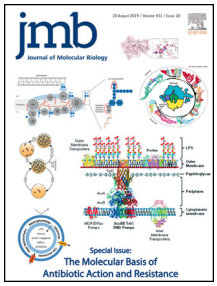
Review



The Microbial Toxin Microcin B17: Prospects for the Development of New Antibacterial Agents

Frederic Collin and Anthony Maxwell

Department Biological Chemistry, *John Innes Centre, Norwich Research Park, Norwich NR4 7UH, UK*

Correspondence to Anthony Maxwell: *tony.maxwell@jic.ac.uk*
https://doi.org/10.1016/j.jmb.2019.05.050
Edited by Christopher G. Dowson

Abstract

Microcin B17 (MccB17) is an antibacterial peptide produced by strains of *Escherichia coli* harboring the plasmid-borne *mccB17* operon. MccB17 possesses many notable features. It is able to stabilize the transient DNA gyrase–DNA cleavage complex, a very efficient mode of action shared with the highly successful fluoroquinolone drugs. MccB17 stabilizes this complex by a distinct mechanism making it potentially valuable in the fight against bacterial antibiotic resistance. MccB17 was the first compound discovered from the thiazole/oxazole-modified microcins family and the linear azole-containing peptides; these ribosomal peptides are post-translationally modified to convert serine and cysteine residues into oxazole and thiazole rings. These chemical moieties are found in many other bioactive compounds like the vitamin thiamine, the anti-cancer drug bleomycin, the antibacterial sulfathiazole and the antiviral nitazoxanide. Therefore, the biosynthetic machinery that produces these azole rings is noteworthy as a general method to create bioactive compounds. Our knowledge of MccB17 now extends to many aspects of antibacterial–bacteria interactions: production, transport, interaction with its target, and resistance mechanisms; this knowledge has wide potential applicability. After a long time with limited progress on MccB17, recent publications have addressed critical aspects of MccB17 biosynthesis as well as an explosion in the discovery of new related compounds in the thiazole/oxazole-modified microcins/linear azole-containing peptides family. It is therefore timely to summarize the evidence gathered over more than 40 years about this still enigmatic molecule and place it in the wider context of antibacterials.

Introduction

In 2017, the World Health Organisation warned that "The world is running out antibiotics," highlighting again the need for new antibacterials to fight bacterial disease and circumvent the worrying extent of antimicrobial-resistant infections [World Health Organisation Report: Antibacterial agents in clinical development (2017)]. Against this backdrop, fewer pharmaceutical companies are carrying out research and development in the antibiotic area [1,2]. Fishing for new targets using large-compound library screening and genomic methods has not yielded the expected results in terms of new clinically relevant antibiotics or new targets [3,4]. In the last 50 years or so, only two new classes of antibiotics have been brought to market [5]. Most successful antibiotics work on only a few validated targets, which are linked to essential bacterial functions [6]. Examples include the following: cell wall synthesis [penicillins (Amoxicillin), carbapenems and cephalosporins (Cephalexin)], cell membrane stability (daptomycin), protein synthesis by targeting the 30S ribosomal subunit [tetracyclines (Doxycycline)], the 50S ribosomal subunit [macrolides (Azithromycin) and chloramphenicol], specific biosynthetic pathways like folate synthesis, (trimethoprim/sulfamethoxazole drug combination), RNA synthesis by targeting RNA polymerase [rifamycins (Rifampicin)], DNA synthesis by targeting DNA gyrase/DNA topoisomerase IV [quinolones (Ciprofloxacin, or CFX)]. Exploiting these validated targets by novel mechanisms might provide a solution to the limited numbers of novel antibacterial

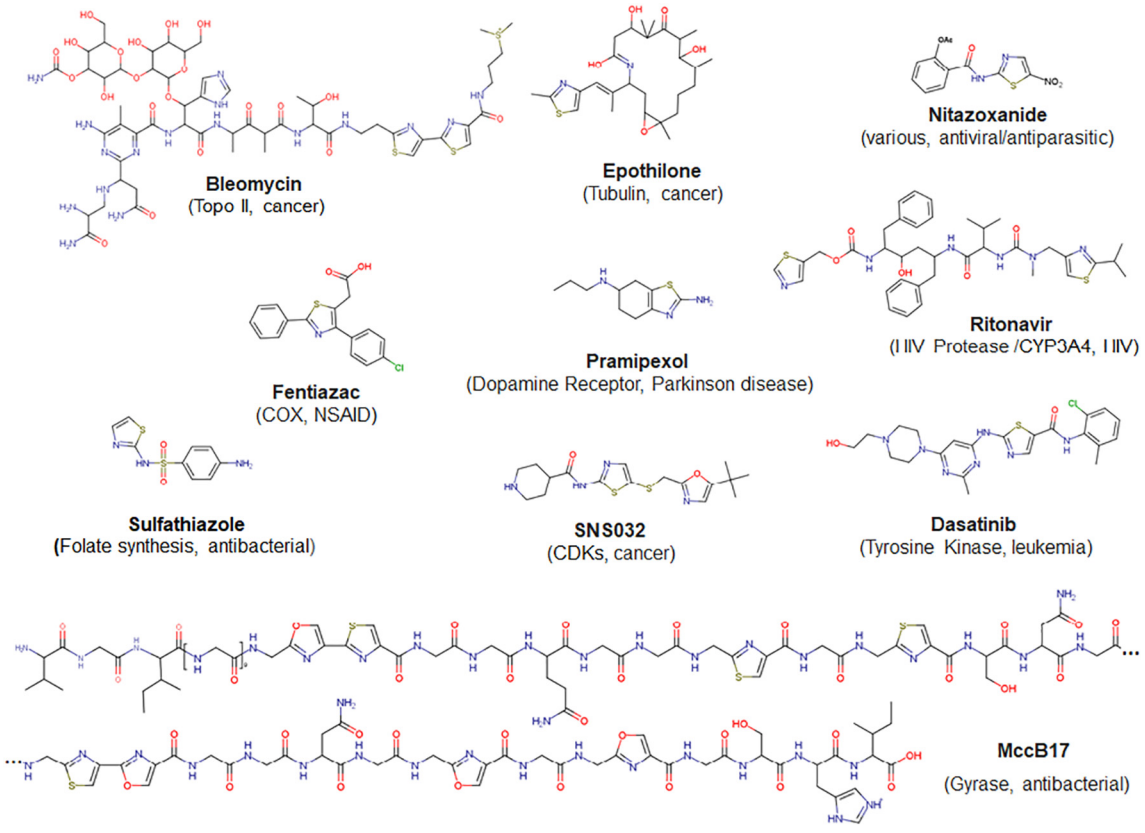


Fig. 1. Oxazole- and thiazole-containing bioactive molecules: drugs containing oxazole and/or thiazole ring; the target and the indication are indicated in brackets. COX, cyclooxygenase; NSAID, nonsteroidal anti-inflammatory drug; CYP3A4, cytochrome P450 3A4; CDK, cyclin-dependent kinase.

drugs in the pipeline and the issue of antibacterial resistance.

Among the current antibiotics, quinolones have a special place. These compounds inhibit bacterial type II topoisomerases: gyrase and topo IV, but have additionally an unusual mode of action that make them particularly effective. Quinolones not only inhibit the regulation of DNA topology in the cell, blocking DNA replication and transcription, but they also generate double-strand-breaks, which are recognized as toxic lesions [7,8]. This is a very efficient mode of action as a small number of events trigger a chain of reactions that will kill the cell. This has led us to refer to quinolones as "topoisomerase poisons."

Microcin B17 (MccB17; Fig. 1) is one of the few other known bacterial topoisomerase poisons. This antibacterial peptide targets bacterial gyrase with a mode of action related to but distinct from quinolones, thus offering potential new inspiration for the design of antibacterial drugs. MccB17 is a bacteriocin, that is, a peptidic toxin produced by bacteria to inhibit the growth of similar bacterial strains. It is more specifically a microcin, having a molecular mass <10 kDa, distinguishing it from the larger colicins [9]. MccB17 is also an archetypal member of the thiazole–oxazole-modified microcins (TOMMs) family and the linear azole-containing peptides (LAPs) (Fig. 2) [10].

MccB17 is produced by strains of *Escherichia coli* carrying the pmcb plasmid, which contains an operon encoding seven proteins: McbA, McbB, McbC, McbD, McbE, McbF and McbG (Fig. 3). The *mcbA* gene is the structural gene, *mcbBCD* encode the components of MccB17 synthase that post-translationally modifies McbA to introduce the thiazole and oxazole moieties in the peptide backbone. The genes *mcbE, mcbF* and *mcbG* are responsible for export and immunity. The machinery responsible for MccB17 biosynthesis illustrates many general concepts valid to the wider TOMMs and LAPs families such as the organization of the operon, the nature of the synthase(s) that will modify the precursor peptide, and the role of the leader peptide. In addition, MccB17 illustrates various molecular aspects of antibiotic activity: transport, drug–target interaction and structure–activity relationships, resistance mechanism by export, target mutation or protective molecules. The mode of action of MccB17 shares many similarities with quinolones allowing parallels relating to topoisomerase targeting and resistance. MccB17 is an inhibitor with relatively modest activity on gyrase [11] but is, like quinolones,

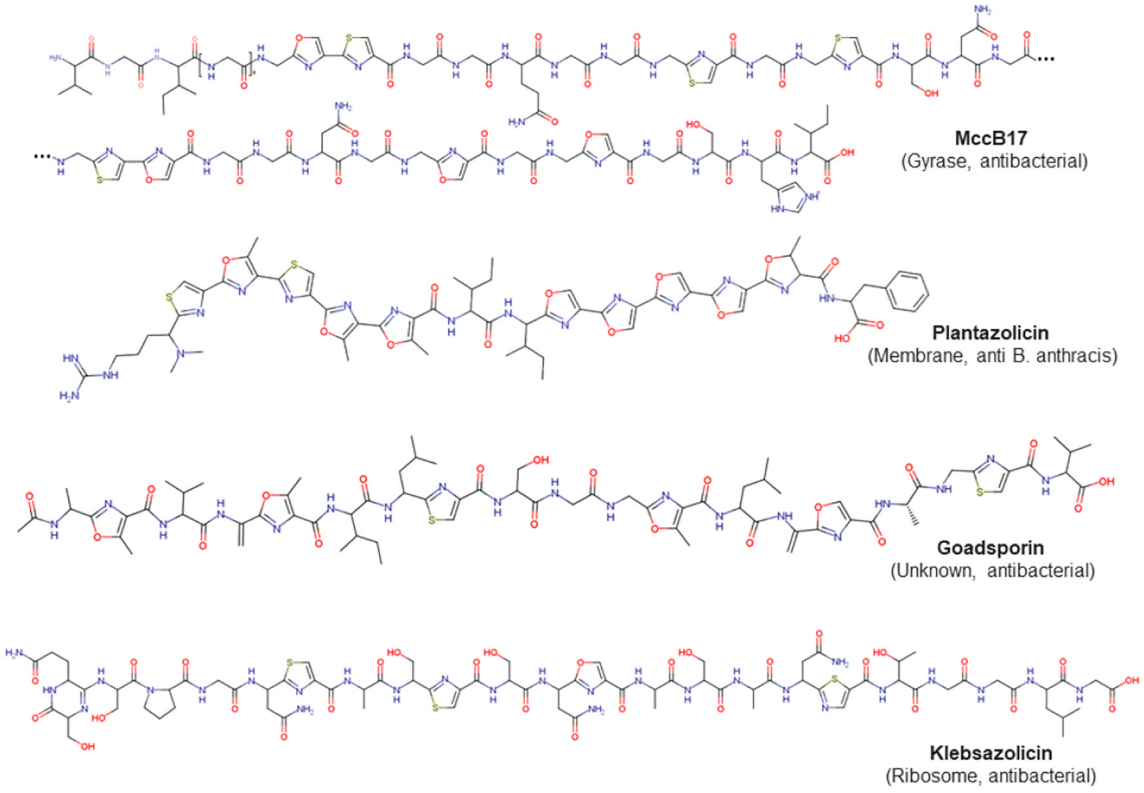


Fig. 2. MccB17 and other LAPs.

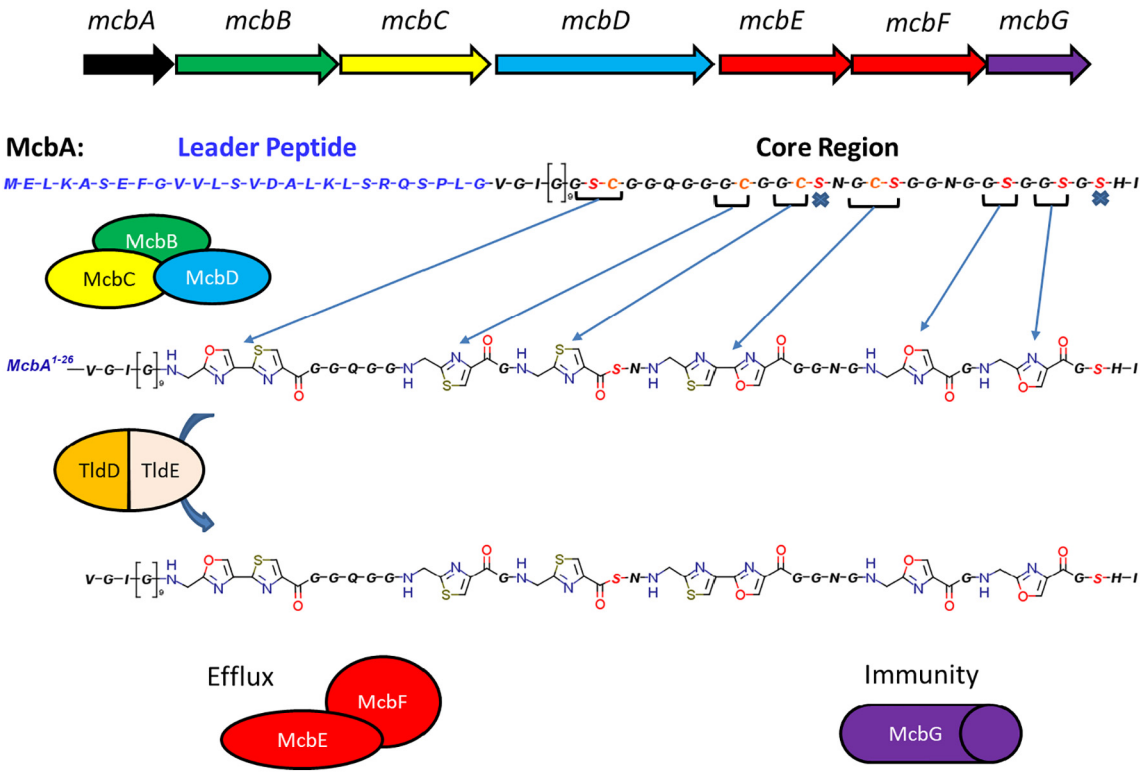


Fig. 3. The MccB17 gene cluster, showing the McbA gene product and how it is processed by the microcin synthase (McbBCD) and protease (TldD/E). McbE and McbF control efflux, and McbG is responsible for immunity.

able to stabilize a transient gyrase–DNA covalent complex, an event that leads to the generation of double-strand DNA breaks that are lethal to the cell. The exact mechanism of MccB17 action is still unknown but is likely to be distinct from that of the quinolones. MccB17 per se is not an easily druggable molecule; however, solving the molecular details of its mechanism to stabilize the gyrase–DNA complex would be invaluable in the quest for novel antibacterial compounds.

In the following paragraphs, we summarize the knowledge accumulated on MccB17 over more than 40 years or so and its relevance in the field of antibacterials with an emphasis on three major aspects: thiazole–oxazole-modified peptide molecules and their biosynthetic machinery as tools for generating novel bioactive molecules; MccB17 as a gyrase poison, structure–activity and potential for novel antibacterial drugs; and MccB17 transport and resistance as a universal concept of antibacterial drugs.

Discovery of MccB17

A systematic search for compounds produced by enteric bacteria with antibacterial activity against *E. coli* using a soft agar double-layer technique with a cellophane film on the surface led to the identification of series of novel compounds. These compounds, having molecular weights of about 1 kDa, thermostability and solubility in 16% methanol, were not part of the bacteriocin family and were therefore grouped as a new family named microcins [12]. MccB17, a highly bactericidal compound, was among them. It was named microcin 17w at the time as it was produced by strain no. 17 and was co-isolated with a closely related compound of MW <1000 named microcin 17e, which was not pursued in later publications. Microcin 17w was shown not to affect RNA or protein synthesis but specifically to inhibit DNA replication leading to DNA degradation and induction of the SOS response, which placed it in the class B microcins (inhibitors of DNA replication) giving it the final name of MccB17. The screen included evaluation of stability and study of the spectrum of activity, which identified the following properties: soluble in methanol, insoluble in ethanol, not absorbed on activated charcoal, loss of activity after exposition for 3 h at pH <1 or >12, stable for 30 min at 100 °C, sensitive to proteolysis by pronase, subtilisin and thermolisin, resistant to trypsin and chemotrypsin. The compound displayed antibacterial activity on some strains of *E. coli*, *Enterobacter*, *Pseudomonas* and *Shigella* [12].

MccB17 Structure

MccB17 is a 43-residue peptide containing oxazole and thiazole heterocycles (Fig. 3). The MccB17 precursor is synthesized by the ribosome as McbA [13], a 69-residue peptide, with the 26 N-terminal residues forming a leader peptide and the 43 C-terminal residues forming a glycine-rich domain that is the MccB17 precursor sequence [13]. Four serines and four cysteines in the 43-residue domain are post-translationally converted, respectively, to oxazole and thiazole heterocycles to form pro-MccB17 [14,15]. Removal of the leader peptide by an endogenous protease generates MccB17 [16]. These modifications are essential for the antibacterial activity of MccB17. MccB17's structure was confirmed by NMR [14,15] and mass spectrometry (MS) [17,18].

Mode of Action

MccB17 was initially identified by its ability to inhibit DNA replication leading to the induction of the SOS system, DNA degradation and cell death [19]. When incubated in a cell-free extract, MccB17 induces double-strand cleavage of plasmid DNA. *E. coli* gyrase was identified as the putative target after mutation of *E. coli* GyrB W751 was linked to resistance to MccB17 [20]. This was further confirmed by biochemical assays. MccB17 was shown to inhibit DNA supercoiling by gyrase [21,22] and to induce DNA cleavage, under appropriate conditions [23]. Gyrase containing the GyrB W751 mutation shows very little DNA cleavage in the presence of MccB17, strongly implicating this residue (Trp751) in interaction with the toxin [23].

The DNA gyrase reaction cycle

As the action of MccB17 interferes with the reaction cycle of DNA gyrase, it is important to describe the mechanism of supercoiling by gyrase [8,24]. The enzyme consists of two subunits, GyrA and GyrB (97 kDa and 90 kDa, respectively, in *E. coli*), which form an A_2B_2 complex with DNA. This complex involves wrapping of a segment of DNA of about 130 bp around the enzyme (Fig. 4). This positions a piece of DNA, the “G” or “gate” segment, at the DNA cleavage active site, comprising residues from both GyrA and GyrB. Another segment, the T or “transported” segment, is poised for capture by the ATPase domains (N-terminal) of GyrB that form the N-Gate. Cleavage of the G segment occurs in both strands with a 4-base stagger and is effected by a tyrosine residue (Y122 in the case of *E. coli* gyrase), which makes a covalent bond, a 5′-phosphotyrosyl linkage, to the broken DNA. In supercoiling, the binding of ATP initiates conformational changes that result in dimerization of the GyrB N-terminal domains and the passage of the T segment through the G segment (strand passage; Fig. 4). ATP hydrolysis completes the reaction cycle and allows resetting of the enzyme. Each cycle leads to the introduction of

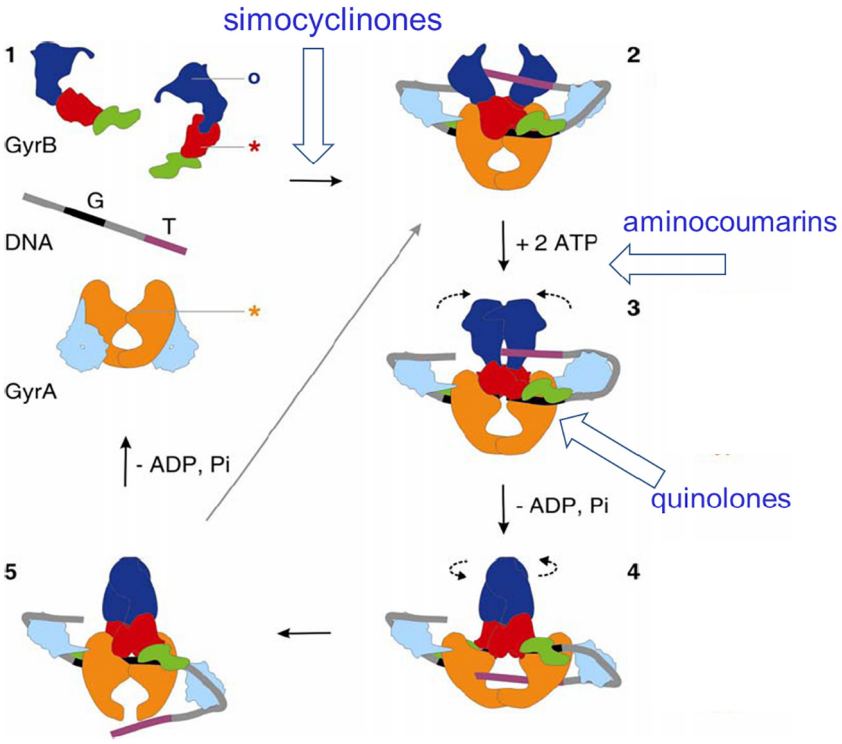


Fig. 4. DNA gyrase catalytic cycle showing points at which inhibitors interact. The GyrA dimer and the GyrB monomers bind to DNA as an A_2B_2 complex. DNA wrapped around the C-terminal domains of GyrA presents the T (transported) segment over the G (gate) segment. The T segment is captured by closure of the N-gate (N-terminal domains of GyrB). Cleavage of the G segment and passage of the T segment through the G segment leads to the introduction of two negative supercoils. Catalysis requires the binding and hydrolysis of 2 molecules of ATP. The sites of action of three antibiotics are shown: simocyclinones prevent the binding of DNA; aminocoumarins prevent the binding of ATP; quinolones interrupt the DNA breakage-reunion cycle by gyrase. Stars indicate the active-site region for DNA cleavage, and the circle indicates the ATP-binding pocket. Adapted from Costenaro and Maxwell [24] with permission.

two negative supercoils into the DNA, that is, the reduction of the linking number of the DNA by two. Supercoiling can be processive, involving multiple strand-passage cycles without dissociation of the enzyme, or distributive. In the absence of ATP, gyrase can relax DNA, albeit more slowly, by carrying out reverse strand passage, where the T segment enters the enzyme from the "exit gate" (Fig. 4) in GyrA and passes through the DNA gate from the other direction [25].

From the foregoing description and Fig. 4, it is clear that the gyrase topoisomerase reactions are very dynamic and involve several conformational changes. This presents opportunities for ligand binding, and a large number of compounds are known to target gyrase and inhibit the supercoiling/relaxation reactions (Fig. 4), these include quinolones, aminocoumarins and simocyclinones. MccB17 also interrupts the gyrase reaction cycle, but exactly how it does so is currently not entirely clear.

Effect of MccB17 on DNA gyrase activity

Initially, no inhibition of gyrase supercoiling of relaxed DNA by MccB17 could be observed in reactions carried out at 37 °C [23]. However, in later work in reactions at 25 °C, it was shown that MccB17 slowed down both the gyrase supercoiling and relaxation reactions by a factor ~3 [22]. Taken together, these data suggest that MccB17 does not block the topoisomerase reaction of gyrase but seems to moderately slow down strand passage. By itself, this would not appear to be a lethal mode of action.

MccB17 and DNA cleavage

A critical step during the supercoiling cycle is the cleavage of both strands of DNA by gyrase (Fig. 4). Quinolone drugs, such as CFX, can arrest the reaction at this step. The stabilization of the cleavage complex is thought to be the cause of lethality of the quinolones [7]. *In vitro*, quinolones can stabilize the cleavage complex with linear DNA substrates as small as 20 bp in length [26]. MccB17 can also stabilize the cleavage complex, but the reaction shows certain differences from CFX. At a given enzyme concentration, MccB17 shows less cleavage than CFX [23]. Originally, the cleavage reaction was thought to be ATP-dependent [23], but later work showed that cleavage also occurs without ATP, albeit at a much lower level [22]. MccB17 requires linear DNA of more than 150 bp [27] in contrast to the short fragments that can be cleaved in the presence of CFX [26]. This length of DNA allows for the formation of the DNA gate, wrapping of the DNA around gyrase and the placement of a DNA-T segment at the N-gate (Fig. 4). Gyrase-mediated DNA cleavage generates a different linear DNA pattern depending on the stabilizing agent used. The cleavage pattern induced by MccB17 is different

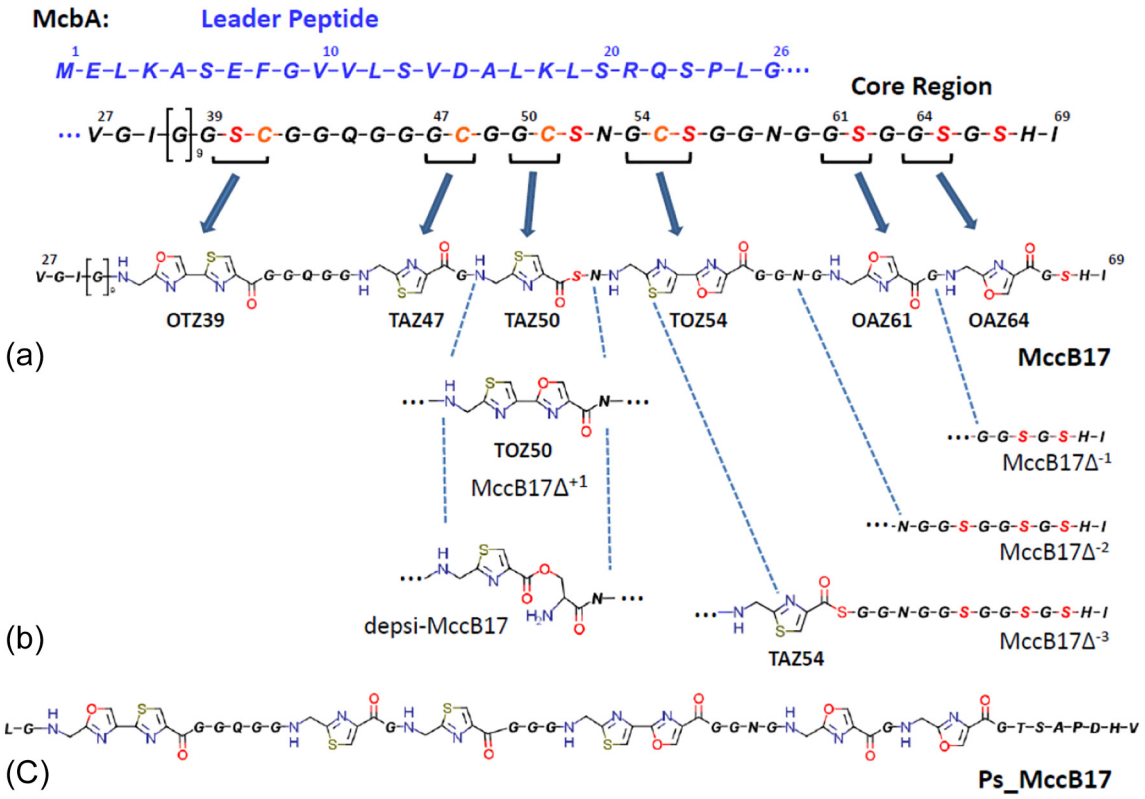


Fig. 5. McbA protein and the nomenclature of MccB17 heterocycles. (A) McbA, the *mcbA* gene product and the nomenclature of the heterocycles contained in MccB17 used in this publication. (B) By-products of MccB17 biosynthesis found in MccB17-producer strain cell extract. Only domains that are different from MccB17 are shown, "…" stand for the rest of the peptide that is identical to MccB17. Corresponding residues in MccB17 and by-products are connected by blue dashed lines. (C) Ps_MccB17, a MccB17 variant produced by *P. syringae*.

from the one induced by quinolones and is closer to the one produced with Ca^{2+}, which is thought to more likely reflect the natural cleavage specificity of gyrase [23]. Kinetic studies on the gyrase cleavage reaction showed that MccB17 primarily increases the forward rate of cleavage, whereas CFX both increases the forward rate of cleavage and decreases the religation rate. MccB17 promotes the conversion of single-stranded cleavage to double-stranded cleavage, that is, promotes the cleavage of the second strand of DNA after the first strand has been cleaved by gyrase [22].

Taken together, these findings suggest that MccB17 binds to the gyrase (A_2B_2)-DNA complex such that it interrupts, but does not completely inhibit, the strand-passage reaction and stabilizes a conformation of the enzyme where the DNA is cleaved and covalently bound to the enzyme. The fact that a significant length of DNA is required for MccB17-promoted cleavage implies that the T-segment is involved in MccB17 binding. It is then likely that MccB17 recognizes a transient conformation of the enzyme that occurs during the strand-passage process when the T-segment is passed through the G-segment. ATP greatly stimulates strand passage, which explains the apparent requirement of ATP for MccB17 action.

MccB17 Isomers and Variants

We use the following nomenclature to describe MccB17 and its variants (Fig. 5). The residues are numbered according to corresponding amino acid in McbA: the first residue of MccB17 is V27 and the last I69. The heterocycles will be referred to by the numbering of the first residue included in the heterocycle: as an example, the first heterocyclic site in MccB17 is (bis-)heterocycle 39 since it results from the modification of G39S40C41. The nature of the heterocycle will be described as follows: oxazole = OAZ, thiazole = TAZ, oxazole–thiazole = OTZ, and thiazole–oxazole = TOZ. So the first (bis-)heterocycle in MccB17 is referred as OTZ39. In addition, variants of MccB17 with bis-oxazole or bis-thiazole will be referred as to OOZ and TTZ, respectively.

Cell extracts from MccB17-producer strains contain a mixture of by-products of MccB17 biosynthesis

that are captured by reverse phase chromatography with a 50% acetonitrile fraction and that can be separated by HPLC. The more polar compounds that elute before MccB17 are partially processed McbA: MccB17Δ^{-1}, MccB17Δ^{-2} and MccB17Δ^{-3}, that contain seven, six and five heterocycles instead of the eight found in MccB17 due to incomplete conversion of S65, S62 and S56 to heterocycles. A more hydrophobic variant migrates after MccB17, named MccB17Δ^{+1}; this compound contains an additional heterocycle resulting from the conversion of S52 into an oxazole ring at site 50. However MccB17Δ^{+1} represents only 16% of the MccB17 cell extract, suggesting that the presence of N53 is unfavorable to the heterocyclization of S52. This disfavored heterocyclization gives rise to another oddity: on the elution profile of MccB17 purification two different peaks share the molecular weight of MccB17, with the area of these peaks being variable depending on the pH of the sample [13]. The second peak has been shown to be MccB17 (Fig. 5), whereas the first peak corresponds to an isomer. This isomer results from an *N*,*O*-peptidyl shift during heterocyclization of site 50 that creates a depsipeptide bond between the carboxyl group of TAZ50 and the hydroxyl group of the next residue S52 [18]. In depsiMccB17, the S52 amino group is free and becomes positively charged in acidic conditions, thus changing the polarity of the molecule and therefore its retention time on HPLC. More than 60% of species with MW = 3093 Da are depsiMccB17, the rest being MccB17, making depsiMccB17 the main product of the MccB17 biosynthetic pathway [18]. However, there is no difference in antibacterial activity and gyrase cleavage activity between MccB17 and depsiMccB17; therefore, we will not discriminate between the two species in the following sections. The antibacterial activity of all variants will be covered in the following section.

MccB17 from other organisms

An operon similar to *mcbABCDEFG* has been identified in *Pseudomonas syringae*. When the operon from *P. syringae pv. Glycinea* was cloned into *E. coli* it enabled the production of the MccB17-related compound Ps_MccB17 that shares the heterocyclic core of MccB17, apart from a substitution of S52 N53 by three G residues, and with significantly different N- and C-termini [28]. Ps_MccB17 promotes *E. coli* gyrase DNA cleavage with 134% of MccB17 activity. However, when tested on lawns of *E. coli*, Ps_MccB17 showed only 30% of MccB17 antibacterial activity. MccB17 and Ps_MccB17 show similar antibacterial activity on *Pseudomonas aeruginosa,* corresponding to about 30% if compared to MccB17 activity against *E. coli*. The discrepancy between *in vitro* and antibacterial activity, and the variation between organisms, highlights the role of N- and C- terminal residues in MccB17 uptake. Hybrids of MccB17 and Ps_MccB17 were created to clarify the role of the N- and C-terminal residues in MccB17 and Ps_MccB17, thus providing invaluable information about the structure–activity relationship for both uptake and *in vitro* cleavage. These results are summarized in the following section (Structure–Activity Relationships). During the production of Ps_MccB17 a variant lacking the five C-terminal residues can be isolated from the cell extract, which is slightly less active than the full-length Ps_MccB17. Another variant of Ps_MccB17 from *P. syringae pv. aesculin,* differing only by the point mutation T60P (Ps_McbA numbering), was also produced in *E. coli* but not investigated further. Genome mining for MccB17-like gene clusters has produced a limited set of hits in *E. coli* with variants with up to six-glycines deleted in the N-terminal polyglycine of MccB17. In other *Pseudomonas* species and *Halomonas anticariensis*, McbA is significantly different with the loss of the heterocyclic core shared by MccB17 and Ps_MccB17 [29].

Structure–Activity Relationships

Alteration to the MccB17 structure influence antibacterial activity in two ways: by altering the uptake of the peptide by bacteria or by modifying its interaction with DNA gyrase. In the following, antibacterial activity is measured by the magnitude of the halo of growth inhibition on a plate at a given concentration of peptide, or by measuring the minimum concentration of peptide producing a clearance zone after serial dilution. *In vitro* activity refers to the ability of the compound to promote DNA cleavage by gyrase. As experimental conditions vary between studies, the inhibitory activity of MccB17 variants will be given as a percentage of the inhibitory activity of wild-type MccB17 given in the relevant article, or as an estimate when quantitative data are not available. We have divided modifications into three sections. The first section comprises point mutations that alter the heterocyclic core of MccB17 (Fig. 6), while the second covers the study of N-terminal and C-terminal modifications (Fig. 6). In a third section we present new results: complementary data on MccB17 C-terminal I69, and novel mutational analysis of the glutamine/asparagine present in MccB17 that help clarify the importance of these residues for activity (Fig. 7).

Residues involved in ring formation

MccB17 contains 8 heterocyclic aromatic rings in the following order from N- to C-terminus: OTZ39 processed from G39S40C41, TAZ47 resulting from G47C48, TAZ50 resulting from G50C51, TOZ54 resulting from G54C55S56, OAZ61 resulting from

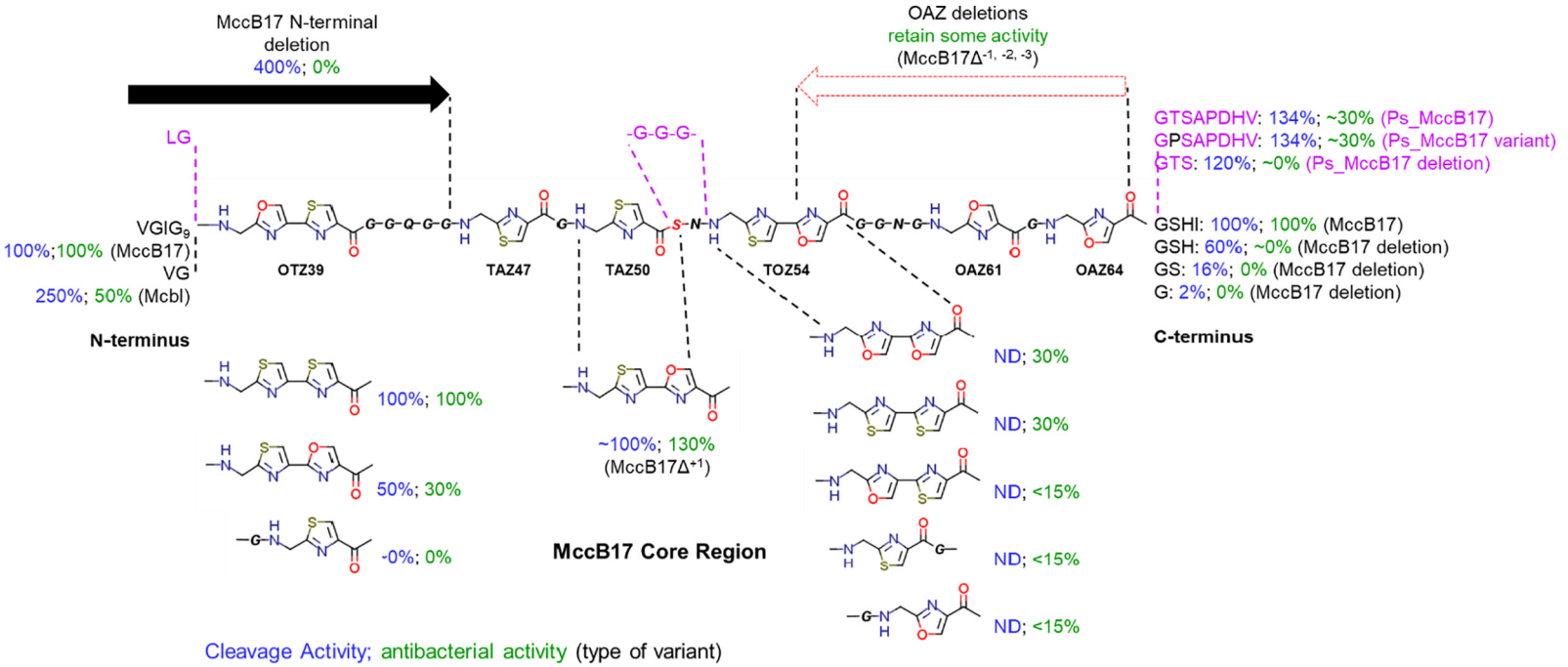


Fig. 6. Effect of variation in the core, N-terminal and C-terminal region of MccB17. The core region containing the heterocyclic moieties of MccB17 is represented in the middle of the figure with heterocycle variations reported in the literature. On the left of the core region, N-terminal variations are depicted, and on the right, C-terminal variations. For each variant, the percentage of MccB17 cleavage activity is shown in blue, and the percentage of MccB17 antibacterial activity is shown in green. The region in PS_MccB17 different from MccB17 is shown in magenta. The black arrow represents deletion of residues up to the dashed line. The dashed red arrows represent unprocessed serines leading to MccB17 variants lacking oxazole (OAZ) heterocycles. ND indicates not determined.

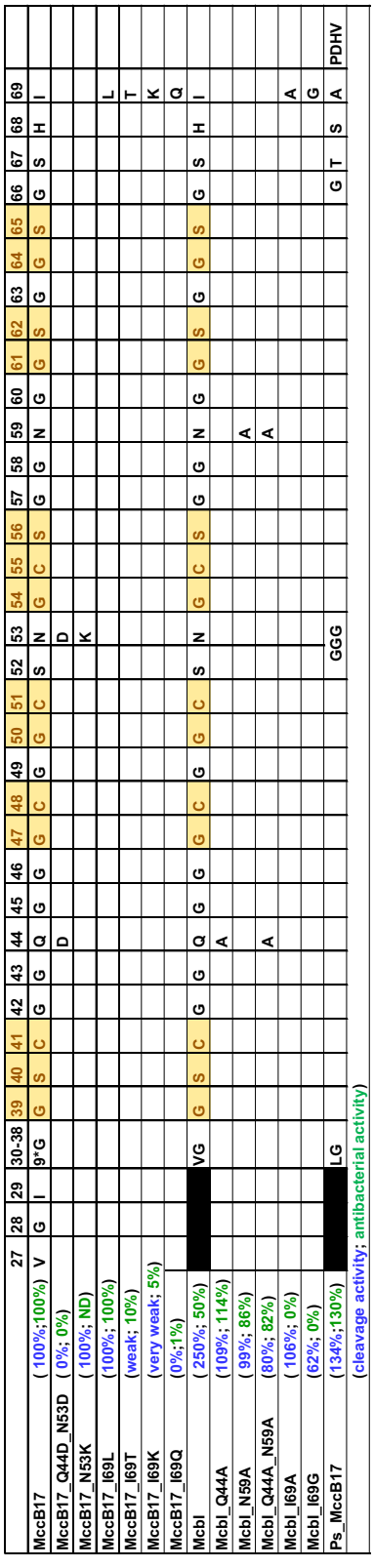


Fig. 7. Effect of point mutations on MccB17 activity. The figure shows amino acids constituting MccB17 with their numbering; post-translationally modified residues are highlighted in orange. Mutations are show underneath the corresponding residue. The effect of each mutation is shown in brackets as a percentage of MccB17 cleavage activity in blue, and a percentage of MccB17 antibacterial activity in green. Similarly, mutants of MccI, a shorter version of MccB17, are shown. Ps_MccB17 is shown to illustrate the effect of the substitution of residues S52 N53 with GGG.

G61S62, and OAZ64 resulting from G64S65. These heterocycles have been identified as essential for MccB17 activity [30]. A significant amount of work has focused on determining the importance of these uncommon amino acids for antibacterial activity and the effect of their variation. The bis-heterocycles OTZ39 and TOZ54 have been shown to be particularly important for MccB17 activity. The conversion of the oxazole heterocycle of OTZ39 to a thiazole (TTZ39) does not affect the antibacterial activity or the stabilization of DNA cleavage *in vitro*, whereas inverting the two heterocycles to TOZ (TOZ39) causes a 30% decrease in antibacterial activity compared to wild-type MccB17, and 50% decrease of cleavage activity [31]. Removal of the oxazole ring from OTZ39 (G39TAZ40) results in a compound with some residual activity (not quantified) [30]. However, MccB17$^{46–69}$, a MccB17 deletion with no OTZ39, has a high cleavage activity (400% of MccB17 *in vitro* cleavage) but no antibacterial activity [31,32]. TOZ54 is even more critical: conversion of the thiazole–oxazole to thiazole–thiazole (TTZ54) or oxazole–oxazole (OOZ54) is very detrimental to antibacterial activity, causing a 70% decrease in antibacterial activity compared to MccB17 [30]. Swapping the two heterocycles of TOZ54 to OTZ54 or removing one of the two heterocycles (TAZ54G56 and G54OAZ55) causes a decrease of antibacterial activity to $<$15% of MccB17. Site 50 is of particular interest, since the corresponding sequence in the precursor peptide is G50C51S52N53, it could be processed to TOZ50N53; however, this site is only processed to TAZ50 in MccB17, with TOZ50N53 being only present in the variant MccB17Δ^{+1}, a compound that represents only 16% of MccB17 cell extract [30]. MccB17Δ^{+1} (TOZ50N53) shows a 30% increase in antibacterial activity [30] but only shows a 10% increase of cleavage compared to MccB17 in *in vitro* assays [21]. However, later work with synthetic MccB17Δ^{+1} showed it to have a decreased ability to stabilize cleavage compared to synthetic and recombinant MccB17 [31]. In the corresponding site, the *Pseudomonas* MccB17 (Ps_MccB17) has three glycines instead of S52N53, meaning that no Ps_MccB17Δ^{+1} can be produced for lack of a Ser to be heterocyclized, suggesting that the additional heterocycle in MccB17Δ^{+1} has a marginal importance. Unfortunately, no mutagenesis data are available for TAZ47 and TAZ50 sites. Due to the N- to C-terminus directionality of MccB17 heterocyclic formation, under-processed MccB17 species lack oxazoles in the C-terminal part of MccB17; MccB17Δ^{-1} is a partially processed MccB17 derivative that has unprocessed G64S65 at site OAZ64, and MccB17Δ^{-2} has additionally unprocessed G61S62 at site OAZ61. These two compounds have been reported to retain antibacterial activity (unpublished data from Roy *et al.* [30] and D. Ghilarov personal communication). Interestingly, mutation of S65N at the site OAZ64 yields a MccB17 derivative lacking OAZ64 with no antibacterial activity

[15]. Since MccB17Δ^{-1} retains antibacterial activity, it would be valuable to know if S65N mutation disrupts the uptake of the compound or causes steric hindrance with the target. The general picture that emerges is that TOZ54 is of central importance and that with the two preceding thiazoles, it forms the essential core of MccB17 necessary for cleavage activity. All the other heterocycles are absent in some variants that still retain cleavage activity. Unfortunately, no variants containing only these three heterocycles have been evaluated so far, the closest to this structure reported was the two halves of MccB17: MccB17$^{1\text{-}52}$K and MccB17$^{53–69}$ that retain some marginal cleavage activity [32]. This suggests that a cleavage promoting motif can be found multiple times in the MccB17 core.

MccB17 N-terminus and C-terminus

Several iterations of N-terminal deletion have been reported: MccB17$^{35–69}$, where 9 residues have been removed, shows no alteration in *in vitro* cleavage activity [32]. A synthetic MccB17$^{38–69}$, where all the residues upstream of the first heterocycle have been deleted, had an *in vitro* cleavage activity of 250% of MccB17, but only retained 50% of the antibacterial activity [31]. Further deletions down to the glycine before TAZ47 in the synthetic MccB17$^{46–69}$ completely abrogate antibacterial activity despite the compound having strong *in vitro* cleavage activity [31]. In Ps_MccB17, only two residues, L and G, precede OTZ39; thus, this variant lacks the whole polyglycine stretch present in MccB17 but retains full cleavage activity with *E. coli* gyrase [28]. A MccB17 variant where residues G28 to G37 were deleted called Ec-McbI retains 85% of MccB17 antibacterial activity [28] and stabilizes *in vitro* DNA cleavage with an efficiency of 200% of MccB17 (Collin and Maxwell, unpublished). Taken together, this shows that the sequence preceding OTZ39 has a marginal role in the stabilization of the cleavage complex but plays an important role in MccB17 uptake as deletion of the N-terminus hinder antibacterial activity, while the ability to stabilize the cleavage complex is maintained or even improved [28].

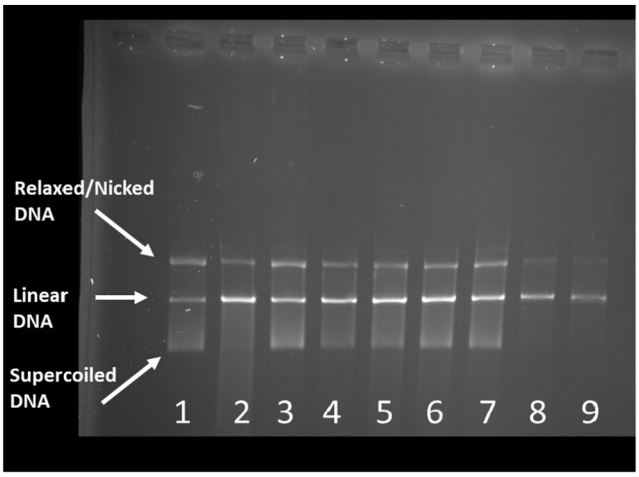
Similarly, the MccB17 C-terminus, consisting of GSHI$^{66–69}$ after the last heterocycle OAZ64, has been extensively studied and shown to be of great importance. The deletion of C-terminal residues is detrimental to both *in vitro* cleavage activity and antibacterial activity. Variants with a deletion of I69 retain only 2% of MccB17 antibacterial activity and the *in vitro* cleavage is reduced to 60% of MccB17 [33]. Furthermore, deletion of H68 decreases the antibacterial activity to 0.04% of MccB17 [33], and *in vitro* cleavage activity to 16% of MccB17 [32], and the additional removal of S67 yields a derivative with no antibacterial activity and 2% of MccB17 *in vitro* cleavage activity [32]. Residues H68 and I69 are required for the toxin to retain its activity. Comparison of deletion mutants MccB17$^{1–63}$ and MccB17$^{1–63}$-H64I65 shows that the presence of the C-terminal HI is not sufficient to maintain antibacterial activity: MccB17$^{1–63}$ had 0.04% of MccB17 antibacterial activity and MccB17$^{1–63}$H64I65 had 0.2% of MccB17 antibacterial activity [33]. Interestingly, a shorter deletion mutant MccB17$^{1–60}$ had only 0.02% of MccB17 antibacterial activity and approximately 20% of MccB17 *in vitro* cleavage activity, suggesting that MccB17$^{1–63}$ and MccB17$^{1–63}$H64I65 probably retain some cleavage-complex stabilization activity. It is notable that Roy *et al.* reported that species with as few as five rings out of the normal complement of eight heterocycles in MccB17 were active as antibiotics; however, this was not substantiated by data [30]. MccB17 C-terminal residue I69 has been the subject of particular attention: mutation I69L did not alter antibacterial activity and *in vitro* cleavage, whereas mutations to more polar residues were detrimental to antibacterial activity: MccB17 I69T had 10% of MccB17 activity and very weak stabilization of *in vitro* cleavage (not quantified); MccB17 I69K had 5% of MccB17 antibacterial activity and very weak stabilization of *in vitro* cleavage (not quantified), MccB17 I69Q had 1% of MccB17 antibacterial activity and no stabilization of *in vitro* cleavage [33]. The overall picture is that the N-terminal sequence preceding MccB17 core is important for uptake and can be significantly altered without hindering activity, whereas the C-terminal portion is essential for uptake and for promoting *in vitro* cleavage and allows little variation.

Investigation of novel mutations of the MccB17 C-terminal I69 and amide residues

In this work, we have used Ec-McbI as our reference. As described before, it is an *E. coli-P. syringae* hybrid of MccB17 that lacks the N-terminal I29 and polyG$^{30–38}$ of *E. coli* MccB17. It possesses an improved ability to stabilize the cleavage complex (200% of MccB17 activity) at the cost of reduced uptake (81% of MccB17 antibacterial activity).

To further our understanding of the importance of I69 and specifically the importance of the hydrophobic side chain, we mutated I69 to A and G: mutation I69A did not alter the *in vitro* cleavage activity (106% of McbI), whereas mutation I69G caused a decrease in the cleavage activity (62% of McbI); however, the antibacterial activity was lost for both mutations (Fig. 8). This is further evidence that I69 is critical for transport and to a lesser extent involved in the interaction with the gyrase–DNA complex.

Amide residues Q44, N53 and N59 are the only other residues present in the MccB17 core apart from the heterocycles and glycines (and unprocessed S52). As these residues can form hydrogen bonds, they might be critical for the interaction of MccB17 with gyrase. Parks *et al.* [11] showed that hydrolysis of



	% of total DNA cleaved	Inhibition of growth of MG1655 (mm)	Inhibition of growth of NR698 (mm)
1-Negative Control	21 ±4	0	0
2-CFX (8 µM)	80 ±4	31 ± 3	48 ± 1
3-MccB17 (50 µM)	41 ±5	30 ± 1	34 ± 1
4-McBI (50 µM)	72 ±7	21 ± 2	22 ± 2
5-McBI_Q44A (50 µM)	69 ±5	27 ± 1	32 ± 3
6-McBI_N59A (50 µM)	63 ±4	19 ± 1	26 ± 2
7-McBI_Q44A_ N59A (50 µM)	55 ±6	19 ± 1	23 ± 2
8-McBI_ I69A (50 µM)	89±1	0	0
9-McBI_ I69G (50 µM)	95±8	0	0

Fig. 8. Evaluation of McbI mutants: McbI is a MccB17 variant lacking G28 to G37; due to its improved cleavage activity, McbI was used as the reference to evaluate the effect of mutation of amide residues Q44 and N59, and the C-terminal I69. Upper panel: *E. coli* DNA–gyrase cleavage assays with McbI mutants. Lower panel: activity of various MccB17 derivatives. Inhibition of *E. coli* growth: Q44A and N59A have a marginal negative effect on the ability to stabilize the cleavage complex or on the inhibition of bacterial growth. McbI I69A and McbI I69G retain the ability to stabilize the cleavage complex; however, they lose their ability to inhibit bacterial growth. Assays were carried out as described previously [31], DNA gyrase was used according to the supplier's protocol (Inspiralis Ltd).

N53 and N59 to D renders MccB17 inactive both for antibacterial activity and *in vitro* cleavage. We have shown as well that replacing N53 with K was only slightly detrimental to *in vitro* cleavage [32]. In addition, we isolated the N59K mutant, which showed *in vitro* cleavage activity similar to MccB17 (unpublished). As mentioned in the previous section, Ps_MccB17 possesses three Glycines instead of S53N53 and has the same *in vitro* cleavage activity with *E. coli* gyrase as MccB17. This, added to the fact that the MccB17 mutant with S52N53 substituted to GGG had the same antibacterial activity on *E. coli,* shows that N53 is required for neither *in vitro* activity nor uptake. To investigate the importance of the other amides present in MccB17, we produced the three McbI mutants: Q44A, N59A and the double mutant Q44A_N59A. These mutants retained strong *in vitro* cleavage activity compared to McbI (109%, 99% and 80%, respectively), and antibacterial activity was similar to McbI (114%, 86% and 82% of McbI activity, respectively) (Fig. 8). The role of these amides might be limited to favoring solubility of MccB17 since their removal has a very limited effect on uptake and stabilization of DNA cleavage by gyrase.

Resistance to MccB17

As a prototype antibiotic, it is important to consider resistance to MccB17. Probably on account of its natural origin, there are a number of ways that bacteria can become resistant to the toxin. This does not preclude its future development as a clinical antibiotic, and knowledge of these resistance mechanisms is important for optimising MccB17-derived compounds.

Transport-based

Three chromosomal genes have been linked to resistance to MccB17: *ompF* and *ompR*, which affect the production of the general outer membrane transporter OmpF, and *sbmA* which encodes the MccB17-specific transporter SbmA (*S*ensitivity to *B*17 *m*icrocin, A) [34]. Efforts to isolate MccB17-resistant *E. coli* have predominantly yielded strains with mutation

in these two transporters. The frequency of these mutations was about 1×10^6; 20% of these were identified as mutations in *ompF*, whereas the remaining 80% were linked to *sbmA*, which appeared to be specific to MccB17 [34].

SbmA

Many MccB17-resistant mutations map to *sbmA*. The SbmA protein is a non-essential inner-membrane transporter [34–36]. Sequence similarity analysis suggested that SbmA is the transmembrane domain of an ABC transporter [37]; however, it was later shown that the peptide transport is driven by the electrochemical gradient instead of ATP hydrolysis, and that SbmA is present as a homodimer in the membrane [38,39]. SbmA is involved in the transport of a wide range of chemically diverse compounds: bleomycin [15], microcin J25 [40], the proline-rich peptides Bac7 [41], PR-39 [36], peptide nucleic acids [42] and the synthetic DNA mimics peptide phosphorodiamidate morpholino oligomers [43].

OmpF

OmpF confers partial resistance to MccB17 [34]. OmpF is, with OmpC, one of the two porins present in the outer membrane of *E. coli*. These trimeric proteins form water-filled channels that allow hydrophilic molecules below a certain molecular weight to diffuse across the outer membrane. OmpF and OmpC expression is regulated by medium osmolarity, being synthesized at low and high osmolarities, respectively [44]. The regulation involves a cytoplasmic regulator encoded by ompR and a cell-envelope sensor encoded by *envZ* [45].

McbE and McbF

These proteins form part of the immunity mechanism for MccB17, along with McbG (discussed below), that prevents producing cells from committing "suicide." Specifically, it has been proposed that McbE/F serves as a "pump" that exports MccB17 out of the producing bacterium [46]. Modeling suggests that McbE is an integral membrane protein that associates with McbF, which is thought to be a nucleotide-binding protein. Together McbE and McbF operate to keep the intracellular concentration of MccB17 low conferring partial resistance to the toxin [46].

Protective molecules

Another (general) mechanism of resistance to antibacterial compounds is the production of a molecule that prevents the toxin from binding to its target; this is also the case with MccB17. The mechanism used by producing strains to protect themselves against endogenous MccB17 involves the protein McbG (Fig. 9), which protects *E. coli* gyrase from MccB17 [46]. McbG belongs to the pentapeptide repeat protein (PRP) family [47]; among this family, various molecules are found that confer resistance to other gyrase poisons: AlbG and CysO have the same role as McbG in the biosynthetic pathway of their respective toxins: albicidin and cystobactamide, two closely related molecules [48,49]. Other proteins include the Qnr family of PRPs, which confer resistance against the quinolone class of drugs to bacteria that harbor them [50], as well as the MfpA proteins that protect mycobacterial gyrase against quinolones [51,52]. There is a potential fitness cost to this type of resistance as PRPs are often also gyrase inhibitors [47,53,54]. SbmC is not a PRP but a smaller protein that confers resistance to MccB17.

McbG

McbG is a PRP that prevents MccB17 from binding to gyrase; this, combined with the efflux pump McbE-McbF that control concentration of MccB17 in the cell, confers immunity to the MccB17. McbG alone provide partial resistance to MccB17 [46].

AlbG

AlbG has the same role as McbG in the albicidin biosynthetic pathway; that is, it protects the producing bacteria from the toxin. This non-ribosomal peptide, produced by *Xanthomonas albilineans,* is a pathogenesis factor and DNA gyrase poison (*E. coli* and *X. albilineans*) [48,55]. X-ray crystallography reveals that the protein has an intriguing right-handed quadrilateral β-helix structure that resembles DNA [56] also seen in MfpA and Qnr, leading to the suggestion that these proteins might act by binding to gyrase at a DNA-binding site.

CysO

Cystobactamides are a family of closely related molecules produced by *Cystobacter* sp.; they are also structurally similar to albicidin [49].

Qnr

The *qnr* gene was discovered on a multi-resistance plasmid (pMG252) carried by a clinical isolate of *Klebsiella pneumoniae* [51]. Qnr is a 218-amino-acid PRP that confers resistance to quinolone drugs [50]. It interacts with gyrase in the absence of DNA and quinolones and decreases DNA binding to gyrase when pre-incubated with the enzyme and added to DNA and ADPNP; this

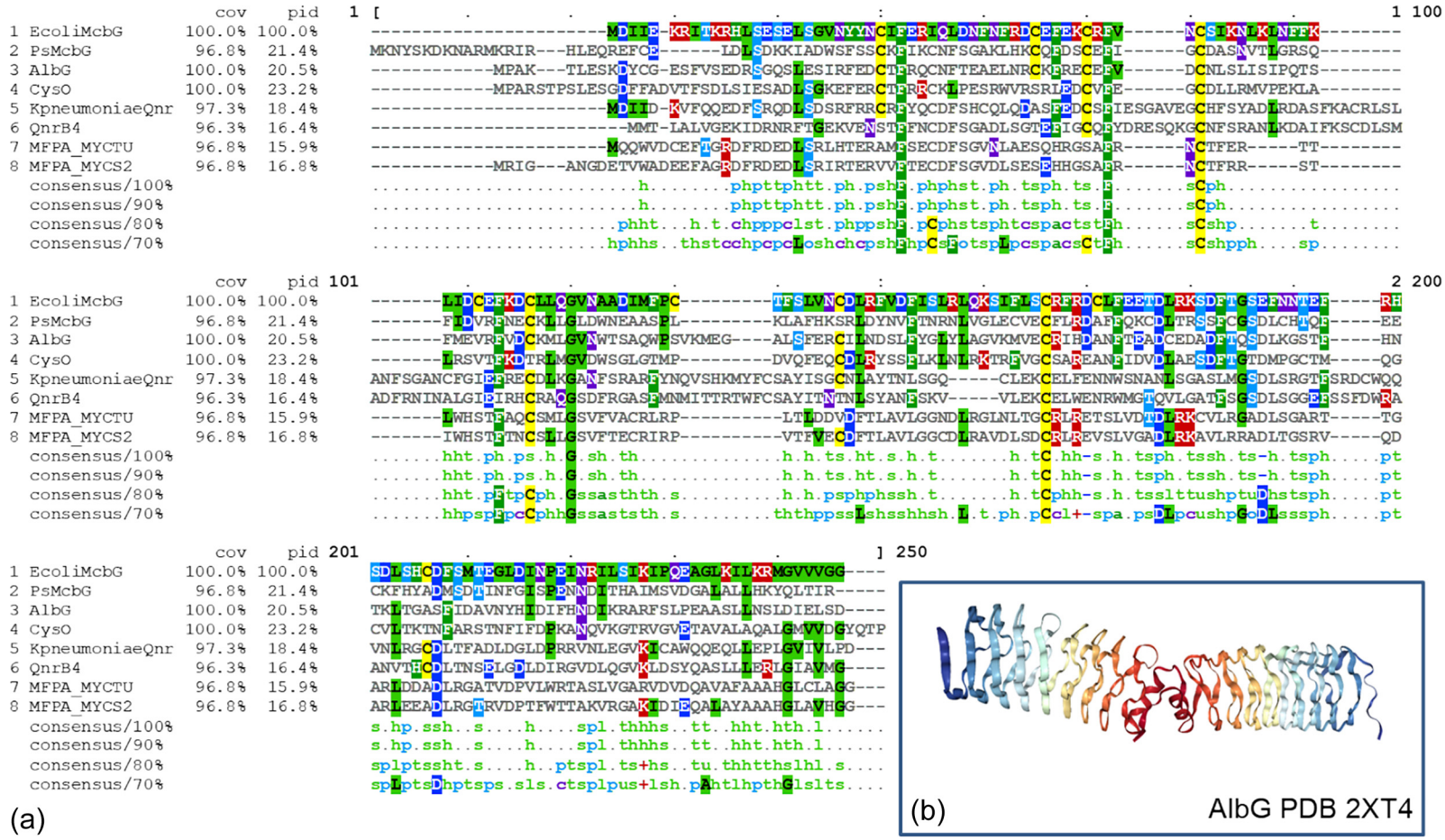


Fig. 9. (a) Alignment of pentapeptide proteins related to McbG: the reference is *E. coli* McbG, and the second is McbG from *P. syringae* that produce a variant of MccB17. AlbG is the immunity protein to Albicidin, an NRP topoisomerase poison produced by *X. albilineans*, the causal agent of leaf scald disease. CysO is the immunity protein to cystobactamide, a compound related to Albicidin produced by *Myxobacterium cystobacter*. Qnr is produced by *K. pneumoniae* and confers resistance to quinolones drugs. MfpA familly proteins confer quinolone resistance to mycobacteria; *M. tuberculosis* and *M. smegmatis* are presented. Alignment generated by Clustal Omega and displayed with Mview 1.63, Copyright © 1997–2018 Nigel P. Brown. Despite their sequence differences, all these proteins have multiple repeating sequences of five amino acids in length and probably adopt a right-handed β-helix fold with a roughly square-shaped cross section as shown by AlbG. (b) the crystal structure of AlbG PDB 2XT4 [56].

suggests that Qnr probably interacts at an early stage of the catalytic cycle when gyrase binds to DNA [57]. Cells overexpressing Qnr are still susceptible to MccB17 [58], suggesting that the activity of one PRP on gyrase does not confer resistance to all related inhibitors.

There are now a large family of Qnr proteins known from a number of bacterial species [59]. The structure of the chromosomally encoded Qnr homologue protein from *Enterococcus faecalis* (*Efs*Qnr) has been determined [54] and shows the right-handed quadrilateral beta-helical fold found on other PRPs (AlbG and MfpA). Aside from protecting gyrase from quinolones *Efs*Qnr was also shown to inhibit gyrase-catalysed supercoiling activity [54].

MfpA

Although it belongs to the PRPs, MfpA was identified during a screen for efflux pumps contributing to quinolone resistance in *Mycobacterium smegmatis* [60]. $MfpA_{Mt}$ is the *Mycobacterium tuberculosis* equivalent of MfpA; it was shown not to protect *M. tuberculosis* gyrase from quinolones on its own *in vitro* [53], but requires the presence of MfpB, a small GTPase to confer resistance to quinolones *in vitro* and *in vivo* [61]. *M. tuberculosis* MfpA was the first PRP whose structure was solved showing the right-handed quadrilateral β-helix structure that has led to the idea that these proteins may act as DNA mimics [52]. It was assumed that MfpA, and by analogy McbG, binds to the DNA gyrase A protein in the DNA-binding saddle [52]. Although MfpA appears to bind to gyrase and inhibit supercoiling, there is currently no direct proof of its mode of action; it is entirely feasible that it and other related PRP proteins bind to another part of gyrase.

SbmC

SbmC is a 157-amino-acid peptide (18 kDa) encoded in the *E. coli* chromosome that confers resistance to MccB17; it is not a PRP protein. It was thought to provide resistance by recognizing and sequestrating MccB17 as it also blocked MccB17 export from producing cells [62]; however, later observation that SbmC (GyrI) co-purified with gyrase and inhibited its supercoiling activity [63]. Further evidence showed that SbmC interacted with the whole gyrase complex and not GyrA or GyrB alone, and that the 8-amino-acid sequence of GyrI $ITGGQYAV^{89–96}$ was sufficient to inhibit DNA gyrase supercoiling activity [64]. A crystal structure of SbmC has been published [65]. Furthermore, SbmC has also been linked to resistance to the toxin CcdB and partial protection against quinolones [66].

Summary

Taken together it seems likely that the PRP proteins, including McbG, act in a similar way, i.e. binding to gyrase and preventing toxin action in doing so. The structural information on MfpA, *Efs*Qnr and AlbG is suggestive that the proteins act as DNA mimics with T-segment mimicry having been suggested [67], but this is by no means proven. There are currently few data on the cross reactivity of these pentapeptide proteins, that is, how effective one PRP protein is at protecting gyrase from a non-cognate toxin, although it appears that Qnr does not confer resistance to MccB17. Interestingly, a plasmid carrying the MccB17 immunity genes *mcbEFG* produced a 2- to 8-fold increase in resistance to various quinolones [60]; unfortunately, the experiment was not extended to single *mcbG* or *mcbEF* gene to address if that was due to efflux or target protection.

Mutations in the MccB17 target, DNA gyrase

The initial identification of the MccB17 target was provided by the isolation of a resistant *E. coli* strain carrying a point mutation in gyrase: GyrB W751R [20]. Various point mutations were constructed to assess the alteration of the position 751 [68]. Replacement by residues with an aromatic side-chain (F, H) conferred only marginal resistance to MccB17, whereas substitution by E or G gave a level of resistance comparable to W751R. Substitution by K improved the resistance compared to W751R. However, some of these mutations were detrimental: E, K, G and H substitution altered gyrase activity, whereas R and F mutants retain normal activity [68]. In-frame deletion of W751 was also evaluated but rendered GyrB inactive [68]. Attempts to isolate other gyrase mutations conferring resistance to MccB17 have thus far been fruitless (Collin and Maxwell, unpublished); identification of further mutations would be useful in better defining the MccB17-binding site.

Given the relatedness between MccB17 action and quinolone action, it is interesting to consider known quinolone-resistance mutations and whether they confer resistance to MccB17. One such study [23] using the quinolone-resistant gyrase mutants GyrB K447E and GyrA S83W suggested that these two mutations also conferred resistance to MccB17-induced gyrase cleavage *in vitro*. Conversely, a CcdB-resistant mutant (GyrB R462C) did not [23]. However, this study did not look at other quinolone-resistance gyrase mutants, nor did it assess the effects on DNA supercoiling. In relation to the GyrB W751R mutant, this shows wild-type sensitivity to quinolones [28]. Taken together, our knowledge of mutations in gyrase conferring resistance to MccB17 is limited; the intriguing cross-resistance with two quinolone-resistant loci is worthy of further

investigation and raises the question as to whether the MccB17- and quinolone-binding sites might overlap (Fig. 10). We speculate below (Future Perspectives) about the possible mode of action of MccB17 on gyrase.

Biosynthesis

In recent years, this is the area where outstanding progress has been made. The crystal structures of two major actors in the biosynthetic pathway have been solved, significantly improving our understanding of the molecular details of MccB17 maturation.

Identification of MccB17 genetic determinants

MccB17 production and immunity was linked to the presence of a plasmid named pRYC17 by using transconjugation between microcinogenic *E. coli* strain LP17 and *E. coli* strain K12, and tracking spontaneous loss of production capacity. Using sedimentation analysis, the plasmid pRYC17 was shown to have a MW = 36×10^6 Da, 70 kilobases and 1–2 copies per chromosome. The plasmid belongs to the incompatibility group FII [69]. The genetic information necessary for MccB17 production and immunity were shown to be present in a BamHI-EcoRI 5.1-kb fragment and further deletion located the genes encoding production of MccB17 in a 3.5-kb segment and the immunity in an adjacent 1-kb segment [70]. Using complementation experiments, it was further shown that this production fragment contained four cistrons A, B, C, D, with A suggested to be the structural gene due to its small size [70]. The *mcbA* gene encodes a 69-amino-acid peptide from which only 43 are found in the final product, which includes 26 glycine residues [13]. The four open reading frames were confirmed later using a LacZ fusion and physical mapping of mRNA start sites, and the genes were sequenced revealing the complete details of the MccB17 operon [71].

MccB17 operon

MccB17 production involves 7 genes: *mcbA*, *mcbB*, *mcbC*, *mcbD*, *mcbE*, *mcbF* and *mcbG* [72]. McbA is the MccB17 structural protein [13], McbB, McbC and McbD form the microcin synthase that carries out the post-translational modification of four serines and four cysteines into the corresponding oxazoles and thiazoles. McbE and McbF are responsible for the export of MccB17, and McbG provides immunity to MccB17. The 26-amino-acid leader peptide of McbA is removed by the endogenous protease TldD/TldE to form MccB17 [15].

Microcin synthase

MccB17 synthase converts serine and cysteine residues in the MccB17 peptide precursor into oxazole and thiazole heterocycles. It is formed by the McbB, McbC and McbD proteins [14,15,73]. The formation of the thiazole and oxazole rings is a two-step process that is achieved through cyclodehydration into azoline rings by McbD followed by the aromatization of the formed azoline (dehydrogenation) by McbC. MccB17 synthase has been subjected to in-depth studies, but the recent publication of the structure of the whole McbBCD complex, determined by x-ray crystallography, represents a significant leap forward in our understanding of microcin synthase structure and mechanism (Fig. 11) [74]. In addition to the three subunits McbB, McbC and McbD, forming the synthase, the crystal structure includes additional elements involved in the heterocycle synthesis: the over-processed bound substrate MccB17Δ^{+1} with the leader peptide still attached, as well as the flavin mononucleotide (FMN) and ADP co-factors. There are some discrepancies between published biochemical studies and crystallographic data, concerning the general organization of the enzyme, which are discussed below; the specific role of each subunit is also discussed.

Organization of the MccB17 synthase

The microcin synthase is composed of three proteins: McbB (MW = 34 kDa), McbC (MW = 31 kDa) and McbD (MW = 45 kDa), all of which are required for MccB17 maturation [75]. Early studies by size exclusion chromatography suggested a 1:1:1 stoichiometry for McbB/McbC/McbD, as the complex was migrating at approximately 100 kDa [76], (MW $McbB_1C_1D_1$ = 110 kDa). Apparent dissociation constants for each constituent of the complex have been measured by titration of one subunit *versus* the two others at fixed concentration, showing dissociation constants (K_ds) between 1 and 2 μM [75]. However, the recent McbBCD crystal structure reveals a more complex organization of the three subunits: two McbBs in different conformations, one McbC and one McbD form a tetrameric asymmetric unit that forms an octamer through 2-fold symmetry (Fig. 11) [74]. To reconcile information that might seem contradictory, it is important to keep in mind that the crystal structure might show a transient state where, in the absence of the TldD/E protease, the final stage of maturation could not be completed leaving the substrate locked onto the synthase. However, when the McbA substrate is removed, the McbBCD complex still appears as an octamer in gel filtration experiments, as also does the homologous Klebsazolicin (KLB) synthase KlpBCD [74]. Taken together, this evidence suggests that the octamer is the biologically relevant form of the MccB17 synthase.

McbB and recognition of the McbA substrate

McbB is a zinc metalloprotein containing two putative Zn^{2+}-binding sites; however, the Zn^{2+}-McbB stoichiometry was found to be 1:1 [77]. Mutational analysis of the Zn^{2+}-binding sites in MBP-McbB showed that only the first site was critical for McbB activity; the MBP-McbB (C181A, C184A) mutant was unable to process McbA but could compete with WT McbB in the synthase complex, whereas MBP-McbB (C266A, C269A) had no altered MccB17 production activity [75]. Finally, Zn^{2+} bound to C192, C195, C290 and C292 is visible in the crystal structure, supporting the evidence from mutational analysis, and confirming the suggested structural role of Zn^{2+} in McbB [74,77]. The crystal structure shows that McbB is the anchoring site for the leader peptide: most of the leader peptide of McbA is resolved and binds to the McbB–McbB interface. McbB can be divided in two domains linked by a loop: an N-terminal domain, McbB-dom1, which is the predominant site of interaction with the leader peptide, and the C-terminal domain McbB-dom2, which contains the structural Zn^{2+}-binding site.

To process McbA into pro-MccB17, the MccB17 synthase requires binding to the leader peptide [76,78]. The study of McbA$^{1–26}$-COOH and McbA$^{1–26}$-$CONH_2$ in buffer pH = 5.7 with 40% TFE by circular dichroism and 2D-NMR showed that the leader peptide adopts a helical conformation (residues 5 to 21) in these conditions. It should be noted that the PatE peptide, the equivalent of McbA in Patellamide biosynthesis, (a RiPP belonging to the cyanobactin family also) shows a helical conformation in solution [79] different from the conformation observed in the crystal structure with its synthase [80]. The helical structure of McbA in solution is as follows: the α-helix is stabilized by two salt bridges: L4–E7 and D15–K18, one side of the helix has a polar surface formed by residues S6, S13 and S20, while the other side possesses a hydrophobic patch consisting of F8 and L12 [81]. The leader peptide-bound state is shown in the crystal structure with residues 4–22 resolved, the peptide is bound at the McbB1-dom1/McbB2-dom2 interface. This bound state presents a different conformation compared to the NMR free structure; it is organized as a β-turn (residues 6–9), β-strand (residues 9–14) and a short α-helix (residues 16–21). In this bound state, the intramolecular salt bridges present in McbA alone between K4–E7 and D15–K18 are disrupted in favor of new ionic interactions with McbB. The salt bridges established between McbA and McbB are D15 to K175 and K18 to E176. E7 and K75 are too far apart to form a salt bridge and K4 (McbA) has no visible ionic interaction with McbB. These two states illustrate conformational changes that take place in the leader peptide upon binding to the synthase. The hydrophobic patch is involved in the binding to the synthase; mutation F8A and L12A in McbA$^{1–46}$ impairs heterocycle formation (detected by anti-MccB17 antibodies) by preventing binding of the substrate to the synthase [81]. This interaction is clearly illustrated in the crystal structure where the side chain of F8 interacts with a hydrophobic pocket at the end of a three-helix bundle from McbB1-dom1. Furthermore, L12 interacts with a hydrophobic pocket formed at the interface of McbB1-dom1 and McbB2-dom2 [74]. Additional mutations were investigated [81]: V11P or V14P significantly decreased processing; this was initially thought to be due to the disruption of the helical structure; however, the crystal structure shows this region to be a β-strand where the side chains of the two V are facing each other. It is unclear why this substitution by P would disrupt the interaction with McbB. Mutation of the polar surface: S6A, S13A or S20A, had no effect on processing. An earlier publication suggested that McbD was the anchoring protein for McbA, as cross-linking experiments between the McbBCD complex and an McbA analogue, where F8 has been replaced with *L*-4-benzoylphenylalanine (BPA), showed cross-linking between McbD and the McbA analogue [75]; we will address this experiment further in the paragraph dedicated to McbD.

Role of the McbA polyglycine linker

McbA possesses a long stretch of 10 glycines (G30–39) between the leader peptide and the first heterocycle to be processed (the tenth glycine being processed into MccB17's OTZ39). The influence of this linker length on heterocycle processing was studied with McbA$^{1–46}$, the substrate for a MccB17 deletion variant with only the first bis-heterocycle OTZ39 followed by five residues. McbA$^{1–46}$ with a 11-G or 9-G linker was processed as wild-type; a 7-G linker yielded only one heterocycle after being processed, whereas a 3-G linker McbA$^{1–46}$ was not processed at all. Kinetic analysis refined this result by showing a dependence between single-heterocycle-processing (immunoblot) velocity and the length of the linker: the velocity with an 11-G linker McbA$^{1–46}$ was twice the velocity of the WT. A 9-G linker caused a decrease to 20% of WT velocity. A 7-G linker decreased further to 10% of the WT velocity. 5- and 3-G linkers were, respectively, close to and at background level. It should be noted that the derivative with a 7-G linker intriguingly had only one heterocycle processed according to MS. Interestingly the *P. syringae* MccB17 variant (cf. section 5: Microcin Variants) has a nearly identical heterocyclic core (residues 39 to 66), a leader peptide of identical length, but an N-terminus lacking the polyglycine stretch, with only LG residues before the first heterocycle. The absence of the polyglycine does not hamper the processing of McbA; the

P. syringae synthase has, respectively, 35% identity for McbB, 55% identity for McbC and 40% identity for McbD. Interestingly, chimeric microcins lacking the polyglycine were processed normally by the *E. coli* synthase [28].

McbD and cyclodehydration

McbD belongs to the YcaO family (reviewed by Burkhart *et al*. [82]); this ATPase is responsible for the cyclodehydration reaction in the complex. MccB17 synthase requires ATP or GTP to process the McbA peptide into LP-MccB17 with the ATP/GTPase activity being substrate dependent. The McbD protein possesses a motif that is reminiscent of the Walker B motif and of some G protein GTPases [75,83], and has been proposed to be the ATP/GTPase in the complex, but did not show any ATP/GTPase activity on its own. The crystal structure confirmed McbD as the ATP/GTPase since ADP bound to McbD was visible. In the crystal structure, McbD is interacting mostly with McbB. There is mounting evidence that the cyclodehydration is supported by activation of the hydroxyl group by phosphorylation, thus converting it to a better leaving group. This mechanism was confirmed by ^{18}O-transfer studies [84], and the presence of ADP in the McbD active site shown in the crystal structure also supports this mechanism; however, no part of the peptide substrate is bound in McbD. A synthase construct where the dehydrogenase was disabled (McbC-Y202A) led to the introduction of a single azoline into McbA; unfortunately, this synthase–substrate system could not be crystallized. Early cross-linking experiments with the McbBCD complex in the presence of an analogue of McbA, biotinylated for detection and with F8 substituted by BPA, detected cross-linking between the McbA analogue and McbD, suggesting McbD as the anchor for the leader peptide; however, in the crystal structure, the leader peptide is shown to only interact with the two McbB subunits. If we look at the F8-binding site in McbB1, this site can only accommodate the phenyl ring; it is possible, the BPA, which could not bind McbB, would flip out of the leader-peptide-binding site on McbB1 and react with residues in close proximity. In the crystal structure, only McbB2, sandwiched between McbB1 and McbC, would be near the bound substrate; however, McbB2-dom1, which can potentially bind McbA, is close to McbD. It is possible that in the cross-linking experiments conditions the modified McbA bind McbB2-dom1 allowing the modified McbA to cross link with McbD. The crystal structure of the thioamide-forming YcaO from *Methanocaldococcus jannaschii* bound to ATP and a peptide substrate has been published recently; this enzyme was shown to perform cyclodehydration like McbD if provided with a suitable substrate [85].

McbC and dehydrogenation

Dehydrogenation is carried out by McbC, a flavoprotein; the co-factor FMN was detected in stoichiometric quantities [75] and is also present in the McbBCD crystal structure [74]. Interestingly, in the crystal structure, FMN is present in close proximity to the G63OAZ64GS residues from the C-terminus of MccB17, which confirms McbC as the dehydrogenase responsible for the aromatization of oxazoline and thiazoline to oxazole and thiazole, respectively. The crystal structure shows an McbC homodimer situated at the center of the octamer; the homodimer is stabilized by each monomer embracing the other monomer with a 50-residue-long loop. Each FMN cofactor interacts with both McbC subunits. Residues L201 and Y202 have been linked to the catalysis, and the crystal structure showing the C-terminal oxazole bound between the two proposed catalytic residues and the flavin supports the mechanism proposed by Melby *et al*. [86]. As mentioned above, mutation of McbC Y202A generates a synthetase that can only process one azoline, confirming Y202 as a critical catalytic residue. In addition to the FMN site, another azole-binding site has been identified in the structure in a region dubbed as "RF-sandwich," as the heterocycle is present between McbA R21 and McbC F43.

Synthase substrate tolerance and directionality

The effect of sequence variation of the precursor sequence G39SCG on the processing of the first heterocyclization sites to give OTZ39 has been investigated by mutagenesis of McbA$^{1-46}$ [87]: the G42A mutant was processed normally, G42N had a slight decrease in processing and only one heterocycle was observed by MS, and G42V was only partially processed with only one heterocycle observed by MS. G39A, S40T, C41T and G42H (data not shown) were not processed. The presence of an asparagine after site 50 (G50C51S52N53) is detrimental to processing: only 16% of the sites are fully processed to form the bis-heterocycle TOZ50N53 found in MccB17Δ^{+1}; the remaining sites are processed to the single heterocycle TAZ50S53N53 present in MccB17. MccB17 N53K mutant is processed like MccB17 [32], showing that K has the same effect as N on the processing of site 50.

McbA processing by microcin synthase is distributive: a time-course of *in vitro* heterocycle formation in McbA with purified microcin synthase showed that intermediates containing one to three heterocycles accumulate in the first 3 h; after 16 h, all intermediates from one to eight heterocycles are present; over the next 14 h, intermediates with six to eight heterocycles accumulate leading to MccB17 as the main product after 30 h [88]. McbA

processing by microcin synthase follows an overall N to C directionality driven by the synthase preference for thiazole formation over oxazole [88]. This selectivity was illustrated by the processing of site 39 mutants in McbA$^{1-46}$: cysteine-containing substrates have a rapid turnover, whereas serine-containing substrates have a slow turnover. Moreover, the G39GC substrate is converted more rapidly than G39CG, highlighting the importance of the position of the cyclised residue. In the context of the bis-heterocycle, the following order for the rate of formation is observed: G39SC > G39CC > G39SS > G39CS. Interestingly, the rate of formation of the first heterocycle is different and occurs in the following order: G39CC > G39CS > G39SC > G39SS. It is interesting to note as well that for G39CS a first heterocycle substrate is cyclized rapidly but then stalls and is not processed further to the bis-heterocycle, whereas the other substrates (G39SS, G39SC, G39CC) produce predominantly the bis-heterocyclic species.

Further MS study of full McbA processing provided the following pattern for heterocyclization: the first site processed is OTZ39, then thiazole from site TAZ47, TAZ50 and TOZ54 are formed, followed by the oxazole at site TOZ54. Finally, the last two oxazoles at site OAZ61 and OAZ64 are processed. The analysis could not discriminate the order of formation between these different elements; for example, it is not clear if thiazole from site 39 is formed before the oxazole of the same site, and TAZ47 formed before TAZ50. To address the importance of upstream heterocycles in the introduction of downstream heterocycles, cysteines at sites 39, 47 and 50 were mutated to prolines [74]: removal of the OTZ39 bis-heterocycle slowed down significantly the processing to a 7-heterocycle-containing molecule after overnight incubation. The additional mutation of C47 slowed the reaction further, and the triple mutant could not be processed. The directionality is partially explained by the kinetics of cysteine processing compared to serine and the environment of the residues, but further molecular details are needed to explain all the observations. The finer details of the tuning of this directionality could not be inferred directly from the crystal structure but prompted two hypotheses: the first suggested was that the fixed point represented by the leader peptide, which is critical for the processing, creates a local concentration that promotes processing of the closest processed site. As the peptide is processed, it gains in rigidity and possibly interacts with secondary heterocyclic binding sites present on McbC promoting further downstream heterocyclization. The second hypothesis is linked with the fact that the leader peptide is resistant to proteolysis when in the context of McbA, suggesting that the folding of McbA protects the leader peptide. The directionality of the processing would then be prompted by the unravelling of McbA upon binding of the leader peptide to McbB, which would expose sequentially the heterocyclization sites.

Coupling of heterocyclization and dehydrogenation

We have mentioned earlier that McbA processing by microcin synthase is distributive, which means that the substrate is released between each introduction of heterocycle; however, no incompletely processed azoline intermediates of MccB17 are detected [89]. In addition, McbA processing by a dehydrogenase-deficient synthase (McbC Y202A) yielded a modified McbA containing only a single azoline [74,84]. Taken together, this evidence suggests that heterocyclization and dehydrogenation are tightly coupled. The crystal structure shows the cyclodehydration and the dehydrogenation sites to be far apart, which leaves the question as to how this tight coupling works and particularly how the newly synthesized azoline switches from McbD to McbC.

MccB17 maturation TldD/TldE (PmbA)

The final step of MccB17 production involves the removal of the leader peptide by the TldD/TldE protease. This protease is composed of two related subunits TldE (also known as PmbA) [72] and TldD [90], that share 20% identity at the protein level [91]. The latter is a metalloprotease that is not specific to MccB17 processing as it has also been linked to the degradation of CcdA, the anti-toxin against CcdB [90]. Deletion of TldD, TldE, or both causes the accumulation of the MccB17 precursor in the cell as the leader peptide is not removed and the precursor cannot be exported [90,92]. Recently, the structure of TldD/E has been solved and reveals a surprising novel proteolytic mechanism dubbed a “molecular pencil sharpener” [92]. The TldD/E complex is a hollow sphere possessing a small entry channel that can only be accessed by unfolded peptides. Once inside the sphere, the peptide is non-specifically cleaved by the thermolysin-type-protease [92]. The conserved HExxxH motif in TldD was confirmed to coordinate a metal ion via two His residues, with an additional ligand provided by a conserved C residue located near the C terminus. The MccB17 precursor McbA is not digested by TldD/E, but once it is processed by the synthase to LP-MccB17, the leader peptide segment becomes susceptible to proteolysis and is degraded to produce mature MccB17. This suggests that McbA is folded in its native form, then it unfolds to interact with the McbBCD complex. Once McbA has been processed to LP-MccB17, the leader peptide is not able to fold with the MccB17 segment and is digested by TldD/E. The presence of species lacking one to three heterocycles in cell extracts of MccB17-producing strains in decreasing concentration

($MccB17\Delta^{-1}$ > $MccB17\Delta^{-2}$ > $MccB17\Delta^{-3}$) [18] suggests that the leader peptide is increasingly destabilized as heterocycles are introduced in McbA and starts to become sensitive to TldD/TldE proteolysis after the addition of five heterocycles.

MccB17 export

McB17 is exported from the producing organism by an inner membrane ABC transporter formed by McbE and McbF; this transporter belongs to the ABC2 type. McbE is a 241-amino-acid hydrophobic protein (MW = 27.8 kDa) containing six transmembrane domains; McbF is a 247-amino-acid nucleotide-binding protein (MW = 28.8 kDa) [46]. McbEF was the first multicomponent ABC transporter involved in export to be identified. McbEF has a double role, enabling the toxin to reach competing organisms and, in combination with McbG, providing immunity to the producing strain [46].

MccB17 and Inflammatory Bowel Disease

Although a major motivation for studying MccB17 has been the hope that its mode of action can be exploited to develop new antibiotics, most work on this toxin has been of a fundamental nature. Therefore, it was very surprising when a link between MccB17 and inflammatory bowel disease (IBD) was recently reported [93]. The importance of the gut microbiome to human health has long been appreciated [94–96], and connections between bacterial toxins and human health have been made. For example, van Hemert *et al*. [97] showed that genes from the bacterial species *Lactobacillus plantarum*, a commensal bacterium, were involved in the modulation of the cytokine response of human peripheral blood mononuclear cells, including genes encoding components of the plantaricin (a bacteriocin) biosynthesis and transport pathway. In other words, toxin production might be responsible for the stimulation of anti- or pro-inflammatory immune responses in the gut [97,98]. A key development has been the use of mouse models in the study of human IBD [99] and showing that chemical agents can induce inflammation [100].

More recent work [93] has shown that oxazoles derived from the diet, industrial sources and microbes can stimulate intestinal inflammation. Using a mouse model, they concluded that breakdown products from MccB17 (but not the intact toxin itself) can trigger inflammation in the gastrointestinal tract, thus linking MccB17 and the commensal bacteria that produce the toxin to IBD and Crohn's disease. The implications of this work are far-reaching and could be important in considering alternative therapies for these chronic diseases.

Future Perspectives

Considerations of the mode of action of McccB17

The precise interaction of MccB17 with gyrase remains elusive; we will try, however, to bring together the fragmented information gathered so far to discuss possible modes of action of MccB17. The first piece of evidence concerns residues conferring resistance to MccB17. Despite significant effort, only one residue has been identified to confer resistance; GyrB W751 confers resistance when mutated to a polar residue or G, whereas gyrase remains susceptible when this residue is mutated to aromatic residues. This suggests that GyrB W751 is a possible a site of interaction with MccB17, and the fact that mutation of this residue to other aromatic residues maintains susceptibility to MccB17 suggests that stacking interaction might be involved; however, since no mutants with lipophilic side chains, like Leu or Ile, were investigated, simple lipophilic interaction cannot be ruled out. Aside from the MccB17 heterocyclic core, the C-terminal triad would be a likely candidate for such interaction since it has been shown to be critical for MccB17's ability to stabilize the cleavage complex. This hypothesis could be investigated further with C-terminal deletion of MccB17 reported by Collin *et al*. [32] and Shkundina *et al*. [33] on the W751R and W751K gyrase mutants: the shift of the negatively charged C-terminal carboxyl could enable the MccB17 variant to form an ionic bridge with the positively charged amine residue present at position 751 in mutants resistant to MccB17. In the available crystal structures of the gyrase core–DNA cleavage complex with CFX (GyrB-CTD + GyrA-NTD), GyrB W751 is positioned on the outside surface of the GyrB dimer at the level of the DNA gate (Fig. 10). It has been shown that mutation in gyrase conferring resistance to quinolones, GyrB K447E and GyrA S83 W, affects MccB17's activity but to a lesser degree than GyrBW751R. This would suggest that the MccB17 site of interaction has some overlap with that of quinolones. Pierrat and Maxwell [27] gathered strong evidence that MccB17 requires DNA strand passage to interact with gyrase. An open DNA-gate conformation would make it possible for MccB17 to interact both near the quinolone site and at GyrB W751. Moreover, CcdB, another bacterial toxin requiring strand passage to promote DNA–gyrase cleavage complex [32], could shed light on MccB17's mode of action. CcdB stabilizes the gyrase–DNA complex by inserting as a dimer in a cavity formed between the two GyrA-NTD during strand passage, thus locking the DNA gate in an open state [101]. Following the same logic, it is possible that

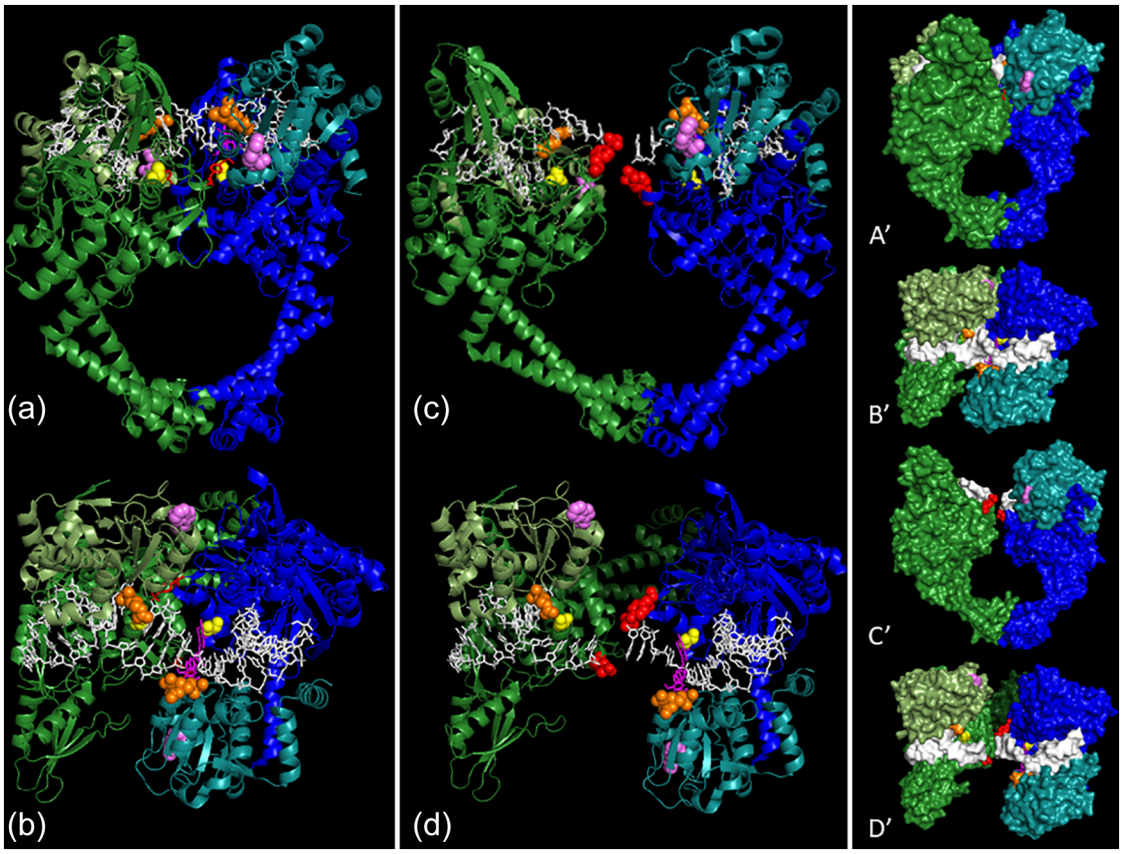


Fig. 10. Mutations conferring resistance to MccB17 and quinolones in the context of the cleavage complex: the gyrase cleavage complex with the topoisomerase poison etoposide is shown with the residues important for MccB17 action. Two GyrB C-terminal domains are shown, respectively, in smudge green and deep teal; two GyrA N-terminal domains are shown in blue and dark green. The DNA is shown as white sticks. Catalytic tyrosines (GyrA Y122) that form a covalent bond with DNA are shown as spheres in red. GyrB W751 (*E. coli*), the residue responsible for specific resistance to MccB17, is shown as violet spheres. GyrB K447 (*E. coli*) shown as orange spheres and GyrA S83 (*E. coli*) shown as yellow spheres are two residues involved in quinolone resistance that also influence MccB17 activity [23]. Etoposide is shown as magenta sticks inserted in one of the cleavage site (only one of its two binding sites is shown for clarity). a and b show, respectively, the crystal structure of *Staphylococcus aureus* gyrase cleavage complex with Etoposide (PDB: 5CDN) [106] seen from the side and from the top. A′ and B′ are the same views but shown as molecular surfaces. c and d are the side and top view of an hypothetical open conformation of the gyrase DNA gate constructed from *S. aureus* gyrase cleavage complex with Etoposide (PDB: 5CDN) and the *B. subtilis* GyrA-N-terminal domain dimer in an open gate conformation (PDB: 4DDQ) [107]. C′ and D′ are the corresponding views shown as molecular surfaces. The position of different residues influencing MccB17 activity on DNA gyrase combined with the position of the topoisomerase poison Etoposide in the cleavage site, particularly its interaction with DNA, shows the potential area of MccB17 interaction with the gyrase–DNA cleavage complex.

MccB17 uses the same "foot-in-the door" mechanism to stabilize the cleavage complex but at a different site. KLB is a RiPP containing three thiazoles, one oxazole and an amidine ring. KLB is an antibacterial that inhibits protein synthesis by blocking the nascent peptide exit tunnel in the 70s ribosome. In the crystal structure with *Thermus thermophilus* 70S ribosomes, KLB shows stacking interactions between two thiazoles residues and the 23S-rRNA [102]. It is possible that some of the heterocyclic moieties of MccB17 have similar interactions with DNA; the cleaved DNA in the gyrase DNA gate would be a likely candidate for such interactions. We suggest that MccB17 could fit in the open space created by the open DNA gate promoted by DNA strand passage' the main interaction of MccB17 would be with the unpaired bases of the DNA gate and GyrB W751. Such an open-gate conformation would be only revealed in the presence of the transported DNA segment and would be consistent with the fact that a relatively long piece of DNA is required for MccB17-induced DNA cleavage [27] and the stimulatory effect of ATP [22]. Figure 11 shows an exaggerated open DNA gate, which has no biological relevance, but allows us to see the sites conferring resistance to

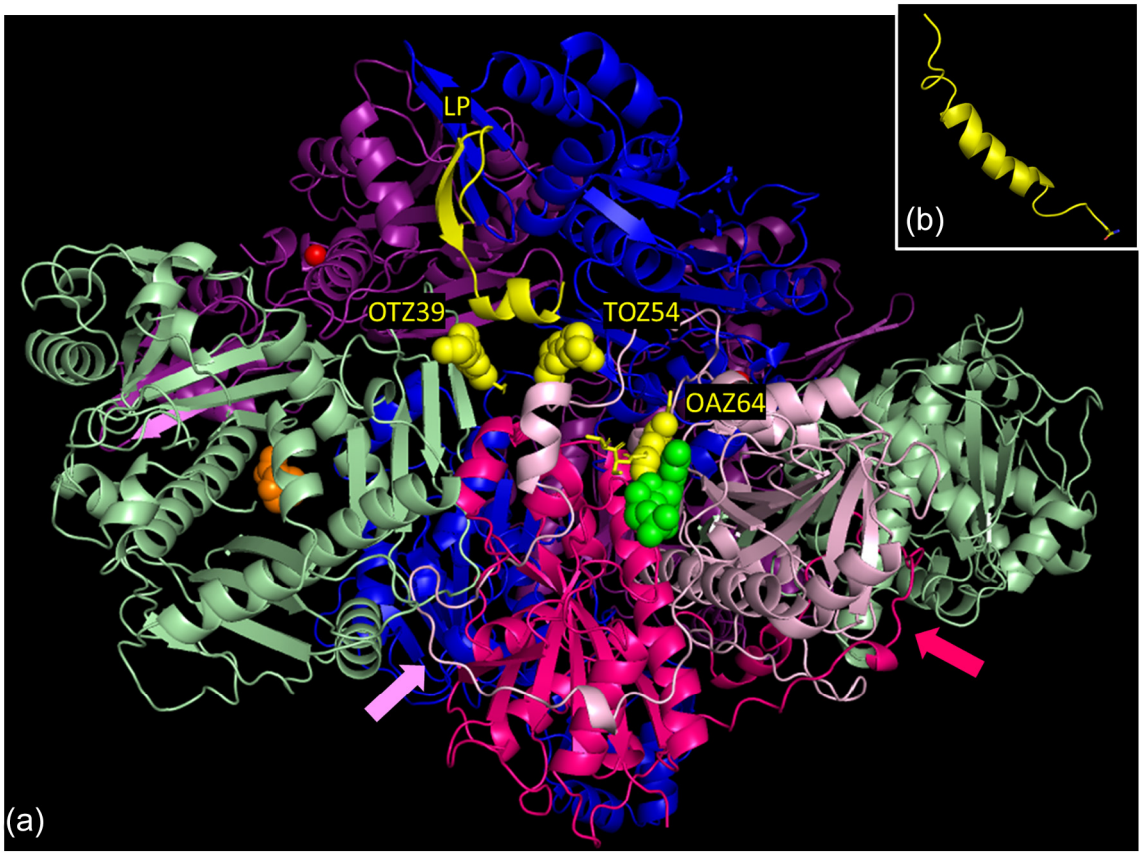


Fig. 11. Structure of the MccB17 synthase [74]. (a) MccB17 synthase is organized as an octamer formed through 2-fold symmetry of the tetramer composed of two McbBs in different conformations, McbB1 (in blue) and McbB2 (in purple), one McbC (in pink or violet) and one McbD (in light green). McbB1 and McbB2 form a dimer in a head to tail arrangement. McbB's structural Zn atoms are show as red spheres. MccB17 is shown in yellow, and the leader peptide (labeled LP) is shown as a yellow β-sheet connected to a α-helix bound to the McbB dimer, mainly interacting with McbB1 (blue). Four heterocycles of MccB17 are shown as yellow spheres labeled with the heterocycle name: OTZ39 TOZ54 OAZ64. MccB17 C-terminal residues G66, S67 and H68 are shown as yellow sticks. No interactions between with the cyclodehydratase McbD and MccB17 are visible, and McbD converts MccB17 Ser and Cys residues into the corresponding azoline. The active site of McbD is highlighted by P396 shown as orange spheres; P396 has been shown to have an important role in the catalysis of the heterocyclization [74]. The dehydrogenase McbC (in pink or violet) converts azolines synthesized by McbD to azole, OAZ64 is visible bound in the active site of McbC, interacting with the FMN co-factor (shown as green spheres). McbC is arranged as a homodimer at the center of the octamer. The dimer is stabilized by one monomer embracing the other monomer with a 50-residue-long loop seen in violet pointed out by a violet arrow on one monomer, and seen in pink pointed out by a pink arrow in the other. For a better visibility MccB17 fragments, FMN, and P396 are only shown for one McbB1B2CD tetramer, but the other is similarly occupied. (b) NMR structure of the leader peptide with a C-terminal amide in solution (PDB: 2MLP) suggests a change of conformation between unbound and bound state.

MccB17 and to quinolones and consider the space where MccB17 could be interacting. If this hypothesis is correct, it would explain why, despite significant efforts over the years, no crystal structures of MccB17 have been obtained with gyrase, since obtaining a gyrase–DNA complex in the right conformation to interact with MccB17 for crystallography trials would require a creative approach. However, obtaining such structure would be a unique chance to capture gyrase in an open DNA-gate conformation.

MccB17 as template for new topoisomerase poisons

MccB17 has been considered as a compound of limited interest due its poor solubility and the restrictions caused by its uptake based on non-essential protein transporters, which enable bacteria to acquire transport-based resistance relatively easily. However, the extended knowledge of the MccB17 biosynthetic pathway offers possibilities to circumvent these limitations in order to

produce a MccB17-inspired antibacterial drug. Producing variants of MccB17 have been a hit or miss matter until the role of the TldD/E protease in MccB17 synthesis was fully understood [92]: mutating the precursor McbA could cause it to become susceptible to the proteolysis by the non-specific protease TldD/E before post-translational modification takes place, and this leads to a shortened version of the desired MccB17 variant or nothing at all. This problem can be overcome by using TldD/E-deficient *E. coli* strains and using an alternative protease to remove the leader peptide; such a TldD/E-deficient strain has been used successfully by Ghilarov *et al*. [74] to isolate Pro-MccB17 for x-ray crystallography with MccB17 synthase. A lysine could be inserted between the leader peptide and the core sequence to enable the removal of the leader peptide by tryptic digest. However, Pro-MccB17 is toxic as it accumulates in the cell so such a method would have limited yield. A more elegant approach would be to insert a specific protease site like TEV or thrombin between the leader peptide and the Core Sequence and co-express the corresponding protease. We have mentioned that the N-terminus of MccB17 is amenable to significant modifications without altering the cleavage activity and that holds true as well for the C-terminus to a lesser degree as shown by the significant difference of sequence between MccB17 and Ps_MccB17 C-terminus. The ability to modify N- and C-terminus provides two handles to improve the solubility and introduce motifs/molecules for alternative uptake of the MccB17 core. Topoisomerase poisons have two fields of application: antibacterial drugs with gyrase and topoisomerase IV, and anti-cancer drugs with human topoisomerase II. All these enzymes share the same mechanism involving double-stranded DNA cleavage and strand passage; once the mechanism employed by MccB17 on *E. coli* gyrase is understood, it might be extended to other type II topoisomerases and organisms. The ability to generate variants of MccB17 provides the potential for selectivity toward a specific topoisomerase. In addition, bacteriocins and microcins have been reported to generally show potential as anti-cancer agents as reviewed by Baindara *et al*. [103], even if MccB17 is not among the compounds that have been tested.

MccB17 biosynthetic pathway as a source of chemical diversity

The concepts exposed for making the MccB17 core druggable can be extended to the generation of designer TOMMs. We have shown that the MccB17 biosynthetic pathway is robust and flexible; however, heterocycle processing has some limitations. The MccB17 synthase can be efficiently isolated to be used *in vitro* to introduce oxazoles and thiazoles in a designed substrate peptide [74,87], thus exploiting the MccB17 biosynthetic pathway to produce novel oxazole–thiazole-containing peptides. Elements from other TOMMs or LAPs biosynthetic pathways could be considered to add chemical diversity such as the amidine formation capacity of KlpD, the equivalent of McbD in Klebsazolicin [104] or the N–C cyclization seen in Cyanobactines [80,105].

Avenues to explore with MccB17

There are still a lot of unknowns surrounding MccB17. Very little is known about the PRP McbG, and, as shown by MfpA, the mode of action of such PRPs might not be straightforward. Even if we consider McbG as a simple DNA mimic, is it substituting for the DNA gate (G) segment or for the transported (T) segment? Similarly, scarce information is available about the nature of McbE/McbF transporter, and the selectivity and mechanism of such efflux pumps would be valuable information for the field of ABC transporters and might prove relevant in the context of antibacterial resistance. Despite significant leaps with the crystal structures that have confirmed the role of the different elements, there are still questions about the mechanism of the MccB17 synthase, McbBCD: there is a tight coordination between the cyclodehydration by McbD and the dehydrogenation by McbC as no azoline intermediates are released; how the substrate shifts from McbD to McbC during MccB17 processing remains to be addressed. There is strong evidence that the synthase biological structure is an octamer comprising two functional tetramers, raising the question of the benefit of this octameric assembly; the homodimerization of McbC provides stability to the complex, but is there a collaboration between the two tetramers for MccB17 processing? Finally, as we mentioned earlier, solving the molecular details of the MccB17 mode of action on gyrase would be of great value both for our understanding of gyrase mechanism and for antibacterial discovery.

Acknowledgments

We thank Dmitry Ghilarov, Jonathan Heddle and Rebecca Lo for helpful comments on the manuscript. F.C. was supported by grant BB/J016853/1 from the Biotechnology and Biosciences Research Council, UK (to A.M.), and work in A.M.'s lab is also funded by the Biotechnology and Biosciences Research Council

Institute Strategic Programme Grants BB/J004561/1 and BB/P012523/1.

Received 5 December 2018;
Received in revised form 28 May 2019;
Available online 08 June 2019

Keywords:
TOMMs;
DNA gyrase;
DNA topoisomerases;
microbial toxins

Present address: F. Collin, Institute for Physical Chemistry, University of Muenster, Correnstrasse 30, 48149 Muenster, Germany.

Abbreviations used:
McCB17, microcin B17; TOMM, thiazole–oxazole-modified microcin; LAP, linear azole-containing peptide; MS, mass spectrometry; CFX, ciprofloxacin; OAZ, oxazole; TAZ, thiazole; OTZ, oxazole–thiazole; TOZ, thiazole–oxazole; BPA, *L*-4-benzoylphenylalanine; FMN, flavin mononucleotide; IBD, inflammatory bowel disease; KLB, Klebsazolicin.

References

[1] R. Bax, S. Green, Antibiotics: the changing regulatory and pharmaceutical industry paradigm, J. Antimicrob. Chemother. 70 (2015) 1281–1284, https://doi.org/10.1093/jac/dku572.
[2] S. O'Brien, Meeting the societal need for new antibiotics: the challenges for the pharmaceutical industry, Br. J. Clin. Pharmacol. 79 (2015) 168–172, https://doi.org/10.1111/bcp.12401.
[3] D.J. Payne, M.N. Gwynn, D.J. Holmes, D.L. Pompliano, Drugs for bad bugs: confronting the challenges of antibacterial discovery, Nat. Rev. Drug Discov. 6 (2007) 29–40, https://doi.org/10.1038/nrd2201.
[4] R. Tommasi, D.G. Brown, G.K. Walkup, J.I. Manchester, A.A. Miller, ESKAPEing the labyrinth of antibacterial discovery, Nat. Rev. Drug Discov. 14 (2015) 529–542, https://doi.org/10.1038/nrd4572.
[5] A.R.M. Coates, G. Halls, Y. Hu, Novel classes of antibiotics or more of the same? Br. J. Pharmacol. 163 (2011) 184–194, https://doi.org/10.1111/j.1476-5381.2011.01250.x.
[6] C. Walsh, T. Wencewicz, Antibiotics: Challenges, Mechanisms, Opportunities, 2nd Edition, American Society of Microbiology 2016https://doi.org/10.1128/9781555819316.
[7] K.J. Aldred, R.J. Kerns, N. Osheroff, Mechanism of quinolone action and resistance, Biochemistry. 53 (2014) 1565–1574, https://doi.org/10.1021/bi5000564.
[8] N.G. Bush, K. Evans-Roberts, A. Maxwell, DNA topoisomerases, EcoSal Plus 6 (2015)https://doi.org/10.1128/ecosalplus.ESP-0010-2014.
[9] S. Duquesne, D. Destoumieux-Garzón, J. Peduzzi, S. Rebuffat, Microcins, gene-encoded antibacterial peptides from enterobacteria, Nat. Prod. Rep. 24 (2007) 708, https://doi.org/10.1039/b516237h.
[10] P.G. Arnison, M.J. Bibb, G. Bierbaum, A.A. Bowers, T.S. Bugni, G. Bulaj, J.A. Camarero, D.J. Campopiano, G.L. Challis, J. Clardy, P.D. Cotter, D.J. Craik, M. Dawson, E. Dittmann, S. Donadio, P.C. Dorrestein, K.-D. Entian, M.A. Fischbach, J.S. Garavelli, U. Göransson, C.W. Gruber, D. H. Haft, T.K. Hemscheidt, C. Hertweck, C. Hill, A.R. Horswill, M. Jaspars, W.L. Kelly, J.P. Klinman, O.P. Kuipers, A.J. Link, W. Liu, M.A. Marahiel, D.A. Mitchell, G.N. Moll, B.S. Moore, R. Müller, S.K. Nair, I.F. Nes, G.E. Norris, B.M. Olivera, H. Onaka, M.L. Patchett, J. Piel, M.J. T. Reaney, S. Rebuffat, R.P. Ross, H.-G. Sahl, E.W. Schmidt, M.E. Selsted, K. Severinov, B. Shen, K. Sivonen, L. Smith, T. Stein, R.D. Süssmuth, J.R. Tagg, G.-L. Tang, A. W. Truman, J.C. Vederas, C.T. Walsh, J.D. Walton, S.C. Wenzel, J.M. Willey, W.A. van der Donk, Ribosomally synthesized and post-translationally modified peptide natural products: overview and recommendations for a universal nomenclature, Nat. Prod. Rep. 30 (2013) 108–160, https://doi.org/10.1039/c2np20085f.
[11] W.M. Parks, A.R. Bottrill, O.A. Pierrat, M.C. Durrant, A. Maxwell, The action of the bacterial toxin, microcin B17, on DNA gyrase, Biochimie. 89 (2007) 500–507, https://doi.org/10.1016/J.BIOCHI.2006.12.005.
[12] C. Asensio, J.C. Perez-Dfaz, M.C. Martlnez, F. Baquero, S. De Bacteriolog, R. Sanitaria, L. Paz, A New Family of Low Molecular Weight Antibiotics from Enterobacteria, https://eurekamag.com/pdf/004/004594197.pdf 1976.
[13] J. Davagnino, M. Herrero, D. Furlong, F. Moreno, R. Kolter, The DNA replication inhibitor microcin B17 is a forty-three-amino-acid protein containing sixty percent glycine, Proteins Struct. Funct. Bioinforma. 1 (1986) 230–238, https://doi.org/10.1002/prot.340010305.
[14] A. Bayer, S. Freund, G. Nicholson, G. Jung, Posttranslational backbone modifications in the ribosomal biosynthesis of the glycine-rich antibiotic microcin B17, Angew. Chemie Int. Ed. English. 32 (1993) 1336–1339, https://doi.org/10.1002/anie.199313361.
[15] P. Yorgey, J. Lee, J. Kördel, E. Vivas, P. Warner, D. Jebaratnam, R. Kolter, Posttranslational modifications in microcin B17 define an additional class of DNA gyrase inhibitor, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 4519–4523, https://doi.org/10.1073/pnas.91.10.4519.
[16] P. Yorgey, J. Davagnino, R. Kolter, The maturation pathway of microcin B17, a peptide inhibitor of DNA gyrase, Mol. Microbiol. 9 (1993) 897–905, https://doi.org/10.1111/j.1365-2958.1993.tb01747.x.
[17] N.L. Kelleher, C.L. Hendrickson, C.T. Walsh, Posttranslational heterocyclization of cysteine and serine residues in the antibiotic microcin B17: distributivity and directionality, Biochemistry. 38 (1999) 15623–15630, https://doi.org/10.1021/bi9913698.
[18] D. Ghilarov, M. Serebryakova, I. Shkundina, K. Severinov, A major portion of DNA gyrase inhibitor microcin B17 undergoes an N,O-peptidyl shift during synthesis., J. Biol. Chem. 286 (2011) 26308–18. https://doi.org/10.1074/jbc.M111.241315.
[19] M. Herrero, F. Moreno, Microcin B17 blocks DNA replication and induces the SOS system in *Escherichia coli*., J. Gen. Microbiol. 132 (1986) 393–402. doi:https://doi.org/10.1099/00221287-132-2-393.
[20] J.L. Vizan, C. Hernandez-Chico, I. del Castillo, F. Moreno, The peptide antibiotic microcin B17 induces double-strand cleavage of DNA mediated by *E. coli* DNA gyrase, Embo J. 10 (1991) 467–476.

[21] D.B. Zamble, D.A. Miller, J.G. Heddle, A. Maxwell, C.T. Walsh, F. Hollfelder, In vitro characterization of DNA gyrase inhibition by microcin B17 analogs with altered bisheterocyclic sites, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 7712–7717, https://doi.org/10.1073/pnas.141225698.

[22] O.A. Pierrat, A. Maxwell, The action of the bacterial toxin microcin B17. Insight into the cleavage-religation reaction of DNA gyrase., J. Biol. Chem. 278 (2003) 35016–23. https://doi.org/10.1074/jbc.M304516200.

[23] J.G. Heddle, S.J. Blance, D.B. Zamble, F. Hollfelder, D.A. Miller, L.M. Wentzell, C.T. Walsh, A. Maxwell, The antibiotic microcin B17 is a DNA gyrase poison: characterisation of the mode of inhibition, J. Mol. Biol. 307 (2001) 1223–1234, https://doi.org/10.1006/jmbi.2001.4562.

[24] L. Costenaro, J.G. Grossmann, C. Ebel, A. Maxwell, Modular structure of the full-length DNA gyrase B subunit revealed by small-angle x-ray scattering, Structure. 15 (2007) 329–339, https://doi.org/10.1016/j.str.2007.01.013.

[25] N.L. Williams, A. Maxwell, Probing the two-gate mechanism of DNA gyrase using cysteine cross-linking, Biochemistry. 38 (1999) 13502–13511, http://www.ncbi.nlm.nih.gov/pubmed/10521257.

[26] M. Cove, A.P. Tingey, A. Maxwell, DNA gyrase can cleave short DNA fragments in the presence of quinolone drugs, Nucleic Acids Res. 25 (1997) 2716–2722, https://doi.org/10.1093/nar/25.14.2716.

[27] O.A. Pierrat, A. Maxwell, Evidence for the role of DNA strand passage in the mechanism of action of microcin B17 on DNA gyrase, Biochemistry. 44 (2005) 4204–4215, https://doi.org/10.1021/bi0478751.

[28] M. Metelev, M. Serebryakov, D. Ghilarov, Y. Zhao, K. Severinov, Structure of microcin B-like compounds produced by *Pseudomonas syringae* and species specificity of their antibacterial action, J. Bacteriol. 195 (2013) 4129–4137, https://doi.org/10.1128/JB.00665-13.

[29] C.L. Cox, J.R. Doroghazi, D.A. Mitchell, The genomic landscape of ribosomal peptides containing thiazole and oxazole heterocycles, BMC Genomics 16 (2015) 778, https://doi.org/10.1186/s12864-015-2008-0.

[30] R.S. Roy, N.L. Kelleher, J.C. Milne, C.T. Walsh, In vivo processing and antibiotic activity of microcin B17 analogs with varying ring content and altered bisheterocyclic sites, Chem. Biol. 6 (1999) 305–318, https://doi.org/10.1016/S1074-5521(99)80076-3.

[31] R.E. Thompson, F. Collin, A. Maxwell, K.A. Jolliffe, R.J. Payne, Synthesis of full length and truncated microcin B17 analogues as DNA gyrase poisons, Org. Biomol. Chem. 12 (2014) 1570–1578, https://doi.org/10.1039/C3OB42516A.

[32] F. Collin, R.E. Thompson, K.A. Jolliffe, R.J. Payne, A. Maxwell, Fragments of the bacterial toxin microcin B17 as gyrase poisons., PLoS One. 8 (2013) e61459. https://doi.org/10.1371/journal.pone.0061459.

[33] I. Shkundina, M. Serebryakova, K. Severinov, The C-terminal part of microcin B is crucial for DNA gyrase inhibition and antibiotic uptake by sensitive cells., J. Bacteriol. 196 (2014) 1759–67. https://doi.org/10.1128/JB.00015-14.

[34] M. Lavina, A.P. Pugsley, F. Moreno, Identification, mapping, cloning and characterization of a gene (sbmA) required for microcin B17 action on *Escherichia coli* K12, Microbiology. 132 (1986) 1685–1693, https://doi.org/10.1099/00221287-132-6-1685.

[35] J. Glazebrook, A. Ichige, G.C. Walker, A *Rhizobium meliloti* homolog of the *Escherichia coli* peptide-antibiotic transport protein SbmA is essential for bacteroid development., Genes Dev. 7 (1993) 1485–97. http://www.ncbi.nlm.nih.gov/pubmed/8393417.

[36] M. Pränting, A. Negrea, M. Rhen, D.I. Andersson, Mechanism and fitness costs of PR-39 resistance in *Salmonella enterica* serovar Typhimurium LT2, Antimicrob. Agents Chemother. 52 (2008) 2734–2741, https://doi.org/10.1128/AAC.00205-08.

[37] K. LeVier, G.C. Walker, Genetic analysis of the *Sinorhizobium meliloti* BacA protein: differential effects of mutations on phenotypes, J. Bacteriol. 183 (2001) 6444–6453, https://doi.org/10.1128/JB.183.21.6444-6453.2001.

[38] G. Runti, M.d.C. Lopez Ruiz, T. Stoilova, R. Hussain, M. Jennions, H.G. Choudhury, M. Benincasa, R. Gennaro, K. Beis, M. Scocchi, Functional characterization of SbmA, a bacterial inner membrane transporter required for importing the antimicrobial peptide Bac7(1–35), J. Bacteriol. 195 (2013) 5343–5351. https://doi.org/10.1128/JB.00818-13.

[39] N. Corbalan, G. Runti, C. Adler, S. Covaceuszach, R.C. Ford, D. Lamba, K. Beis, M. Scocchi, P.A. Vincent, Functional and structural study of the dimeric inner membrane protein SbmA, J. Bacteriol. 195 (2013) 5352–5361, https://doi.org/10.1128/JB.00824-13.

[40] R.A. Salomon, R.N. Farias, The peptide antibiotic microcin 25 is imported through the tonB pathway and the sbmA protein, J. Bacteriol. 177 (1995) 3323–3325, https://doi.org/10.1128/jb.177.11.3323-3325.1995.

[41] M. Mattiuzzo, A. Bandiera, R. Gennaro, M. Benincasa, S. Pacor, N. Antcheva, M. Scocchi, Role of the Escherichia coli SbmA in the antimicrobial activity of proline-rich peptides, Mol. Microbiol. 66 (2007) 151–163, https://doi.org/10.1111/j.1365-2958.2007.05903.x.

[42] A. Ghosal, A. Vitali, J.E.M. Stach, P.E. Nielsen, Role of SbmA in the uptake of peptide nucleic acid (PNA)–peptide conjugates in *E. coli*, ACS Chem. Biol. 8 (2013) 360–367. doi:https://doi.org/10.1021/cb300434e.

[43] S.E. Puckett, K.A. Reese, G.M. Mitev, V. Mullen, R.C. Johnson, K.R. Pomraning, B.L. Mellbye, L.D. Tilley, P.L. Iversen, M. Freitag, B.L. Geller, Bacterial resistance to antisense peptide phosphorodiamidate morpholino oligomers, Antimicrob. Agents Chemother. 56 (2012) 6147–6153, https://doi.org/10.1128/AAC.00850-12.

[44] M. Sato, K. Machida, E. Arikado, H. Saito, T. Kakegawa, H. Kobayashi, Expression of outer membrane proteins in *Escherichia coli* growing at acid pH, Appl. Environ. Microbiol. 66 (2000) 943–947https://www.ncbi.nlm.nih.gov/pmc/articles/PMC91927/pdf/am000943.pdf.

[45] R. Benz, Structure and function of porins from gram-negative bacteria, Annu. Rev. Microbiol. 42 (1988) 359–393, https://doi.org/10.1146/annurev.mi.42.100188.002043.

[46] M.D.C. Garrido, M. Herrero, R. Kolter, F. Moreno, The export of the DNA replication inhibitor microcin B17 provides immunity for the host cell; the export of the DNA replication inhibitor Microcin B17 provides immunity for the host cell., EMBO J. 7 (1988) 853–1862. https://doi.org/10.1002/j.1460-2075.1988.tb03018.x.

[47] M.W. Vetting, S.S. Hegde, J.E. Fajardo, A. Fiser, S.L. Roderick, H.E. Takiff, J.S. Blanchard, Pentapeptide repeat proteins, Biochemistry. 45 (2006) 1–10, https://doi.org/10.1021/bi052130w.

[48] S.M. Hashimi, M.K. Wall, A.B. Smith, A. Maxwell, R.G. Birch, The phytotoxin albicidin is a novel inhibitor of DNA

gyrase, Antimicrob. Agents Chemother. 51 (2007) 181–187, https://doi.org/10.1128/AAC.00918-06.
[49] S. Baumann, J. Herrmann, R. Raju, H. Steinmetz, K.I. Mohr, S. Hüttel, K. Harmrolfs, M. Stadler, R. Müller, Cystobactamids: myxobacterial topoisomerase inhibitors exhibiting potent antibacterial activity, Angew. Chemie Int. Ed. 53 (2014) 14605–14609, https://doi.org/10.1002/anie.201409964.
[50] J.H. Tran, G.A. Jacoby, Mechanism of plasmid-mediated quinolone resistance, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 5638–5642, https://doi.org/10.1073/pnas.082092899.
[51] C. Montero, G. Mateu, R. Rodriguez, H. Takiff, Intrinsic resistance of *Mycobacterium smegmatis* to fluoroquinolones may be influenced by new pentapeptide protein MfpA, Antimicrob. Agents Chemother. 45 (2001) 3387–3392, https://doi.org/10.1128/AAC.45.12.3387-3392.2001.
[52] S.S. Hegde, M.W. Vetting, S.L. Roderick, L.A. Mitchenall, A. Maxwell, H.E. Takiff, J.S. Blanchard, A fluoroquinolone resistance protein from Mycobacterium tuberculosis that mimics DNA, Science 308 (5727) (2005) 1480–1483, https://doi.org/10.1126/science.1110699.
[53] A. Mérens, S. Matrat, A. Aubry, C. Lascols, V. Jarlier, C.J. Soussy, J.D. Cavallo, E. Cambau, The pentapeptide repeat proteins MfpAMt and QnrB4 exhibit opposite effects on DNA gyrase catalytic reactions and on the ternary gyrase–DNA–quinolone complex, J. Bacteriol. 191 (2009) 1587–1594, https://doi.org/10.1128/JB.01205-08.
[54] S.S. Hegde, M.W. Vetting, L.A. Mitchenall, A. Maxwell, J.S. Blanchard, Structural and biochemical analysis of the pentapeptide repeat protein EfsQnr, a potent DNA gyrase inhibitor, Antimicrob. Agents Chemother. 55 (2011) 110–117, https://doi.org/10.1128/AAC.01158-10.
[55] S. Cociancich, A. Pesic, D. Petras, S. Uhlmann, J. Kretz, V. Schubert, L. Vieweg, S. Duplan, M. Marguerettaz, J. Noëll, I. Pieretti, M. Hügelland, S. Kemper, A. Mainz, P. Rott, M. Royer, R.D. Süssmuth, The gyrase inhibitor albicidin consists of p-aminobenzoic acids and cyanoalanine, Nat. Chem. Biol. 11 (2015) 195–197, https://doi.org/10.1038/nchembio.1734.
[56] M.W. Vetting, S.S. Hegde, Y. Zhang, J.S. Blanchard, Pentapeptide-repeat proteins that act as topoisomerase poison resistance factors have a common dimer interface, Acta Crystallogr. Sect. F. Struct. Biol. Cryst. Commun. 67 (2011) 296–302, https://doi.org/10.1107/S1744309110053315.
[57] J.H. Tran, G.A. Jacoby, D.C. Hooper, Interaction of the plasmid-encoded quinolone resistance protein Qnr with *Escherichia coli* DNA gyrase, Antimicrob. Agents Chemother. 49 (2005) 118–125, https://doi.org/10.1128/AAC.49.1.118-125.2005.
[58] G.A. Jacoby, M.A. Corcoran, D.C. Hooper, Protective effect of qnr on agents other than quinolones that target DNA gyrase, Antimicrob. Agents Chemother. 59 (2015) 6689–6695, https://doi.org/10.1128/AAC.01292-15.
[59] D.C. Hooper, G.A. Jacoby, Topoisomerase inhibitors: fluoroquinolone mechanisms of action and resistance, Cold Spring Harb. Perspect. Med. 6 (2016) a025320, https://doi.org/10.1101/cshperspect.a025320.
[60] O. Lomovskaya, F. Kawai, A. Matin, Differential regulation of the mcb and emr operons of *Escherichia coli*: role of mcb in multidrug resistance, Antimicrob. Agents Chemother. 40 (1996) 1050–1052, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC163261/pdf/401050.pdf.
[61] J. Tao, J. Han, H. Wu, X. Hu, J. Deng, J. Fleming, A. Maxwell, L. Bi, K. Mi, Mycobacterium fluoroquinolone resistance protein B, a novel small GTPase, is involved in the regulation of DNA gyrase and drug resistance, Nucleic Acids Res. 41 (2013) 2370–2381, https://doi.org/10.1093/nar/gks1351.
[62] M.R. Baquero, M. Bouzon, J. Varea, F. Moreno, sbmC, a stationary-phase induced SOS *Escherichia coli* gene, whose product protects cells from the DNA replication inhibitor microcin B17, Mol. Microbiol. 18 (1995) 301–311. https://doi.org/10.1111/j.1365-2958.1995.mmi_18020301.x.
[63] A. Nakanishi, T. Oshida, T. Matsushita, S. Imajoh-Ohmi, T. Ohnuki, Identification of DNA gyrase inhibitor (GyrI) in *Escherichia coli*, J. Biol. Chem. 273 (1998) 1933–1938, https://doi.org/10.1074/jbc.273.4.1933.
[64] A. Nakanishi, S. Imajoh-Ohmi, F. Hanaoka, Characterization of the interaction between DNA gyrase inhibitor and DNA gyrase of *Escherichia coli*, J. Biol. Chem. 277 (2002) 8949–8954, https://doi.org/10.1074/jbc.M111278200.
[65] M.J. Romanowski, S.A. Gibney, S.K. Burley, Crystal structure of the *Escherichia coli* SbmC protein that protects cells from the DNA replication inhibitor microcin B17, Proteins. 47 (2002) 403–407, http://www.ncbi.nlm.nih.gov/pubmed/11948793.
[66] M. Chatterji, S. Sengupta, V. Nagaraja, Chromosomally encoded gyrase inhibitor GyrI protects *Escherichia coli* against DNA-damaging agents, Arch. Microbiol. 180 (2003) 339–346, https://doi.org/10.1007/s00203-003-0598-4.
[67] S. Shah, J.G. Heddle, Squaring up to DNA: pentapeptide repeat proteins and DNA mimicry, Appl. Microbiol. Biotechnol. 98 (2014) 9545–9560, https://doi.org/10.1007/s00253-014-6151-3.
[68] F.J. Del Castillo, I. Del Castillo, F. Moreno, 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 (2001) 2137–2140, https://doi.org/10.1128/JB.183.6.2137-2140.2001.
[69] F. Baquero, D. Bouanchaud, M.C. Martinez-Perez, C. Fernandez, Microcin plasmids: a group of extrachromosomal elements coding for low-molecular-weight antibiotics in *Escherichia coli*, J. Bacteriol. 135 (1978) 342–347, http://www.ncbi.nlm.nih.gov/pubmed/355221.
[70] J.L. San Millan, R. Kolter, F. Moreno, Plasmid genes required for microcin B17 production, J. Bacteriol. 163 (1985) 1016–1020http://jb.asm.org/content/163/3/1016.full.pdf.
[71] O. Genilloud, F. Moreno, R. Kolter, DNA sequence, products, and transcriptional pattern of the genes involved in production of the DNA replication inhibitor microcin B17, J. Bacteriol. 171 (1989) 1126–1135, https://doi.org/10.1128/JB.171.2.1126-1135.1989.
[72] M.C. Rodríguez-Sáinz, C. Hernández-Chico, F. Moreno, Molecular characterization of pmbA, an *Escherichia coli* chromosomal gene required for the production of the antibiotic peptide MccB17, Mol. Microbiol. 4 (1990) 1921–1932, https://doi.org/10.1111/j.1365-2958.1990.tb02041.x.
[73] A. Bayer, S. Freund, G. Jung, Post-translational heterocyclic backbone modifications in the 43-peptide antibiotic microcin B17. Structure elucidation and NMR study of a 13c,18N-labelled gyrase inhibitor, Eur. J. Biochem. 234 (1995) 414–426. https://doi.org/10.1111/j.1432-1033.1995.414_b.x.
[74] D. Ghilarov, C.E.M. Stevenson, D.Y. Travin, J. Piskunova, M. Serebryakova, A. Maxwell, D.M. Lawson, K. Severinov, Architecture of Microcin B17 Synthetase: An Octameric Protein Complex Converting a Ribosomally Synthesized Peptide into a DNA Gyrase Poison, Mol Cell 73 (4) (2019) 749–762 e745. https://doi.org/10.1016/j.molcel.2018.11.032 .
[75] J.C. Milne, R. Sinha Roy, A.C. Eliot, N.L. Kelleher, A. Wokhlu, B. Nickels, C.T. Walsh, 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 (1999) 4768–4781, https://doi.org/10.1021/bi982975q.
[76] Y.M. Li, J.C. Milne, L.L. Madison, R. Kolter, C.T. Walsh, From peptide precursors to oxazole and thiazole-containing peptide antibiotics: microcin B17 synthase, Science (80-.). 274 (1996) 1188–1193. https://doi.org/10.1126/science.274.5290.1188.
[77] D.B. Zamble, C.P. McClure, J.E. Penner-Hahn, C.T. Walsh, The McbB component of microcin B17 synthetase is a zinc metalloprotein, Biochemistry. 39 (2000) 16190–16199, https://doi.org/10.1021/bi001398e.
[78] L.L. Madison, E.I. Vivas, Y.-M. Li, C.T. Walsh, R. Kolter, The leader peptide is essential for the post-translational modification of the DNA–gyrase inhibitor microcin B17, Mol. Microbiol. 23 (1997) 161–168, https://doi.org/10.1046/j.1365-2958.1997.2041565.x.
[79] W.E. Houssen, S.H. Wright, A.P. Kalverda, G.S. Thompson, S.M. Kelly, M. Jaspars, Solution structure of the leader sequence of the patellamide precursor peptide, PatE1–34, ChemBioChem. 11 (2010) 1867–1873, https://doi.org/10.1002/cbic.201000305.
[80] J. Koehnke, G. Mann, A.F. Bent, H. Ludewig, S. Shirran, C. Botting, T. Lebl, W. Houssen, M. Jaspars, J.H. Naismith, Structural analysis of leader peptide binding enables leader-free cyanobactin processing, Nat. Chem. Biol. 11 (2015) 558–563, https://doi.org/10.1038/nchembio.1841.
[81] R. Sinha Roy, S. Kim, J.D. Baleja, C.T. Walsh, Role of the microcin B17 propeptide in substrate recognition: solution structure and mutational analysis of McbA1–26, Chem. Biol. 5 (1998) 217–228, https://doi.org/10.1016/S1074-5521(98)90635-4.
[82] B.J. Burkhart, C.J. Schwalen, G. Mann, J.H. Naismith, D.A. Mitchell, YcaO-dependent posttranslational amide activation: biosynthesis, structure, and function., Chem. Rev. 117 (2017) 5389–5456. https://doi.org/10.1021/acs.chemrev.6b00623.
[83] J.C. Milne, A.C. Eliot, N.L. Kelleher, C.T. Walsh, ATP/GTP hydrolysis is required for oxazole and thiazole biosynthesis in the peptide antibiotic microcin B17, Biochemistry. 37 (1998) 13250–13261, https://doi.org/10.1021/bi980996e.
[84] K.L. Dunbar, D.A. Mitchell, Insights into the mechanism of peptide cyclodehydrations achieved through the chemoenzymatic generation of amide derivatives, J. Am. Chem. Soc. 135 (2013) 8692–8701, https://doi.org/10.1021/ja4029507.
[85] S.-H. Dong, A. Liu, N. Mahanta, D.A. Mitchell, S.K. Nair, Mechanistic basis for ribosomal peptide backbone modifications, ACS Cent. Sci. (2019) acscentsci.9b00124. https://doi.org/10.1021/acscentsci.9b00124.
[86] J.O. Melby, X. Li, D.A. Mitchell, Orchestration of enzymatic processing by thiazole/oxazole-modified microcin dehydrogenases, Biochemistry. 53 (2014) 413–422, https://doi.org/10.1021/bi401529y.
[87] R. Sinha Roy, P.J. Belshaw, C.T. Walsh, 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 (1998) 4125–4136, https://doi.org/10.1021/bi9728250.
[88] N.L. Kelleher, P.J. Belshaw, C.T. Walsh, Regioselectivity and chemoselectivity analysis of oxazole and thiazole ring formation by the peptide-heterocyclizing microcin B17 synthetase using high-resolution MS/MS [19], J. Am. Chem. Soc. 120 (1998) 9716–9717, https://doi.org/10.1021/ja9822097.
[89] R. Sinha Roy, A.M. Gehring, J.C. Milne, P.J. Belshaw, C.T. Walsh, Thiazole and oxazole peptides: biosynthesis and molecular machinery, Nat. Prod. Rep. 16 (1999) 249–263, https://doi.org/10.1039/a806930a.
[90] N. Allali, H. Afif, M. Couturier, L. Van Melderen, The highly conserved TldD and TldE proteins of *Escherichia coli* are involved in microcin B17 processing and in CcdA degradation, J. Bacteriol. 184 (2002) 3224–3231, https://doi.org/10.1128/JB.184.12.3224-3231.2002.
[91] N. Murayama, H. Shimizu, S. Takiguchi, Y. Baba, H. Amino, T. Horiuchi, K. Sekimizu, T. Miki, Evidence for involvement of Escherichia coli genes pmbA, csrA and a previously unrecognized gene tldD, in the control of DNA gyrase by letD (ccdB) of sex factor F, J. Mol. Biol. 256 (1996) 483–502, https://doi.org/10.1006/jmbi.1996.0103.
[92] D. Ghilarov, M. Serebryakova, C.E.M. Stevenson, S.J. Hearnshaw, D.S. Volkov, A. Maxwell, D.M. Lawson, K. Severinov, The origins of specificity in the microcin-processing protease TldD/E., Structure. 25 (2017) 1549–1561.e5. https://doi.org/10.1016/j.str.2017.08.006.
[93] S.S. Iyer, T. Gensollen, A. Gandhi, S.F. Oh, J.F. Neves, F. Collin, R. Lavin, C. Serra, J. Glickman, P.S.A. de Silva, R.B. Sartor, G. Besra, R. Hauser, A. Maxwell, A. Llebaria, R.S. Blumberg, Dietary and microbial oxazoles induce intestinal inflammation by modulating aryl hydrocarbon receptor responses., Cell. 173 (2018) 1123–1134.e11. https://doi.org/10.1016/j.cell.2018.04.037.
[94] M.-C. Arrieta, L.T. Stiemsma, N. Amenyogbe, E.M. Brown, B. Finlay, The intestinal microbiome in early life: health and disease, Front. Immunol. 5 (2014) 427, https://doi.org/10.3389/fimmu.2014.00427.
[95] Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome., Nature. 486 (2012) 207–14. https://doi.org/10.1038/nature11234.
[96] Human Microbiome Project Consortium. A framework for human microbiome research., Nature. 486 (2012) 215–21. https://doi.org/10.1038/nature11209.
[97] S. van Hemert, M. Meijerink, D. Molenaar, P.A. Bron, P. de Vos, M. Kleerebezem, J.M. Wells, M.L. Marco, Identification of *Lactobacillus plantarum* genes modulating the cytokine response of human peripheral blood mononuclear cells, BMC Microbiol. 10 (2010) 293, https://doi.org/10.1186/1471-2180-10-293.
[98] R.A. Flaherty, S.D. Freed, S.W. Lee, The wide world of ribosomally encoded bacterial peptides, PLoS Pathog. 10 (2014), e1004221. https://doi.org/10.1371/journal.ppat.1004221.
[99] S. Wirtz, M.F. Neurath, Mouse models of inflammatory bowel disease, Adv. Drug Deliv. Rev. 59 (2007) 1073–1083, https://doi.org/10.1016/J.ADDR.2007.07.003.
[100] S. Wirtz, C. Neufert, B. Weigmann, M.F. Neurath, Chemically induced mouse models of intestinal inflammation, Nat. Protoc. 2 (2007) 541–546, https://doi.org/10.1038/nprot.2007.41.
[101] L. Van Melderen, Molecular interactions of the CcdB poison with its bacterial target, the DNA gyrase, Int. J. Med. Microbiol. 291 (2001) 537–544, https://doi.org/10.1078/1438-4221-00164.
[102] M. Metelev, I.A. Osterman, D. Ghilarov, N.F. Khabibullina, A. Yakimov, K. Shabalin, I. Utkina, D.Y. Travin, E.S. Komarova, M. Serebryakova, T. Artamonova, M. Khodorkovskii, A.L. Konevega, P.V. Sergiev, K. Severinov, Y.S. Polikanov, Klebsazolicin inhibits 70S ribosome by obstructing the peptide exit tunnel., Nat. Chem. Biol. 13 (2017) 1129–1136. https://doi.org/10.1038/nchembio.2462.

[103] P. Baindara, S. Korpole, V. Grover, Bacteriocins: perspective for the development of novel anticancer drugs, Appl. Microbiol. Biotechnol. 102 (2018) 10393–10408, https://doi.org/10.1007/s00253-018-9420-8.

[104] D.Y. Travin, M. Metelev, M. Serebryakova, E.S. Komarova, I.A. Osterman, D. Ghilarov, K. Severinov, Biosynthesis of translation inhibitor klebsazolicin proceeds through heterocyclization and N-terminal amidine formation catalyzed by a single YcaO enzyme, J. Am. Chem. Soc. 140 (2018) 5625–5633, https://doi.org/10.1021/jacs.8b02277.

[105] J.A. McIntosh, C.R. Robertson, V. Agarwal, S.K. Nair, G.W. Bulaj, E.W. Schmidt, Circular logic: nonribosomal peptide-like macrocyclization with a ribosomal peptide catalyst, J. Am. Chem. Soc. 132 (2010) 15499–15501, https://doi.org/10.1021/ja1067806.

[106] P.F. Chan, V. Srikannathasan, J. Huang, H. Cui, A.P. Fosberry, M. Gu, M.M. Hann, M. Hibbs, P. Homes, K. Ingraham, J. Pizzollo, C. Shen, A.J. Shillings, C.E. Spitzfaden, R. Tanner, A.J. Theobald, R.A. Stavenger, B.D. Bax, M.N. Gwynn, Structural basis of DNA gyrase inhibition by antibacterial QPT-1, anticancer drug etoposide and moxifloxacin, Nat. Commun. 6 (2015) 10048, https://doi.org/10.1038/ncomms10048.

[107] M.G. Rudolph, D. Klostermeier, Mapping the spectrum of conformational states of the DNA- and C-gates in *Bacillus subtilis* gyrase, J. Mol. Biol. 425 (2013) 2632–2640, https://doi.org/10.1016/J.JMB.2013.04.010.