al*., 1998a,b). Cross-linking experiments indicate that the McbBCD synthetase recognizes the leader sequence through the McbD subunit (Madison *et al*., 1997).

The N-terminal fused heterocycle of MccB17 is called site A; the C-terminal fused heterocycle is called site B. A fragment containing the first 46 amino acids of pro-MccB17 (and therefore including site A only) is modified by McbBCD *in vitro* and *in vivo* (Li *et al*., 1996; Madison *et al*., 1997). Therefore, C-terminal modification sites are not required for initiation of the maturation reaction. McbBCD acts in an N-to-C-terminus direction, and site A fused heterocycle is therefore the first to be synthesized in the context of full-sized premicrocin. Kinetic studies show that site A thiazole ring is synthesized ~100 times faster than the oxazole ring, and the synthesis of the A site fused heterocycle therefore proceeds in an orderly fashion (Belshaw *et al*., 1998). Despite the directionality of the McbBCD action, the maturation process is distributive rather than processive, and partially mature MccB17 species lacking one or more C-terminal heterocycles (as well as a species containing one extra oxazole ring, which leads to formation of an additional fused heterocycle between sites A and B, Fig. 3A) were detected by mass spectrometry (Kelleher *et al*., 1999).

Microcin B17 targets DNA gyrase (Vizan *et al*., 1991). Based on the fact that DNA binding drug bleomycin contains a fused thiazole heterocycle, it has been suggested that fused heterocycles are also responsible for the inhibitory activity of MccB17 (Li *et al*., 1996). Indeed, a point mutation leading to an S39N substitution in McbA that should abolish formation of site A fused heterocycle was reported to result in a > 99% reduction of antibiotic activity of MccB17 (Yorgey *et al*., 1994). Conversely, a derivative containing an extra fused heterocycle between sites A and B is ~40% more active than standard MccB17 (Kelleher *et al*., 1999).

A systematic structure-functional analysis of MccB17 has not been performed. Mutational analysis of McbA tripeptides that are converted to site A and site B heterocycles showed that derivatives containing bisoxazole- or bisthiazol-fused cycles (produced respectively by cells expressing *mcbA* coding for GlySerSer or GlyCysCys tripeptides in either of the two sites) are active, although mutations in the B site decrease the activity several fold (Roy *et al*., 1999a). The yield of MccB17 containing bisoxazole in site A was very poor, presumably because oxazole synthesis by McbBCD is slow and incomplete modification of site A acts as a kinetic trap and

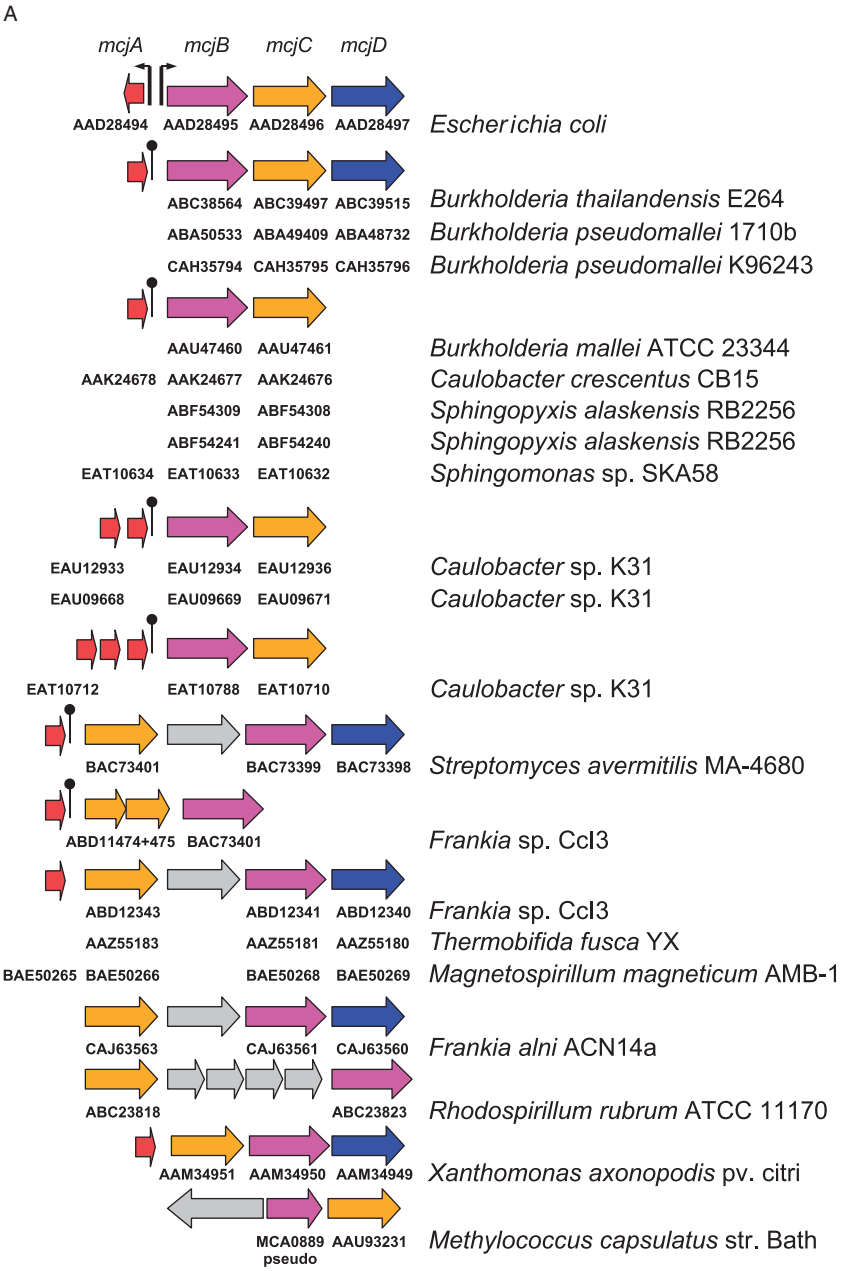
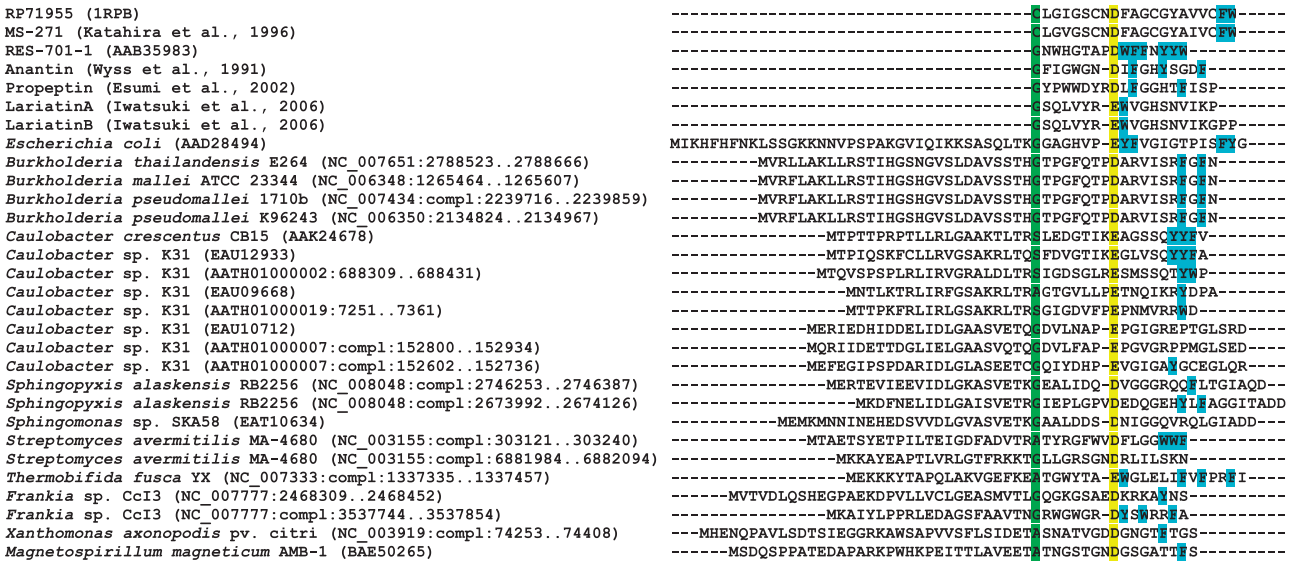


Fig. 2. Evolutionary conservation of gene clusters coding for possible MccJ25 homologues.
A. Genetic organization of predicted *mcj* loci. Block arrows denote genes, with arrow directions indicating the direction of transcription. Homologous genes are indicated by matching colours. Thin arrows above the representation of *E. coli mcj* genes indicate promoters. Putative transcription terminators containing stem-loop structures are also indicated as vertical hairpins. Accession numbers for protein sequences from the NCBI Entrez database are indicated for each genome. For the *mcjB* pseudogene in the *Methylococcus capsulatus* genome, Entrez gene identifier is indicated.
B. Manual alignment of known threaded-lasso peptides, pro-MccJ25 and predicted threaded-lasso peptide precursors. Residues that participate or might participate in the β-lactam bond formation or lock the tail inside the ring are highlighted by colours matching those used in Fig. 1. Accession numbers for protein (where available) or nucleotide sequences from the NCBI Entrez database are indicated for each genome. Numbers after nucleotide sequence accession numbers refer to the start and end co-ordinates of a coding segment; 'compl' indicates reverse direction of translation.
C. A phylogenetic tree of McjC homologues from candidate *mcj* loci. The tree was constructed by the maximum likelihood algorithm implemented in the Phylip package (Felsenstein, 1996).

B



C

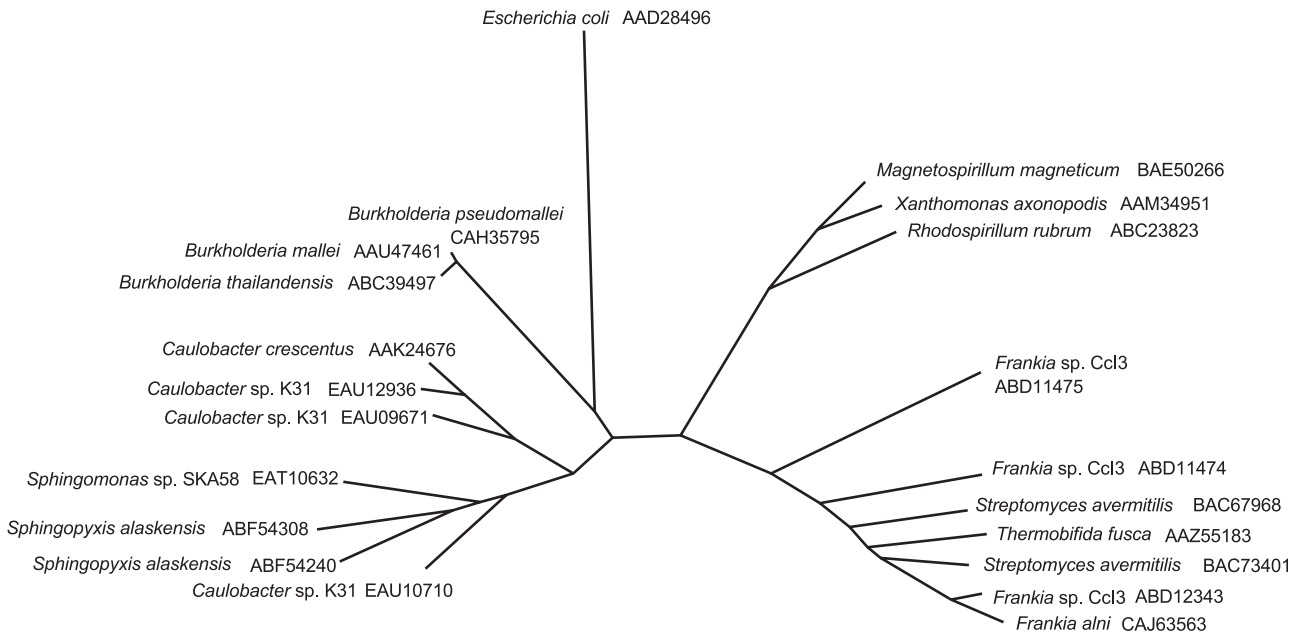


Fig. 2. *cont.*

prevents efficient modification of the remaining sites. A $Gly^{39}Gly^{40}Cys^{41}$ substitution that created a single thiazole heterocycle in site A resulted in a severe reduction of mature MccB17 production. However, a reduction in the production of mature MccB17 with the previously mentioned S39N substitution in site A was not reported (Yorgey *et al*., 1994), suggesting that effects of substitutions in site A on modification of downstream sites might depend on the nature of the substitution. A fully mature compound containing a single thiazole ring in site A was prepared *in vitro* by prolonged incubation of appropriate recombinant precursor with the McbBCD enzyme and was found to possess antimicrobial activity, in apparent contradiction of earlier analysis of the S39N variant, which was found inactive (Yorgey *et al*., 1994). Nevertheless, Roy *et al*. (1999b) concluded that sites other than site A are required for antibiotic activity, while site A controls orderly maturation of MccB17. Derivatives containing single cycles in the B site were obtained with normal yield (Roy *et al*., 1999a). However, their antibacterial and *in vitro* activities were severely reduced (less than 10% of the wild-type MccB17 activity, Zamble *et al*., 2001),

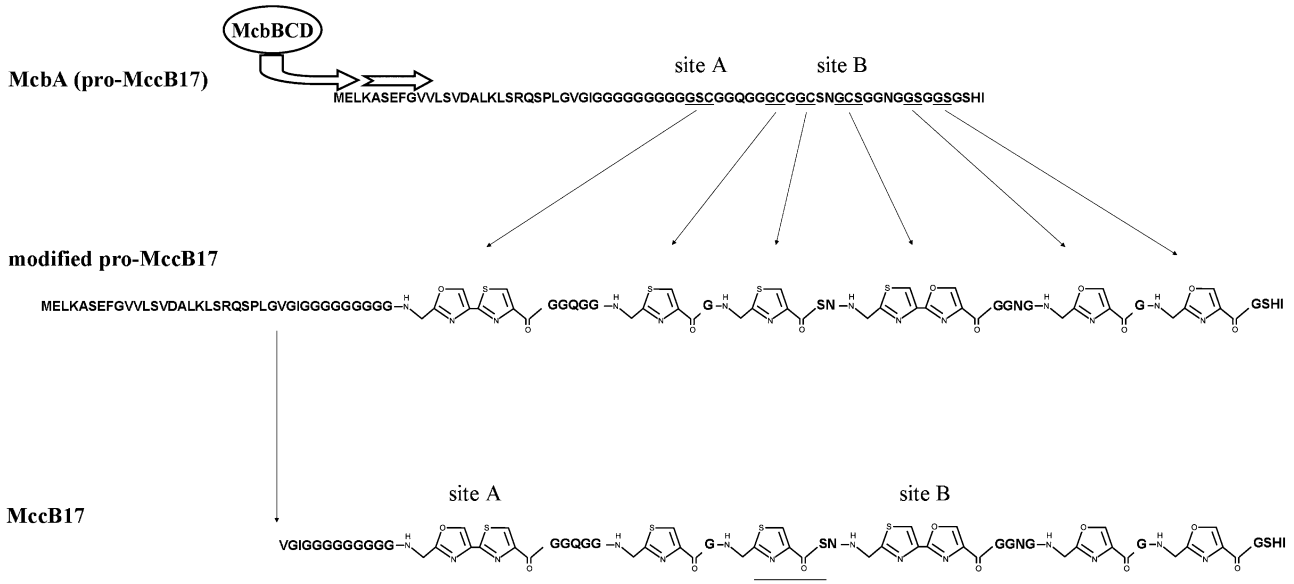


Fig. 3. Maturation and structure of MccB17, a thiazole/oxazole heterocycle containing peptide. The amino acid sequence of pro-MccB17, the product of the *mcbA* gene, is shown at the top. The structures of modified pro-MccB17 and mature MccB17 are shown below, with an additional fused heterocycle found in a minor fraction of MccB17 below the MccB17 sequence indicated by a horizontal line. The products of *mcb* genes involved in the maturation process are shown.

supporting the notion that the fused heterocycle in site B is important for activity. Mutations destroying both site B heterocycles cause loss of activity (Zamble *et al*., 2001).

Point mutations that substituted McbA glycine 48 or 49 for aspartates have been reported to abolish the antibiotic activity of MccB17 (Yorgey *et al*., 1993). The substituted residues are located outside of both sites A and B (Fig. 3A). Gly^{48} is not subject to post-translational modifications, while Gly^{49} becomes part of a thiazole ring that is converted to the third fused heterocycle in 'overmodified' MccB derivative (Roy *et al*., 1999a). Based on reactivity with an MccB17-specific antibody, the altered microcins appeared to contain post-translational modifications, but the extent of these modifications was not determined (Yorgey *et al*., 1993). Given the postulated importance of fused heterocycles for MccB17 activity, it is not clear why substitutions at these positions had such a strong effect. Most recently, Parks *et al*. (2007) have shown that treatment of MccB17 with high pH, which converts, by deamidation, two MccB17 asparagine residues to aspartates abolishes the *in vivo* and *in vitro* activity of MccB17 without affecting the heterocycles. Overall, it appears that the data on mutational analysis of MccB17 maturation and activity are incomplete, and further systematic structure–activity analyses are clearly warranted.

Microcin B17 causes rapid cessation of DNA replication in sensitive cells and subsequent SOS response (Herrero *et al*., 1986). It also induces accumulation of double-stranded breaks in plasmids incubated in the presence of sensitive cell extracts (Vizan *et al*., 1991). Cells harbouring a specific mutation in the *gyrB* gene that codes for subunit B of the heterotetrameric (subunit composition A_2B_2, see below) DNA gyrase are resistant to MccB17 (Vizan *et al*., 1991). Moreover, DNA gyrase purified from the mutant cells is resistant to MccB17 *in vitro*, while the enzyme from the wild-type cells is sensitive (Heddle *et al*., 2001). Thus, the gyrase is the cellular target of MccB17.

DNA gyrase is a bacterial type II DNA topoisomerase that introduces negative supercoils into DNA (see Roca *et al*., 1996 and references cited therein). It functions by wrapping a ~150 bp DNA fragment around itself, introducing a double-stranded break in the DNA duplex called the G segment, passing the other duplex (the T segment) through the break and then sealing the break. The reaction involves formation of a covalent enzyme–G-segment intermediate and requires ATP hydrolysis. The gyrase also catalyses relaxation of supercoiled DNA. The A subunit of the gyrase is responsible for wrapping of DNA around the enzyme and for G-segment breakage and ligation after the strand passage reaction. The B subunit is an ATPase that captures the T segment and passes it through the G segment hydrolysing ATP in the process. Structural data suggest that the gyrase functional cycle involves opening and closing of several molecular 'gates' that allow the movement of DNA duplexes through the enzyme (Roca *et al*., 1996).

The mechanism of MccB17 inhibition of gyrase activity is not fully understood, and the site of its interaction with the enzyme is not determined. At optimal *in vitro* conditions for gyrase function, MccB17 does not have any

effect on gyrase-catalysed supercoiling/relaxation reactions, suggesting that it works with low efficiency and that the enzyme is able to perform several catalytic cycles before being inhibited (Heddle *et al*., 2001). Indeed, modest (~3-fold) inhibition of either reaction is observed when gyrase-catalysed reactions are attenuated by lowering the reaction temperature (Pierrat and Maxwell, 2003). In the presence of MccB17, complexes containing gyrase bound to DNA cleaved at both strands accumulate over time but only in the presence of ATP (Vizan *et al*., 1991; Heddle *et al*., 2001). These complexes impede the movement of DNA polymerase *in vitro* (Heddle *et al*., 2001) and might be responsible for triggering the SOS response that is observed when cells are treated with MccB17. Cleaved DNA complexes are not formed when gyrase from MccB17-resistant cells is used *in vitro* (Heddle *et al*., 2001), suggesting that accumulation of complexes containing cleaved DNA is ultimately responsible for the antibiotic action of MccB17.

Microcin B17 does not appear to interact with free gyrase, nor does it increase gyrase affinity for DNA (Pierrat and Maxwell, 2005). The MccB17-stabilized complex can be reversed by heating and therefore is not covalent. *In vitro*, DNA fragments longer than 150 bp are needed for efficient inhibition of DNA gyrase function by MccB17 (Pierrat and Maxwell, 2005). However, because the C-terminal DNA wrapping domain of GyrA is not needed for MccB17 inhibition (Pierrat and Maxwell, 2005), the requirement for long DNA fragments probably indicates that the passage needs to be initiated before MccB17 can manifest its inhibitory activity (i.e. MccB17 may inhibit strand passage by interacting with the gate region of the gyrase).

Clinically important quinolines also target gyrase and stabilize a covalent complex between the gyrase and cleaved DNA (Heddle *et al*., 2001 and references therein). Unlike MccB17, quinolines efficiently block the supercoiling reaction and the cleaved complex accumulates even in the absence of ATP (Heddle *et al*., 2001). While cells harbouring the *gyrB* mutation that leads to MccB17 resistance are sensitive to quinolines, some quinoline-resistant gyrase mutants with substitutions in the A subunit of the gyrase show weak level of MccB17 resistance *in vitro* (Heddle *et al*., 2001), suggesting that the two drugs might have partially overlapping binding sites. However, a systematic mutagenesis of gyrase genes is needed to map the MccB17 interaction site and to test this conjecture. The only spontaneous *gyrB* mutation that leads to MccB17-resistance substitutes Trp^{751} in the C-terminal domain of the B subunit for Arg (Vizan *et al*., 1991). Limited site-specific mutagenesis of the corresponding codon of *gyrB* reveals that not every substitution of this position leads to MccB17 resistance (del Castillo *et al*., 2001). In the presence of DNA, the C-terminal domain of the B subunit is protected from limited proteolysis by quinolines. MccB17 also protects this domain, but only in the presence of both DNA and ATP (Pierrat and Maxwell, 2005), lending further support to the idea that the binding sites of the two drugs overlap. However, the strict requirement of ATP (and DNA) for MccB17 binding and function might indicate that quinolines and MccB17 act at different stages of the gyrase functional cycle.

The McbC dehydrogenase is highly similar to TfxC, a protein involved in biosynthesis of trifolitoxin, a peptide antibiotic produced by some strains of *Rhizobium leguminosarum bv. trifolii* (Scupham and Triplett, 2006). Like MccB17, trifolitoxin contains a fused thiazole–oxazole heterocycle and is produced from a longer ribosomally synthesized precursor. However, with the exception of TfxC, trifolitoxin biosynthesis genes products do not have any similarity to the *mcb* genes. The cellular target of trifolitoxin is also unknown at present.

Bioinformatic analysis of publicly available sequences reveals the presence of a complete set of MccB17 maturation and export genes in *Pseudomonas putida* (Fig. 4), suggesting that an *mcbA* may also be present. Indeed, an ORF coding for a 69-amino-acid glycine-rich peptide is found appropriately positioned upstream of the *mcbB* homologue (Fig. 4). The putative MccB17 from *P. putida* contains numerous GlySer and GlyCys dipeptides that can be converted to oxazole and thiazole heterocycles respectively. However, tripeptides that could be converted to fused heterocycles are absent. Given the reported significance of fused heterocycles for MccB17 activity, one has to wonder how the *P. putida* MccB17 homologue (assuming it targets DNA gyrase) acts. Clearly, further studies of this interesting system are warranted.

Microcin C7-C51

Microcin C7-C51 is a heptapeptide containing a modified adenosine monophosphate covalently attached to its C-terminus through an N-acyl phosphoramidate linkage (Guijarro *et al*., 1995; Fig. 5). The nucleotide part of MccC7-MccC51 is additionally modified by a propylamine group attached to the phosphate. The peptide moiety of MccC7-MccC51 is encoded by a 21 bp *mccA* gene, one of the shortest genes known (González-Pastor *et al*., 1994). Although the last residue of peptide moiety of MccC7-MccC51 is an aspartic acid, the last codon of *mcjA* codes for an asparagine (Fig. 5).

Microcin C7-C51 is specifically processed inside the target cell (Metlitskaya *et al*., 2006). The product of such processing is a structural analogue of aspartyl-adenylate, an intermediate of the reaction catalysed by aspartyl–tRNA synthetase (Fig. 5). Processed MccC7-MccC51 prevents the synthesis of aminoacylated $tRNA^{Asp}$ by aspartyl–tRNA synthetase *in vitro*, which in turn leads to inhibition of

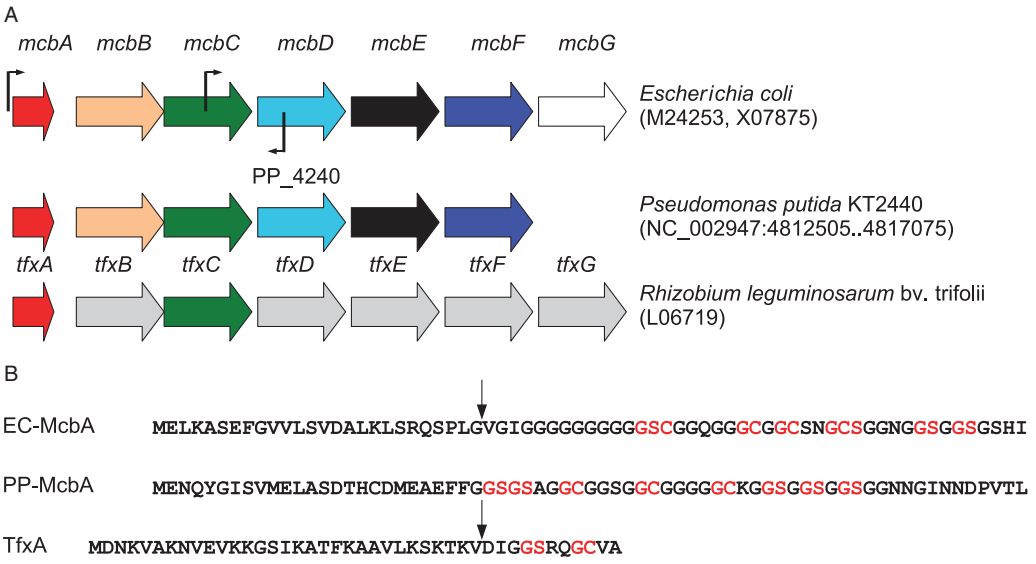


Fig. 4. A cluster of MccB17 biosynthesis genes homologues from *P. putida*.
A. Genetic organization of the *E. coli mcb* locus and a cluster of homologous genes from *P. putida* is compared with matching colours indicating homologous genes. Thin arrows above and below the *E. coli mcb* genes indicate promoters. The cluster of genes coding for trifolitoxin, a thiazole-containing peptide antibiotic produced by some strains of *Rhizobium leguminosarum*, is shown at the bottom. Homologous genes are indicated by matching colours. In this figure and in Figs 2A and 6A, genes coding for microcin precursors and genes coding for immunity pumps are indicated in red and blue, respectively, to indicate common functions (in the absence of evolutionary relationship). Other matching colours between Figs 2A, 4A and 6A do not imply any evolutionary or functional similarity of the genes. The product of the *tfxB* gene is highly similar to McbC; other trifolitoxin biosynthesis genes are not related to the *mcb* genes. Accession numbers for nucleotide sequences from the NCBI Entrez database are indicated for each locus. Numbers after the *P. putida* sequence accession number indicate the start and end point coordinates of the locus.
B. Sequence comparison of *E. coli* pro-MccB17 with a homologue from *P. putida* and trifolitoxin precursor. Di- and tripeptides that are modified by the McbBCD synthetase in *E. coli* pro-MccB17 and likely modification sites in the *P. putida* peptide are highlighted in red. The proteolysis sites in *E. coli* pro-MccB17 and trifolitoxin are indicated by arrows.

translation (Metlitskaya *et al.*, 2006). Unprocessed MccC7-MccC51 does not have any effect on the aminoacylation reaction. Conversely, processed MccC7-MccC51, while active against aspartyl–tRNA synthetase *in vitro*, does not have any effect on MccC7-MccC51-sensitive cells. However, overproduction of aspartyl–tRNA synthetase protects cells from MccC7-MccC51, proving that aspartyl–tRNA synthetase is indeed the target of the drug. Thus, MccC7-MccC51 is a Trojan-horse inhibitor: the peptide moiety allows MccC7-MccC51 to enter sensitive cells, where an as-yet-unidentified peptidase(s) performs a suicidal act of MccC7-MccC51 processing that liberates the inhibitory aminoacyl-nucleotide part of the drug.

The mechanism of the MccC7-MccC51 action made it attractive to try to generate derivatives with altered C-terminal amino acids in the peptide part, for such compounds should target aminoacyl–tRNA synthetases other than the aspartyl–tRNA synthetase targeted by the wild-type MccC7-MccC51. However, saturating mutagenesis of the *mccA* gene failed to generate mature MccC7-MccC51 with substitutions in the last position (Kazakov *et al.*, 2007). Even an Asn^7 to Asp substitution that introduces an amino acid found in mature MccC7-MccC51, instead of genetically encoded Asn, prevented production of mature microcin. The result might indicate that the amide group of the Asn^7 side-chain is incorporated into the modified nucleotide moiety of MccC7-MccC51 to become part of either the phosphoramidate linkage or the propylamine group.

The *mcc* genes are transcribed from a single promoter (Fomenko *et al.*, 2003). The first gene, *mccA*, encodes the pro-MccC7-MccC51 heptapeptide. The second gene, *mccB*, is homologous to E1 ubiquitin-activating enzymes that covalently attach AMP to proteins. Therefore, MccB might attach AMP to pro-MccC7-MccC51. The product of *mccD* is homologous to protein methylases. Cells lacking functional *mccD* produce antibiotically active derivative that, according to mass spectrometry, lacks the aminopropyl group present in MccC7-MccC51 (Fomenko *et al.*, 2003; T. Kazakov and K. Severinov, unpubl. obs.). Thus, MccD is responsible for the transfer of propylamine on the phosphate group of modified pro-MccC7-MccC51 synthesized by MccB. MccE is a two-domain protein, with the N-terminal domain being similar to decarboxylases, while the C-terminal domain being similar to RimI, an acetylase of ribosomal proteins. The *mccE* gene appears to be required for both the production of mature MccC and immunity towards it (Fomenko *et al.*, 2003). The mechanisms of MccE action in either of these processes remain undefined. However, none of the MccE functions are critical because *E. coli* cells with disrupted *mccE* produce an

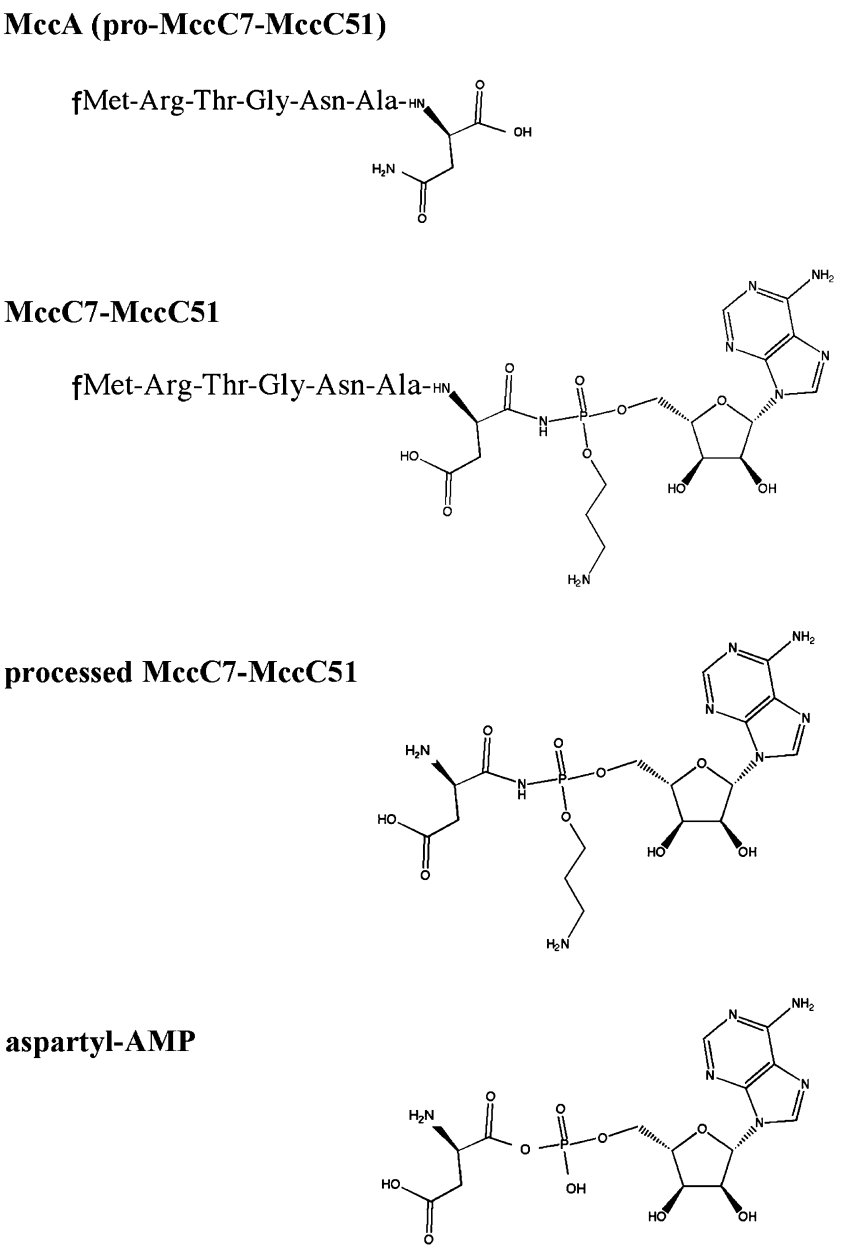


Fig. 5. Maturation, structure and processing of MccC7-MccC51, a peptide-nucleotide. The amino acid sequence of pro-MccC7-MccC51, the product of the *mccA* gene, is presented at the top in three-letter code, with the chemical structure of the terminal amino acid, Asn, shown. Mature MccC7-MccC51, whose peptide moiety contains a terminal Asp (chemical structure shown), and processed MccC7-MccC51 are shown below. Aspartyl-adenylate, an intermediate of the aminoacylation reaction catalysed by aspartyl–tRNA synthetase, is shown at the very bottom.

MccC7-MccC51 derivative that is antibiotically active (Fomenko *et al.*, 2003, T. Kazakov and K. Severinov, unpubl. obs.). Thus, *mccB* and *mccC* are the only two genes required for MccC7-MccC51 production and immunity.

Paired genes encoding *mccB* and *mccC* homologues could be identified in several organisms (Fig. 6A). In some cases, the *mccBC* pair is followed by additional genes homologous to other known MccC7-MccC51 maturation genes (*mccDEF* in *Yersinia pseudotuberculosis*, *mccDE* in *Synechococcus* sp. CC9605). In both of these organisms, the two domains of the MccE homologue are encoded by separate genes (Fig. 6A). The clustering of essential MccC7-MccC51 maturation (*mccB*) and immunity (*mccC*) genes suggests that genes coding for pro-MccC7-MccC51 might be located nearby. Indeed, analysis of upstream sequences reveals appropriately positioned ORFs encoding, in most cases, heptapeptides with terminal asparagines (Fig. 6B). The candidate *mccA* genes are preceded by plausible Shine-Dalgarno sequences and, in most cases, are separated from the *mccB* homologue by ~100 bp of non-coding DNA that contains putative transcription terminators, as is also the case with the *E. coli mcc* genes. In all cases, putative pro-MccC7-MccC51 heptapeptides contain residues (italicized in Fig. 6B) that, when introduced in *E. coli* pro-MccC7-MccC51 as

A

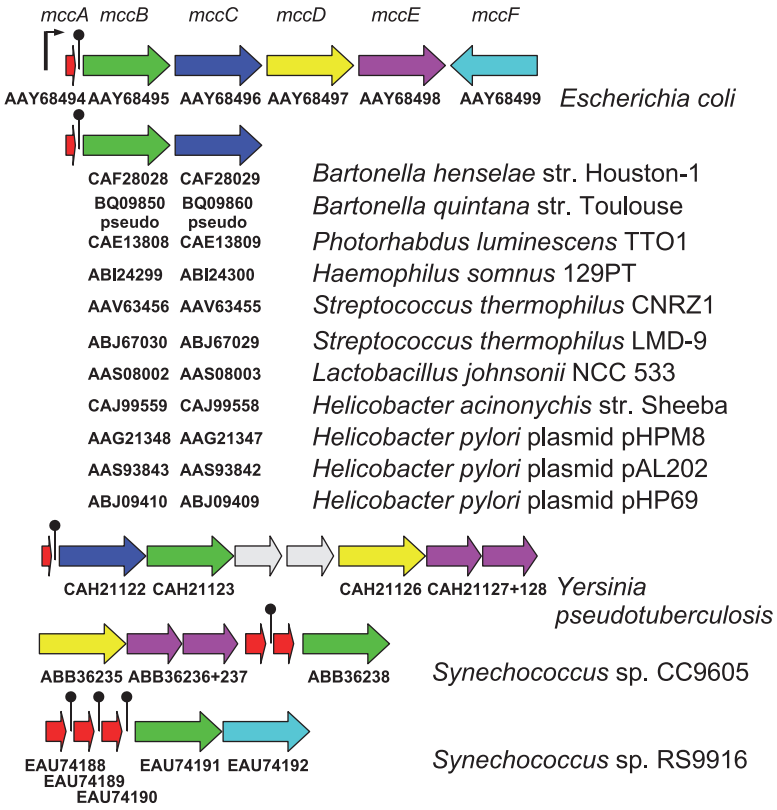


Fig. 6. Evolutionary conservation of gene clusters coding for possible MccC7-Mcc51 homologues.
A. Genetic organization of predicted *mcc* loci. Block arrows denote genes, with arrows pointing the directions of transcription. Homologous genes are indicated by matching colours. A thin arrow above the representation of *E. coli mcc* genes indicates a promoter. Putative intrinsic transcription terminators are also indicated. Accession numbers for proteins sequences from the NCBI Entrez database are indicated for each genome. For pseudogenes in the *B. quintana* genome, Entrez gene identifiers are given. The *mccF* gene is found in the MccC7 gene cluster (González-Pastor *et al*., 1995) but not in the otherwise virtually identical MccC51 cluster (Fomenko *et al*., 2003).
B. Candidate pro-MccC7-MccC51 peptides. The *E. coli* pro-MccC7-MccC51 sequence is shown at the top. Candidate pro-MccC7-MccC51 peptides and their origins are listed below. Accession numbers for proteins (where available) or nucleotide sequences from the NCBI Entrez database are indicated for each genome. Numbers after nucleotide sequence accession numbers refer to the start and end co-ordinates of a coding segment; 'compl' indicates reverse direction of translation. In candidate pro-MccC7-MccC51 heptapeptides, amino acids that, when introduced into corresponding positions of *E. coli* pro-MccC51, abolish maturation are italicized.

B

Genome	MccA sequence	Sequence reference
Escherichia coli	MRTGNAN*	AAY68494
Bartonella quintana str. Toulouse	*MDHIGFN**	NC_005955:1161818..1161841
Bartonella henselae str. Houston-1	MDYRNYNA*	NC_005956:1386616..1386642
Photorhabdus luminescens TTO1	*MISSGSN**	NC_005126:compl:1815670..1815693
Haemophilus somnus 129PT	*MRGRRIN**	NC_008309:18910..18933
Streptococcus thermophilus CNRZ-1	*MKGTILN**	NC_006449:compl:1720776..1720799
Streptococcus thermophilus LMD-9	*MKGTILN**	NC_008532:compl:1778422..1778445
Lactobacillus johnsonii NCC 533	*MHRIMKN**	NC_005362:25488..25511
Helicobacter acinonychis str. Sheeba	*MKLSYRN**	NC_008229:compl:666577..666600
Helicobacter pylori plasmid pHPM8	*MKLSYRN**	NC_004845:compl:5661..5684
Helicobacter pylori plasmid pAL202	*MKLSYRN**	NC_005917:compl:7024..7047
Helicobacter pylori plasmid pHP69	*MKLSYRN**	DQ915941:compl:5231..5254
Yersinia pseudotuberculosis	MYQVGIILSIKCN*	NC_006155:2224961..2225002
Synechococcus sp. RS9916 (3 copies)	MKNLNHELTEKELQSIAGGFRA LSFSRRSSKLQAPRLLQSTKLK VAPKASIWQDMMN*	EAU74188, EAU74189, EAU74190
Synechococcus sp. CC9605 (2 copies)	MTQPNDRQLSNEELSDVAAGLF RRTFFKPRTSRKTLLQPKRLDK VAKNQLWADMMN*	NC_007516:2313861..2314031, 2314244..2314414

point substitutions, disrupt MccC7-MccC51 maturation (Kazakov *et al*., 2007). Thus, either the candidate pro-MccC7-MccC51 peptides co-evolved with cognate maturation proteins, or multiple substitutions compensate each other. It is noteworthy that with one exception (see below), no plausible pro-MccC7-MccC51 heptapeptides with terminal encoded residues other than Asn were found. This agrees well with the results of structure–activity analysis of *E. coli* MccC7-MccC51, which show that the C-terminal Asn is the only residue that allows mature MccC7-MccC51 production (Kazakov *et al*., 2007).

In *Bartonella quintana*, an *mccA*-like ORF (MDHIGFN) is located upstream of non-functional *mccB* and *mccC* homologues each disrupted by stop codons and frameshifting deletions. In closely related *Bartonella henselae*, the *mccB* and *mccC* homologues are intact, but the candidate *mccA* ORF encodes an octapeptide MDYRNINA. A trivial explanation of this observation is that *mccA* of *B. henselae* is not functional and thus the *mcc* cluster is inactivated in both *Bartonella* species. A more interesting possibility is that the C-terminal alanine of the pro-MccC7-MccC51 octapeptide is removed prior to maturation or that *B. henselae* MccC7-MccC51 targets alanyl–tRNA synthetase.

In *Synechococcus* spp. CC9605 and RS9916, and in *Y. pseudotuberculosis*, no candidate *mccA* heptapeptide ORFs could be found upstream of apparently functional

mccBCE clusters. In *Y. pseudotuberculosis*, a 13-codon-long ORF with a terminal Asn codon may code for a pro-MccC7-MccC51-like polypeptide (Fig. 6B). In *Synechococci*, two (CC9605) and three (RS9916) direct repeats, each encoding 56- to 57-amino-acid polypeptides, are present in front of the *mcc*-biosynthesis/resistance genes homologues (Fig. 6). The ORFs are sufficiently similar between the two *Synechococcus* strains to infer homology. Notably, the C-terminal amino acids of these peptides are asparagines, and the C-terminal heptapeptides are almost identical between the two species: (L/I)W(*A/Q*)DMMN, suggesting that the products of these ORFs might be subject to modification by MccB homologues and act as microcins.

Concluding remarks

With the exception of MccB17, the first post-translationally modified microcin to be studied by modern biochemical methods, research conducted in recent years has increased our understanding of post-translationally modified microcin structure and function. Bioinformatic analyses indicate that peptides structurally similar to low-molecular-weight post-translationally modified microcins first identified in *E. coli* might be produced by a variety of bacteria. Future research should illuminate the biochemical and physiological functions of these compounds. Bioinformatics will also be helpful in establishing structure–activity relationship of these interesting molecules.

Notes

In two recent articles, Duquesne *et al*., 2007 and Clarke *et al.* (2007) report the establishment of an in vitro maturation systems for MccJ25 that opens the way for future mechanistic analysis of threaded-lasso peptide generation.

Acknowledgements

This work was supported by NIH R01 Grant GM64530, Northeast Biodefense Center Research Center of Excellence in Biodefense and Infectious Diseases development grant, and Russian Academy of Sciences grant (program “Cellular and Molecular Biology”) (to K.S.) and Russian Foundation for Basic Research Grant 06-04-48865. A.K. and M.S.G. were partially supported by grants from the Howard Hughes Medical Institute (55001056), INTAS (05–8028) and the Russian Academy of Sciences (programme ‘Molecular and Cellular Biology’).

References

Adelman, K., Yuzenkova, J., La Porta, A., Zenkin, N., Lee, J., Lis, J.T., *et al.* (2004) Molecular mechanism of transcription inhibition by peptide antibiotic Microcin J25. *Mol Cell* **14:** 753–762.

Allali, N., Afif, H., Couturier, M., and Van Melderen, L. (2002) The highly conserved TldD and TldE proteins of *Escherichia coli* are involved in Microcin B17 processing and in CCdA degradation. *J Bacteriol* **184:** 3224–3231.

Baquero, F., Bouanchaud, D., Martinez-Perez, M.C., and Fernandez, C. (1978) Microcin plasmids: a group of extrachromosomal elements coding for low-molecular-weight antibiotics in *Escherichia coli*. *J Bacteriol* **135:** 342–347.

Bayro, M.J., Mukhopadhyay, J., Swapna, G.V., Huang, J.Y., Ma, L.C., Sineva, E., *et al.* (2003) Structure of antibacterial peptide microcin J25: a 21-residue lariat protoknot. *J Am Chem Soc* **125:** 12382–12383.

Belshaw, P.J., Roy, R.S., Kelleher, N.L., and Walsh, C.T. (1998) Kinetics and regioselectivity of peptide-to-heterocycle conversions by microcin B17 synthetase. *Chem Biol* **5:** 373–384.

Blond, A., Péduzzi, J., Goulard, C., Chiuchiolo, M.J., Barthélémy, M., Prigent, Y., *et al.* (1999) The cyclic structure of microcin J25, a 21-residue peptide antibiotic from *Escherichia coli*. *Eur J Biochem* **259:** 747–755.

Bohannon, D.E., Connell, N., Keener, J., Tormo, A., Espinosa-Urgel, M., Zambrano, M.M., and Kolter, R. (1991) Stationary-phase-inducible ‘gearbox’ promoters: differential effects of *katF* mutations and role of sigma 70. *J Bacteriol* **173:** 4482–4492.

Borukhov, S., Lee, J., and Laptenko, O. (2005) Bacterial transcription elongation factors: new insights into molecular mechanism of action. *Mol Microbiol* **55:** 1315–1324.

del Castillo, F.J., del Castillo, I., and Moreno, F. (2001) Construction and characterization of mutations at codon 751 of the *Escherichia coli gyrB* gene that confer resistance to the antimicrobial peptide microcin B17 and alter the activity of DNA gyrase. *J Bacteriol* **183:** 2137–2140.

del Castillo, I., Goméz, J.M., and Moreno, F. (1990) *mprA*, an *Escherichia coli* gene that reduces growth-phase-dependent synthesis of microcins B17 and C7 and blocks osmoinduction of *proU* when cloned on a high-copy-number plasmid. *J Bacteriol* **172:** 437–445.

Chiuchiolo, M.J., Delgado, M.A., Farías, R.N., and Salomón, R.A. (2001) Growth-phase-dependent expression of the cyclopeptide antibiotic microcin J25. *J Bacteriol* **183:** 1755–1764.

Chokekijchai, S., Kojima, E., Anderson, S., Nomizu, M., Tanaka, M., Machida, M., *et al.* (1995) NP-06: a novel anti-human immunodeficiency virus polypeptide produced by a *Streptomyces* species. *Antimicrob Agents Chemother* **39:** 2345–2347.

Clarke, D.J., and Campopiano, D.J. (2007) Maturation of McjA precursor peptide into active MccJ25. *Org Biomol Chem* **5:** 2564–2566.

Connell, N., Han, Z., Moreno, F., and Kolter, R. (1987) An *E. coli* promoter induced by the cessation of growth. *Mol Microbiol* **1:** 195–201.

Cristóbal, R.E., Solbiati, J.O., Zenoff, A.M., Vincent, P.A., Salomón, R.A., Yuzenkova, J., *et al.* (2006) Microcin J25 uptake: His5 of the MccJ25 lariat ring is involved in interaction with the inner membrane MccJ25 transporter protein SbmA. *J Bacteriol* **188:** 3324–3328.

Delgado, M.A., Rintoul, M.R., Farías, R.N., and Salomón, R.A. (2001) *Escherichia coli* RNA polymerase is the target

of the cyclopeptide antibiotic microcin J25. *J Bacteriol* **183:** 4543–4550.

Destoumieux-Garzón, D., Duquesne, S., Péduzzi, J., Goulard, C., Desmadril, M., Letellier, L., *et al.* (2005) The iron-siderophore transporter FhuA is the receptor for the antimicrobial peptide microcin J25: role of the microcin Val[11]-Pro[16] β-hairpin region in the recognition mechanism. *Biochem J* **389:** 859–876.

Duquesne, S., Destoumieux-Garzon, D., Zirah, S., Goulard, C., Peduzzi, J., and Rebuffat, S. (2007) Two Enzymes Catalyze the Maturation of a Lasso Peptide in Escherichia coli. *Chem Biol* **14:** 793–803.

Esumi, Y., Suzuki, Y., Itoh, Y., Uramoto, M., Kimura, K., Goto, M., *et al.* (2002) Propeptin, a new inhibitor of prolyl endopeptidase produced by microbispora II. Determination of chemical structure. *J Antibiot (Tokyo)* **55:** 296–300.

Felsenstein, J. (1996) Inferring phylogenies from protein sequences by parsimony, distance, and likelihood methods. *Methods Enzymol* **266:** 418–427.

Fomenko, D., Veselovskii, A., and Khmel, I. (2001) Regulation of microcin C51 operon expression: the role of global regulators of transcription. *Res Microbiol* **152:** 469–479.

Fomenko, D.E., Metlitskaya, A.Z., Péduzzi, J., Goulard, C., Katrukha, G.S., Gening, L.V., *et al.* (2003) Microcin C51 plasmid genes: possible source of horizontal gene transfer. *Antimicrob Agents Chemother* **47:** 2868–2874.

Frechet, D., Guitton, J.D., Herman, F., Faucher, D., Helynck, G., Monegier du Sorbier, B., *et al.* (1994) Solution structure of RP 71955, a new 21 amino acid tricyclic peptide active against HIV-1 virus. *Biochemistry* **33:** 42–50.

Garrido, M.C., Herrero, M., Kolter, R., and Moreno, F. (1988) The export of the DNA replication inhibitor Microcin B17 provides immunity for the host cell. *EMBO J* **7:** 1853–1862.

Genilloud, O., Moreno, F., and Kolter, R. (1989) DNA sequence, products, and transcriptional pattern of the genes involved in production of the DNA replication inhibitor microcin B17. *J Bacteriol* **171:** 1126–1135.

González-Pastor, J.E., San Millán, J.L., and Moreno, F. (1994) The smallest known gene. *Nature* **369:** 281.

González-Pastor, J.E., San Millán, J.L., Castilla, M.A., and Moreno, F. (1995) Structure and organization of plasmid genes required to produce the translation inhibitor microcin C7. *J Bacteriol* **177:** 7131–7140.

Guijarro, J.I., González-Pastor, J.E., Baleux, F., San Millán, J.L., Castilla, M.A., Rico, M., *et al.* (1995) Chemical structure and translation inhibition studies of the antibiotic microcin C7. *J Biol Chem* **270:** 23520–23532.

Heddle, J.G., Blance, S.J., Zamble, D.B., Hollfelder, F., Miller, D.A., Wentzell, L.M., *et al.* (2001) The antibiotic microcin B17 is a DNA gyrase poison: characterisation of the mode of inhibition. *J Mol Biol* **307:** 1223–1234.

Hernández-Chico, C., San Millán, J.L., Kolter, R., and Moreno, F. (1986) Growth phase and *ompR* regulation of transcription of microcin B17 genes. *J Bacteriol* **167:** 1058–1065.

Herrero, M., Kolter, R., and Moreno, F. (1986) Effects of microcin B17 on microcin B17-immune cells. *J Gen Microbiol* **132:** 403–410.

Iwatsuki, M., Tomoda, H., Uchida, R., Gouda, H., Hirono, S., and Omura, S. (2006) Lariatins, antimycobacterial peptides produced by *Rhodococcus sp*. K01-B0171, have a lasso structure. *J Am Chem Soc* **128:** 7486–7491.

Katahira, R., Shibata, K., Yamasaki, M., Matsuda, Y., and Yoshida, M. (1995) Solution structure of endothelin B receptor selective antagonist RES-701-1 determined by ^{1}H NMR spectroscopy. *Bioorg Med Chem* **3:** 1273–1280.

Katahira, R., Yamasaki, M., Matsuda, Y., and Yoshida, M. (1996) MS-271, a novel inhibitor of calmodulin-activated myosin light chain kinase from *Streptomyces* sp. II. Solution structure of MS-271: characteristic features of the 'lasso' structure. *Bioorg Med Chem* **4:** 121–129.

Kazakov, T., Metlitskaya, A., and Severinov, K. (2007) Amino acid residues required for maturation, cell uptake, and processing of translation inhibitor Microcin C. *J Bacteriol* **189:** 2114–2118. December 8; [Epub ahead of print].

Kelleher, N.L., Hendrickson, C.L., and Walsh, C.T. (1999) Posttranslational heterocyclization of cysteine and serine residues in the antibiotic microcin B17: distributivity and directionality. *Biochemistry* **38:** 15623–15630.

Larsen, T.M., Boehlein, S.K., Schuster, S.M., Richards, N.G., Thoden, J.B., Holden, H.M., and Rayment, I. (1999) Three-dimensional structure of *Escherichia coli* asparagine synthetase B: a short journey from substrate to product. *Biochemistry* **38:** 16146–16157.

Li, Y.M., Milne, J.C., Madison, L.L., Kolter, R., and Walsh, C.T. (1996) From peptide precursors to oxazole and thiazole-containing peptide antibiotics: microcin B17 synthase. *Science* **274:** 1188–1193.

Madison, L.L., Vivas, E.I., Li, Y.M., Walsh, C.T., and Kolter, R. (1997) The leader peptide is essential for the post-translational modification of the DNA-gyrase inhibitor microcin B17. *Mol Microbiol* **23:** 161–168.

Makarova, K.S., Aravind, L., and Koonin, E.V. (1999) A superfamily of archaeal, bacterial, and eukaryotic proteins homologous to animal transglutaminases. *Protein Sci* **8:** 1714–1719.

Metlitskaya, A., Kazakov, T., Kommer, A., Pavlova, O., Praetorius-Ibba, M., Ibba, M., *et al.* (2006) Aspartyl-tRNA synthetase is the target of peptide nucleotide antibiotic Microcin C. *J Biol Chem* **281:** 18033–18042.

Miller, M.T., Bachmann, B.O., Townsend, C.A., and Rosenzweig, A.C. (2001) Structure of beta-lactam synthetase reveals how to synthesize antibiotics instead of asparagine. *Nat Struct Biol* **8:** 684–689.

Miller, M.T., Bachmann, B.O., Townsend, C.A., and Rosenzweig, A.C. (2002) The catalytic cycle of beta-lactam synthetase observed by x-ray crystallographic snapshots. *Proc Natl Acad Sci USA* **99:** 14752–14757.

Milne, J.C., Eliot, A.C., Kelleher, N.L., and Walsh, C.T. (1998) ATP/GTP hydrolysis is required for oxazole and thiazole biosynthesis in the peptide antibiotic microcin B17. *Biochemistry* **37:** 13250–13261.

Milne, J.C., Roy, R.S., Eliot, A.C., Kelleher, N.L., Wokhlu, A., Nickels, B., and Walsh, C.T. (1999) Cofactor requirements and reconstitution of microcin B17 synthetase: a multienzyme complex that catalyzes the formation of oxazoles and thiazoles in the antibiotic microcin B17. *Biochemistry* **38:** 4768–4781.

Moreno, F., González-Pastor, J.E., Baquero, M.-R., and Bravo, D. (2002) The regulation of microcin B, C and J operons. *Biochimie* **84:** 521–529.

Mukhopadhyay, J., Sineva, E., Knight, J., Levy, R.M., and Ebright, R.H. (2004) Antibacterial peptide microcin J25 inhibits transcription by binding within and obstructing the RNA polymerase secondary channel. *Mol Cell* **14:** 739–751.

Osterman, A., and Overbeek, R. (2003) Missing genes in metabolic pathways: a comparative genomics approach. *Curr Opin Chem Biol* **7:** 238–251.

Overbeek, R., Fonstein, M., D'Souza, M., Pusch, G.D., and Maltsev, N. (1999) The use of gene clusters to infer functional coupling. *Proc Natl Acad Sci USA* **96:** 2896–2901.

Parks, W.M., Botrill, A.R., Pierrat, O.A., Durrant, M.C., and Maxwell, A. (2007) The action of the bacterial toxin, microcin B17, on DNA gyrase. *Biochimie* **89:** 500–507.

Pierrat, O.A., and Maxwell, A. (2003) The action of the bacterial toxin microcin B17. Insight into the cleavage-religation reaction of DNA gyrase. *J Biol Chem* **278:** 35016–35023.

Pierrat, O.A., and Maxwell. A. (2005) Evidence for the role of DNA strand passage in the mechanism of action of microcin B17 on DNA gyrase. *Biochemistry* **44:** 4204–4215.

Potterat, O., Stephan, H., Metzger, J.W., Gnau, V., Zähner, H., and Jung, G. (1994) Aborycin – a tricyclic 21-peptide antibiotic isolated from *Streptomyces griseoflavus*. *Liebigs Ann Chem* **1994:** 741–743.

Roca, J., Berger, J.M., Harrison, S.C., and Wang, J.C. (1996) DNA transport by a type II topoisomerase: direct evidence for a two-gate mechanism. *Proc Natl Acad Sci USA* **93:** 4057–4062.

Rodríguez-Sáinz, M.C., Hernández-Chico, C., and Moreno, F. (1990) Molecular characterization of *pmbA*, an *Escherichia coli* chromosomal gene required for the production of the antibiotic peptide MccB17. *Mol Microbiol* **4:** 1921–1932.

Rosengren, K.J., Clark, R.J., Daly, N.L., Göransson, U., Jones, A., and Craik, D.J. (2003) Microcin J25 has a threaded sidechain-to-backbone ring structure and not a head-to-tail cyclized backbone. *J Am Chem Soc* **125:** 12464–12474.

Rosengren, K.J., Blond, A., Afonso, C., Tabet, J.C., Rebuffat, S., and Craik, D.J. (2004) Structure of thermolysin cleaved microcin J25: extreme stability of a two-chain antimicrobial peptide devoid of covalent links. *Biochemistry* **43:** 4696–4702.

Roy, R.S., Kim, S., Baleja, J.D., and Walsh, C.T. (1998a) Role of the microcin B17 propeptide in substrate recognition: solution structure and mutational analysis of McbA1–26. *Chem Biol* **5:** 217–228.

Roy, R.S., Belshaw, P.J., and Walsh, C.T. (1998b) Mutational analysis of posttranslational heterocycle biosynthesis in the gyrase inhibitor microcin B17: distance dependence from propeptide and tolerance for substitution in a GSCG cyclizable sequence. *Biochemistry* **37:** 4125–4136.

Roy, R.S., Kelleher, N.L., Milne, J.C., and Walsh, C.T. (1999a) In vivo processing and antibiotic activity of microcin B17 analogs with varying ring content and altered bisheterocyclic sites. *Chem Biol* **6:** 305–318.

Roy, R.S., Allen, O., and Walsh, C.T. (1999b) Expressed protein ligation to probe regiospecificity of heterocyclization in the peptide antibiotic microcin B17. *Chem Biol* **6:** 789–799.

Schneider, T.L., Shen, B., and Walsh, C.T. (2003) Oxidase domains in epothilone and bleomycin biosynthesis: thiazoline to thiazole oxidation during chain elongation. *Biochemistry* **42:** 9722–9730.

Scupham, A.J., and Triplett, E.W. (2006) Determination of the amino acid residues required for the activity of the antirhizobial peptide antibiotic trifolitoxin. *J Appl Microbiol* **100:** 500–507.

Semenova, E., Yuzenkova, Y., Péduzzi, J., Rebuffat, S., and Severinov, K. (2005) Structure-activity analysis of microcinJ25: distinct parts of the threaded lasso molecule are responsible for interaction with bacterial RNA polymerase. *J Bacteriol* **187:** 3859–3863.

Solbiati, J.O., Ciaccio, M., Farías, R.N., and Salomón, R.A. (1996) Genetic analysis of plasmid determinants for microcin J25 production and immunity. *J Bacteriol* **178:** 3661–3663.

Solbiati, J.O., Ciaccio, M., Farías, R.N., González-Pastor, J.E., Moreno, F., and Salomón, R.A. (1999) Sequence analysis of the four plasmid genes required to produce the circular peptide antibiotic microcin J25. *J Bacteriol* **181:** 2659–2662.

Vizan, J.L., Hernández-Chico, C., del Castillo, I., and Moreno, F. (1991) The peptide antibiotic microcin B17 induces double-strand cleavage of DNA mediated by *E. coli* DNA gyrase. *EMBO J* **10:** 467–476.

Wilson, K.A., Kalkum, M., Ottesen, J., Yuzenkova, J., Chait, B.T., Landick, R., *et al.* (2003) Structure of microcin J25, a peptide inhibitor of bacterial RNA polymerase, is a lassoed tail. *J Am Chem Soc* **125:** 12475–12483.

Wyss, D.F., Lahm, H.W., Manneberg, M., and Labhardt, A.M. (1991) Anantin – a peptide antagonist of the atrial natriuretic factor (ANF). II. Determination of the primary sequence by NMR on the basis of proton assignments. *J Antibiot (Tokyo)* **44:** 172–180.

Yee, V.C., Pedersen, L.C., Le Trong, I., Bishop, P.D., Stenkamp, R.E., and Teller, D.C. (1994) Three-dimensional structure of a transglutaminase: human blood coagulation factor XIII. *Proc Natl Acad Sci USA* **91:** 7296–7300.

Yorgey, P., Davagnino, J., and Kolter, R. (1993) The maturation pathway of microcin B17, a peptide inhibitor of DNA gyrase. *Mol Microbiol* **9:** 897–905.

Yorgey, P., Lee, J., Kordel, J., Vivas, E., Warner, P., Jebaratnam, D., and Kolter, R. (1994) Posttranslational modifications in microcin B17 define an additional class of DNA gyrase inhibitor. *Proc Natl Acad Sci USA* **91:** 4519–4523.

Yuzenkova, J., Delgado, M., Nechaev, S., Savalia, D., Epshtein, V., Artsimovitch, I., *et al.* (2002) Mutations of bacterial RNA polymerase leading to resistance to microcin J25. *J Biol Chem* **277:** 50867–50875.

Zamble, D.B., McClure, C.P., Penner-Hahn, J.E., and Walsh, C.T. (2000) The McbB component of microcin B17 synthetase is a zinc metalloprotein. *Biochemistry* **39:** 16190–16199.

Zamble, D.B., Miller, D.A., Heddle, J.G., Maxwell, A., Walsh, C.T., and Hollfelder, F. (2001) *In vitro* characterization of DNA gyrase inhibition by microcin B17 analogs with altered bisheterocyclic sites. *Proc Natl Acad Sci USA* **98:** 7712–7717.

Zhang, G., Campbell, E.A., Minakhin, L., Richter, C., Severinov, K., and Darst, S.A. (1999) Crystal structure of *Thermus aquaticus* core RNA polymerase at 3.3 Å resolution. *Cell* **98:** 811–824.

