# The Structure and Biological Aspects of Peptide Antibiotic Microcin J25

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**Abstract:** Microcin J25 (MccJ25) is a plasmid-encoded peptide of 21 L-amino acids (G1-G-A-G-H5-V-P-E-Y-F10-V-G-I-G-T15-P-I-S-F-Y20-G), excreted to the medium by an *Escherichia coli* strain. MccJ25 is active on Gram-negative bacteria related to the producer strain, including some pathogenic strains. The four-plasmid genes *mcjABCD*, are involved in MccJ25 production: *mcjA* encodes a 58-residue precursor, *mcjB* and *mcjC* codify two processing enzymes required for the *in vivo* synthesis of the mature peptide and *mcjD* encodes the immunity protein (McjD), a member of the super family of ABC transporters. Immunity is mediated by active efflux of the peptide, keeping its intracellular concentration below a critical level. YojI, a chromosomal protein with ATP-binding-cassette-type exporter homology, is also able to export MccJ25. The *E. coli* outer membrane protein, TolC, is necessary for MccJ25 secretion mediated by either McjD or YojI. The uptake of MccJ25 is dependent on the outer-membrane receptor FhuA and the four inner-membrane proteins TonB, ExbD, ExbB and SbmA.

At least two mechanisms described the action of MccJ25 on the target cells: (1) inhibition of the RNA-polymerase (RNAP) activity by obstructing the secondary channel, and consequently, preventing the access of the substrates to its active sites; and (2) operating on the cell membrane, MccJ25 disrupts the electric potential inhibiting the oxygen consumption in *Salmonella enterica*. MccJ25 also inhibits oxygen consumption and the respiratory chain enzymes in *E. coli* throughout the increasing of ROS concentration. Nevertheless the exact mechanism of this phenomenon must be elucidated.

The MccJ25 exhibits a prolonged antimicrobial activity in a mouse infection model, suggesting a noteworthy potential for therapeutic uses.

**Keywords:** Microcins, antibiotics, peptide, enterobacteriaceae.

## INTRODUCTION

Microcins are a miscellaneous group of low-molecular weight antibiotics produced by a number of *Enterobacteriaceae*, mostly *Escherichia coli* strains [1]. Several individualities distinguish them from the more widely studied colicins. Microcins are considerably smaller, their synthesis is neither lethal nor controlled by the SOS regulatory circuit, and synthesis takes place during the stationary phase of growth, except microcin E492 from *Kiebsiella pneumoniae*, which is mainly produced during the early log phase [2]. Moreover, colicins are bactericidal proteins, whereas microcins may exhibit bactericidal or bacteriostatic modes of action. At least for the most extensively characterized microcins, B17 and C7, the genetic systems involved in their production and immunity are considerably more complex than the contiguous arrangement of three genes (synthesis, immunity, and release via lysis) which is a common feature of colicin-producing plasmids [3, 4]. Like those of colicins, the genetic determinants for microcins seem to be invariably plasmid borne. However, Lavinia *et al.* have described a member of this family, microcin H47, which is chromosomally encoded [5]. Microcins are a promising model system for the study of peptide transport mechanisms and the regulation of gene expression in non-proliferating cells. These studies may also contribute to a deeper understanding of the structure-function relationships of antimicrobial peptides. This review introduces the study of a peptide antibiotic which is excreted into the culture medium by a fecal *E. coli* strain named AY25. It has been called microcin J25 (MccJ25) and was the research subject of many laboratories over the past decade.

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## GENETIC DETERMINANTS OF MccJ25

*E. coli* AY25 was found to be resistant to ampicillin but sensitive to other conventional antibiotics. Physical analysis of this *E. coli* strain revealed the presence of five plasmids. Plasmid-curing experiments with ethidium bromide allowed isolating microcin-non producing variants with high efficiency. Supernatants from cultures of these cured strains showed no detectable antibiotic activity, and loss of microcin production was concurrent with loss of immunity to the antibiotic. Gel electrophoresis analysis showed that cured strains had lost a plasmid of about 60 kb. To confirm that the MccJ25$^+$ phenotype was plasmid determined, plasmid DNA was extracted from strain AY25 and used to transform *E. coli* DH5$\alpha$ strain. Microcin-producing transformants were selected on the basis of the immunity conferred by the plasmid. Plasmid DNA analysis showed that they harbored a single plasmid of about 60 kb, which was called pTUC100. The plasmid was subsequently transferred by transformation to strain MC4100, where it was stably maintained. Production of MccJ25 by strains transformed with pTUC100 was comparable to that of the original isolated [6].

A 5.2-kb region conferring MccJ25 production and immunity was subsequently cloned from the wild-type plasmid pTUC100 into the vectors pACYC184 and pBR322. In order to map the MccJ25 genes, the recombinant derivatives were subjected to transposon Tn*5* mutagenesis. By using genetic complementation analysis, it was demonstrated that the various microcin mutations could be classified into three differ-

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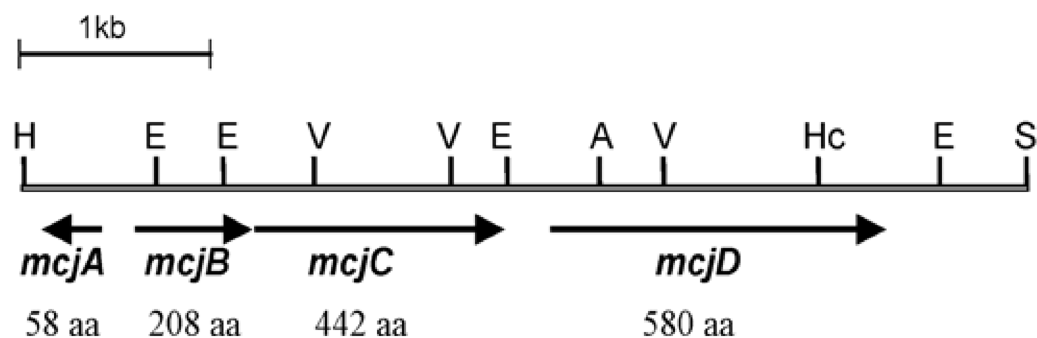


**Fig. (1).** Genetic organization of the HindIII-SalI fragment containing the MccJ25 system. The dotted line below the restriction map shows the sequenced region. Arrows indicate the extension and transcriptional direction of the genes identified by nucleotide sequence analysis. Abbreviations for restriction endonuclease sites: H, HindIII; E, EcoRI; V, EcoRV; A, AccI; Hc, HincII; S, SalI.

ent complementation groups, which defined three genes necessary and sufficient for MccJ25 synthesis (Fig. (**1**)). They were designated as *mcjA*, *mcjB* and *mcjC* [7]. The smallest gene *mcjA* (80-280 bp in size, as estimated by transposon mutagenesis and complementation) was considered a strong candidate for being the structural gene (this prediction was fulfilled, as we shall see below). Tn*5* insertions were not obtained in a segment just downstream from *mcjC*, and it was reasoned that this region could contain an immunity determinant. This region was subcloned and it was demonstrated that contains a gene, named *mcjD*, which confers immunity to MccJ25 [7]. It is not surprising that no insertions were obtained in *mcjD* since they should be lethal to the producing cell.

Nucleotide sequencing of the DNA fragment containing the genetic information for MccJ25 production and immunity revealed the presence of four open reading frames [8], whose size and location corresponded to the four genes previously deduced from complementation experiments. The genes *mcjB*, *mcjC*, and *mcjD* apparently form an operon, while mcjA is transcribed in the opposite direction. The sequence is identified in GenBank by the accession number AF061787. A summary of the sequence information is provided in Fig. (**1**).

## STRUCTURE

It was shown that MccJ25, the antibacterial peptide with the following primary structure: $V_1$GIGTPISF$Y_{10}$ GGGAGHVPE$Y_{20}$F, does not have a head-to-tail cyclized backbone as originally reported [9]. Instead, on the basis of biochemical studies, mass spectrometry, and NMR, it was shown that the peptide has an extraordinary structural fold. MccJ25 is a typical class II lasso peptide [10-12].

The MS/MS results were consistent with a "lariat" structure, consisting of an 8-residue cyclic segment; with a backbone-side chain amide linkage between $Gly^1$ and $Glu^8$, followed by a 13-residue linear segment as shown in Fig. (**2-A**).

Triple-resonance NMR directly confirmed the presence of a backbone-side-chain amide linkage. The tail ($Tyr^9$-$Gly^{21}$) passes through the ring ($Gly^1$-$Glu^8$), with $Phe^{19}$ and $Tyr^{20}$ straddling each side of the ring, sterically trapping the tail in a noncovalent interaction called a lassoed tail [10]. Unthreading does not occur even when the molecule is enzymatically digested with thermolysin. It was concluded that $Phe^{19}$ and $Tyr^{20}$ bracket the cycle, with the aromatic side-chain of $Phe^{19}$ being located on one face of the cycle, and the aromatic side-chain of $Tyr^{20}$ being located on the other face. The steric constraints imposed by the side-chains of $Phe^{19}$ and $Tyr^{20}$ lock the register of the threaded segment relative to the cycle, irreversibly trapping the threaded segment within the cycle [13]. This structural characteristic generates three implications: first, it accounts for the observations that led to the incorrect proposal that MccJ25 was a cyclic peptide; second, it accounts for the observed exceptional stability of MccJ25 to denaturation [7]; and third, it implies that during biosynthesis of MccJ25, the MccJ25 precursor must pre-fold at least transiently adopting a native or near-native conformation.

In general, the peptide adopts a well-defined compact structure. As shown in Fig. (**2-B**), the fold is characterized by two small antiparallel β-sheets. The first sheet comprises residues 6-7 and 18-19 and is formed between part of the ring and the penetrating C-terminal segment. The second sheet, which involves residues 10-11 and 15-16, is associated with a β-turn involving residues 11-14, and forms a hairpin like structure. The geometry of the turn can be classified as a type I β-turn. The main interactions stabilizing the fold are Van der Waals interactions between the amino acid side chains, which are predominantly hydrophobic in nature, and between polar groups in the backbone. Two main hydrophobic patches are present on the surface, with the first patch involving $Tyr^{20}$, $Val^6$, and the methylene groups of the $Glu^8$ side chain and the second patch formed by the side chains of $Pro^7$, $Phe^{10}$, $Pro^{16}$, and $Phe^{19}$. MccJ25 carries only two charges, $His^5$ and the C-terminus. Interestingly, the structure reveals that these charges are very close, suggesting an electrostatic interaction, possibly in the form of a salt bridge. Such interaction might play a major role in stabilizing the threaded structure.

Due to the poor solubility of MccJ25 in water the structure was determined in methanol; however the threaded arrangement and compact fold suggests that the structure will not be significantly affected by solvent. This assumption is supported by NMR spectra that showed no significant change in αH chemical shifts on titration of 40% $H_2O$ into the methanol solution [11]. In summary, it was shown that MccJ25 is not backbone cyclic but belongs to a family of bacterial proteins containing internal linkages between the

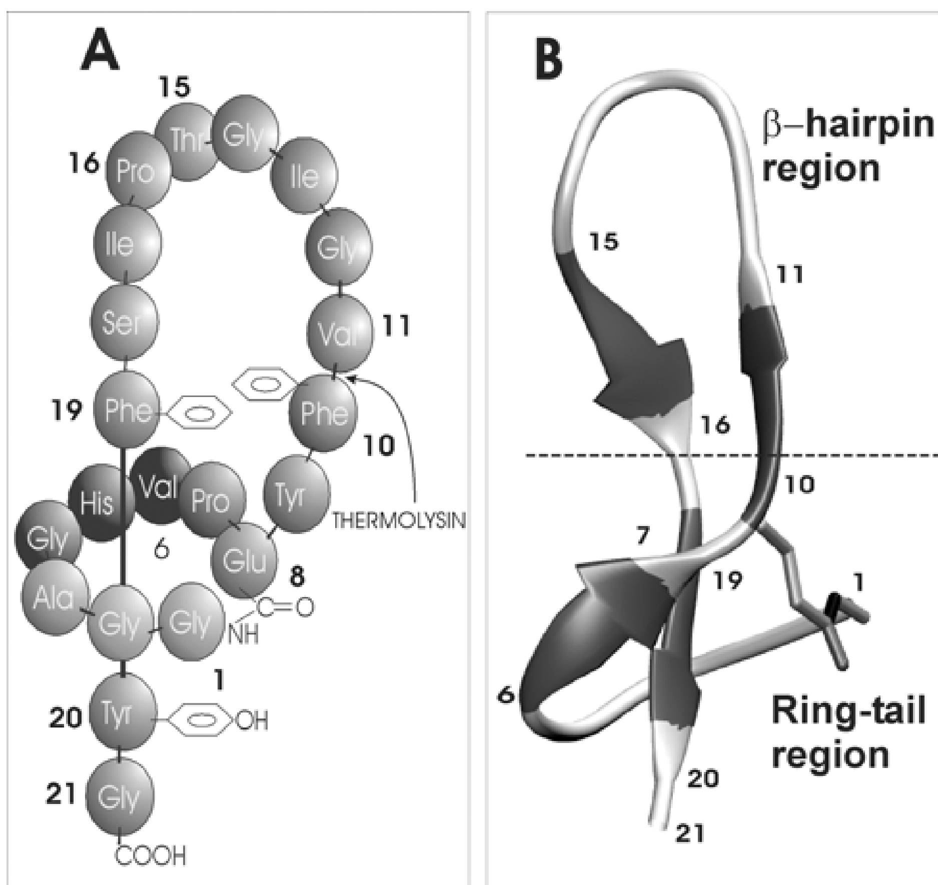


**Fig. (2).** Illustration of the schematic MccJ25 representation indicating the amino acid residues sequence, the lasso structure (**A**) and the ribbon representation of the lowest energy structure of MccJ25 showing the main elements of secondary structure; two small antiparallel β-sheets (**B**).

N-terminus and the side chain of a Glu residue. Bacterial proteins containing such linkages have been shown to exhibit resistance to protease digestion, suggesting that this "lasso" structure may be present to provide stability to otherwise small, and in the case of those not containing disulfide bonds, unconstrained peptides. On the basis of the three-dimensional structure this linkage is likely to occur after appropriate folding and it appears that some chaperone-like folding mechanism may be required to maintain the structure prior to formation of the internal linkage. This might involve either facilitation from the leader sequence in the precursor protein for MccJ25, or an external chaperone, or both [12].

## BIOSYNTHESIS OF MccJ25

It was shown that the four genes *mcjABCD* are necessary and sufficient to confer on a bacterial host the ability to produce mature extracellular microcin [8]. The gene *mcjA* encodes the linear 58 residue precursor of MccJ25, whereas *mcjD* encodes an ATP-binding cassette (ABC) transporter involved in both the exportation of MccJ25 and the self-protection of the producing strain against the deleterious effects of its own microcin. The remaining two genes, *mcjB* and *mcjC*, encode proteins whose functions are actually under study. It is well known that they are necessary for the production of active MccJ25 and have been proposed to encode the enzymes converting McjA into MccJ25 [8]. This process would involve the cleavage of the precursor, the side chain to backbone cyclization of the resulting C-terminal peptide and the three-dimensional structure acquisition of the MccJ25 (Fig. (**2**)). Presumably, the initial event would be a proteolytic cleavage between $Lys^{-1}$ and $Gly^{1}$ of the linear precursor (see Fig. (**3**)) by a leader peptidase, followed by folding of the C-terminal tail and ring. In some cases, processing is carried out by a specific serine protease [14, 15], which belongs to the antibiotic gene cluster [16]. It was observed no significant similarity between the *mcjB* and *mcjC* products and known processing peptidases, although it cannot be excluded the possibility that they have proteolytic activity. On the other hand, for some lantibiotics, most non-lantibiotics from Gram-positive bacteria and also microcin V from *E. coli*, proteolytic processing is carried out concomitant with exportation by the corresponding ABC transporter [17]. However, this does not seem to be the case for MccJ25, for the following reasons: i) the MccJ25 leader peptide does not share consensus sequences with those of the substrates of these ABC transporters; ii) active endogenous MccJ25 is generated in the absence of its ABC exporter, McjD; iii) MccJ25 export seems to be distinct from leader peptide removal, as exogenously added MccJ25 is pumped out of target bacteria containing the McjD pump, and (iv) the MccJ25 exporter lacks a conserved N-terminal extension of about 150 amino acids, which is present in the ABC exporters with processing activity and has been proposed to have leader

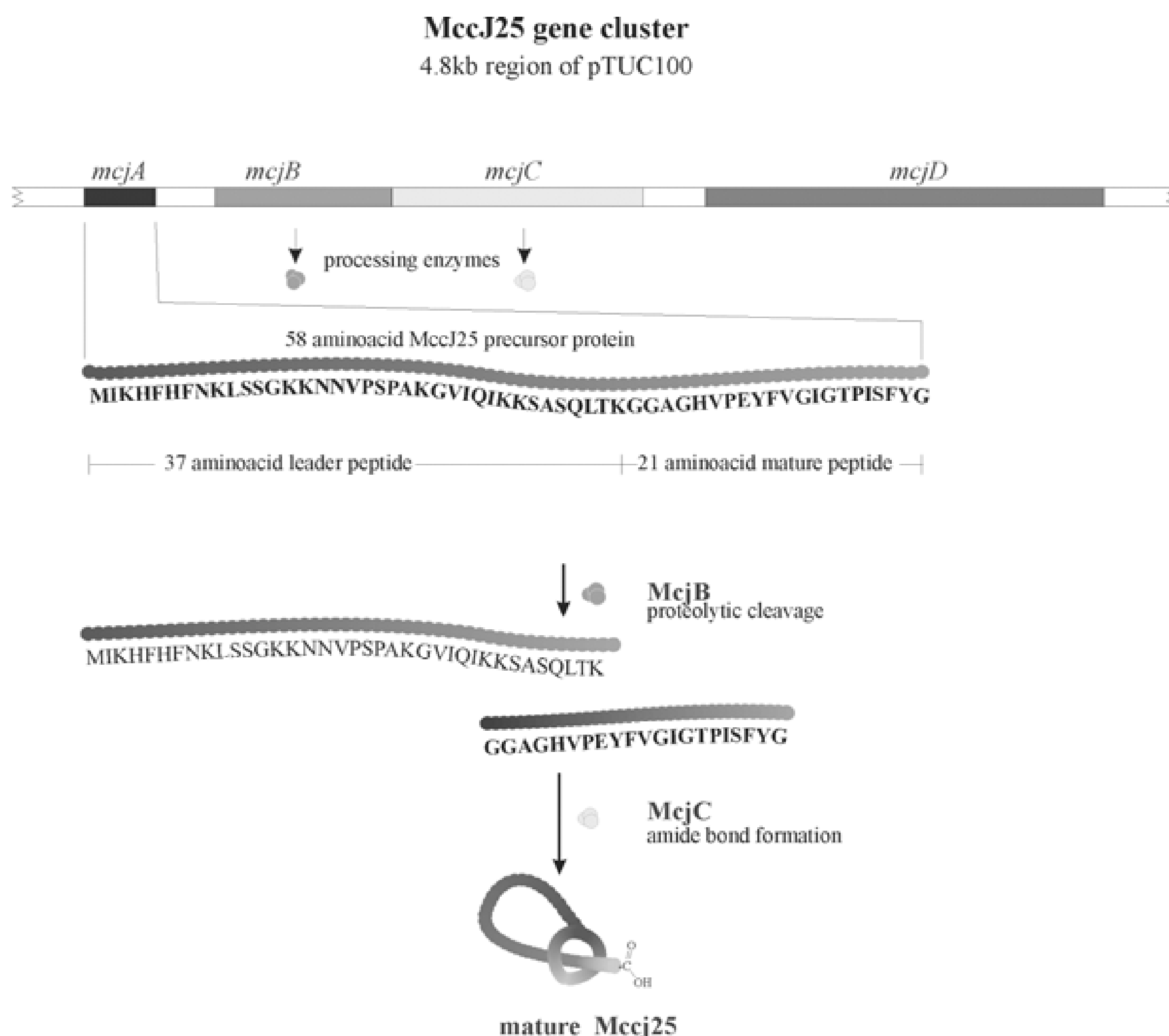


**Fig. (3).** Sequence of the antimicrobial peptide microcin J25 and representation of its encoding genes. The 21 amino acid mature peptide is excised from a 58 amino acid precursor protein. Coded on the same plasmid as the microcin precursor *mcjA* are several other genes associated with maturation (*mcjB* and *mcjC*) and export (*mcjD*) of the mature peptide.

peptidase activity [17]. Another possibility is that a chromosomally-encoded, hitherto unknown *E. coli* leader peptidase is capable of carrying out correct processing of the MccJ25 precursor. In this connection, a chromosomal gene called *pmbA* was suggested to be involved in the maturation of Microcin B17 [18].

Very recently, Duquesne *et al.* [19] demonstrated that McjB and McjC catalyze the maturation of MccJ25. The conversion of the linear precursor McjA into mature MccJ25 was obtained *in vitro* in the presence of McjB and McjC, all proteins being produced by recombinant expression in *E. coli.* Analysis of the amino acid sequences revealed that McjB could have proteolytic activity, whereas McjC would be the ATP/$Mg^{2+}$-dependent enzyme responsible for the formation of the $Gly^1$-$Glu^8$ amide bond. A major result from this article is that no other protein but McjB and McjC is necessary to convert McjA into MccJ25 and a relevant inference from their in vitro data is that MccJ25 maturation is completely independent of the export mechanism.

In further recent work, a recombinant McjA precursor peptide has been maturated into active MccJ25 by using a membrane protein extract from *E. coli* expressing the *mcjABCD* operon [20]. This result suggested that McjB and McjC proteins are located in the inner membrane fraction. Although no membrane-spanning motifs were found in McjB and McjC, would be possible that both proteins could be associated to the membrane by forming a complex with the cytoplasmic domain of McjD. This would allow efficient shuttling of mature MccJ25 from a McjB–McjC complex to the exporter, thus reducing the amount of potentially harmful free microcin in the cytoplasm.

## REGULATION OF MccJ25 PRODUCTION

Production of the best known plasmid-encoded microcins (MccB, and MccC) switches on when cells reach the stationary growth phase. This production is doubly regulated at transcriptional level by (a) the growth phase: microcin operons silent/repressed during exponential growth are induced/de-repressed when cells sense nutrient starvation and stop exponential growth, and (b) global bacterial regulators (CRP, EmrR, IHF, H-NS, LRP, OmpR, Sigma-38 and SpoT)

acting as inducers or repressors of operon expression. As described previously, MccJ25 production —biosynthesis, maturation and secretion to the medium— is also encoded by gene clusters organized in operons which is diagramed in Fig. (**1**). The isolated *lacZ* fusions to *mcjA*, *mcjB* and mcjC, and the regulation of these fusions were used to identify factors that control the expression of these genes [21]. The *mcjA* gene was found to be dramatically induced as cells entered the stationary phase. Expression of *mcjA* could be induced by resuspending non-induced exponential-phase cells in spent supernatant obtained from an early-stationary-phase culture. Induction of *mcjA* expression was not dependent on high cell density, pH changes, anaerobiosis, or the buildup of some inducer.

A starvation for carbon and inorganic phosphate induced *mcjA* expression, while under nitrogen limitation there was no induction at all. These results taken together suggest that stationary-phase induction of *mcjA* is triggered by nutrient depletion. The *mcjB* and *mcjC* genes were also regulated by the growth phase of the culture, but in contrast to *mcjA*, they showed substantial expression already during exponential growth. Induction of the MccJ25 genes was demonstrated to be independent of RpoS, the cyclic AMP-Crp complex, OmpR, and H-NS. Instead, it was found that the growth-phase-dependent expression of *mcjA*, *mcjB*, and *mcjC* may be explained by the concerted action of the positively acting transition state regulators ppGpp, Lrp, and integration host factor. Measurements of MccJ25 production by strains defective in these global regulators showed a good correlation with the reduced expression of the fusions in such mutant backgrounds [21].

## MICROCIN PURIFICATION

MccJ25 was purified from cultures of *E. coli* K-12 strain MC4100 ($F^-$ *ara D139* $\Delta$(*argF-lac*)205$\lambda^-$ *rpsL150 flbB5301 relA1 deoC1 pstF25*) harboring pTUC100, a high-copy-number recombinant plasmid [6]. Cells are grown until stationary phase in M9 minimal medium and the peptide present in the supernatant is applied to a preparative C8 cartridge. After washing the column, MccJ25 is eluted with a mixture of methanol/water (80/20). This crude preparation is purified by reverse phase-high pressure liquid chromatography on a semipreparative μBundapack C18 column with acetonitrile: water gradient as eluent. The purification procedure is simple and yields a preparation, which appeared homogeneous in a different solvent system chromatography [9].

## EXPORTATION AND IMPORTATION OF McCJ25

A schematic view of exportation and importation of MccJ25 from *Enterobacteriaceae* can be seeing in Fig. (**4**). The *mcjD* gene encodes a putative exporter which is required for MccJ25 secretion. Computer aided analysis of the amino acid sequence deduced from the *mcjD* gene showed that it contains all of the typical structural characteristics of known bacterial ABC exporters [7]. The strongest similarities were confined to the C-terminal portion of McjD surrounding the nucleotide-binding fold. The amino half of McjD is predicted to span the inner membrane six times and the carboxy half contains an ATP-binding domain, which includes the highly conserved Walker A and B motifs [22]. Plasmid pTUC203 contains the four genes *mcjABCD*. When it was mutagenized with transposon Tn*5*, many insertion mutations impairing microcin production were obtained in genes *mcjABC*, but none was isolated within *mcjD* [7]. This suggest that inactivation of *mcjD* could be lethal for cells expressing the other three genes; in other words, the activity of the latter would result in the biosynthesis of a toxic compound (i.e., microcin) which, in the absence of McjD, would probably accumulate in the cells and kill them. Hence, McjD could be a component of the pump that extrudes MccJ25 out of producing cells. This view is consistent with the findings that McjD confers resistance (immunity) to exogenous microcin [7].

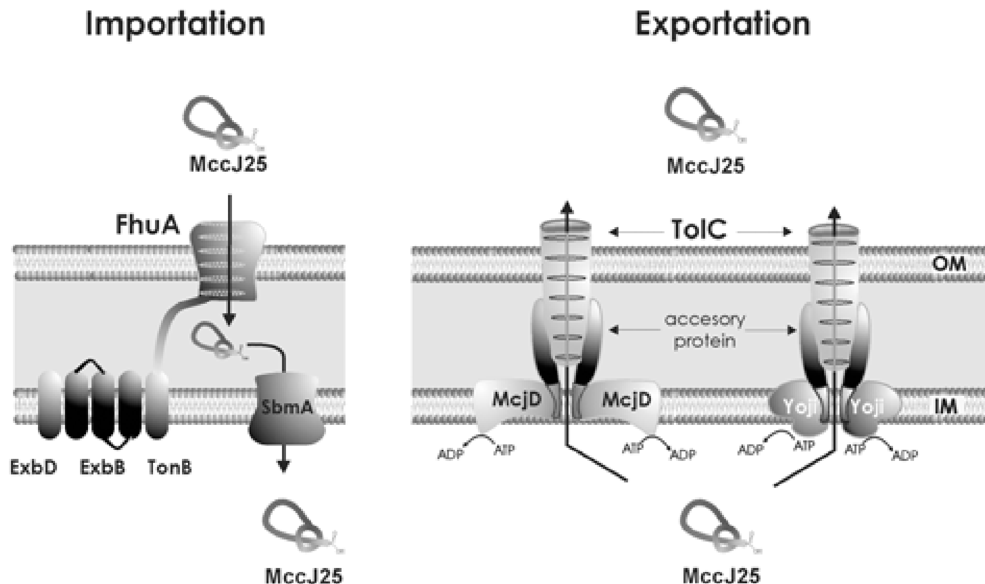


**Fig. (4).** Schematic representation of exportation/importation of MccJ25 from *Enterobacteriaceae*.

MccJ25 is destined for the surrounding medium, so it must cross the cytoplasmic (inner) and outer membranes, and the intervening periplasmic space. Generally, ABC export systems for protein secretion in Gram-negative bacteria require two proteins in addition to the transporter itself. One is an accessory protein of the so-called membrane fusion protein (MFP) family [22] which is anchored in the inner membrane, and the other is an outer membrane protein. It has been hypothesized that the accessory protein is trimeric and functions as an adaptor that connects the substrate-specific inner membrane transporter with the outer membrane component to facilitate the passage of substrates [23].

Secretion of MccJ25 is severely impaired in TolC–depleted cells [24], indicating that TolC, a chromosomally encoded minor outer-membrane protein, is clearly an integral part of the MccJ25 export machinery (Fig. (**4**)). A TolC homotrimer forms a channel through the outer membrane and a tunnel that spans the periplasm [25], providing a long hollow conduit for protein export and efflux of small noxious compounds, mainly detergents and a wide range of antibacterial drugs [26, 27].

What about the accessory protein? A gene that encodes this component in the MccJ25 genetic system has not been detected [8]. Similarly, the MccB17 operon, which also comprises an ABC exporter, does not include an associated accessory factor [28]. It may be that in these cases the *E. coli* host provides such factor.

In the course of experiments aimed at cloning a MccJ25-resistant mutation, a recombinant plasmid able to confer resistance to the antibiotic was found, but unexpectedly, this plasmid did not contained the mutation. Analysis of this plasmid led to the identification of a previously described chromosomal locus, *yojI*, which, when present in multiple copies, protected cells from MccJ25 [29]. Gene disruption experiments showed that a protective effect against MccJ25 was exerted by the single-copy chromosomal gene *yojI*. On the basis of sequence similarities, yojI had been assumed to encode an ABC-type exporter [30]. One obvious explanation for the protective effect of YojI against MccJ25 is that it is capable of pumping out MccJ25 molecules, which would keep the intracellular concentration of the peptide below a toxic level. It was also demonstrated that YojI could substitute McjD, the natural MccJ25 exporter, in secreting MccJ25 [29].

The *yojI* gene did not appear to be associated with any gene coding for an MFP, but it is possible that YojI may interact with a member of the MFP family and an outer membrane channel encoded elsewhere on the chromosome as it was above described for McjD. Evidence which strongly suggests that the resistance to MccJ25 mediated by YojI involves extrusion of the peptide and that YojI is assisted by the multifunctional outer membrane protein TolC was also provided [29]. MccJ25 is the first substrate known for the YojI pump. The specificity of YojI is not known, but it was shown that overproduction of YojI did not alter the resistance to any of twenty six representative antimicrobial agents and chemical compounds translocated by AcrAB or other major drug transporters [31]. This suggests that it could be quite specific in its choice of substrate.

The study of the MccJ25 translocation machinery components has been greatly facilitated by the isolation of mutants resistant to the antibiotic. Most *E. coli* mutations leading to resistance against extracellular MccJ25 map in genes encoding the outer-membrane protein FhuA, and the inner-membrane proteins TonB, ExbB, ExbD, and SbmA (see Fig. (**4**)) [32, 33]. FhuA is a multifunctional protein in the outer membrane of *E. coli*. Although its physiological function is the transport of the siderophore ferrichrome, it also serves as a receptor for several bacteriophages (T1, T5, ϕ80, and UC-1), and it mediates the passage of albomycin, rifamycin CGP 4832, colicin M [34] and, as described herein, MccJ25. The *E. coli* FhuA crystal structure consists of 22 antiparallel transmembrane β-strands that form a C-terminal β–barrel domain (residues 161 to 723) which is obstructed by a globular N-terminal domain (residues 1 to 160) known as the "cork" or "plug" [35, 36]. MccJ25 is unique among the FhuA ligands in that it requires both the β–barrel and cork domains to gain entry into the cell [37]. The only other microcin that uses FhuA as a receptor is Mcc24 [38].

The activity of FhuA is dependent on energy input that is provided by the proton motive force of the cytoplasmic membrane because there is no energy source in the outer membrane. Energy is transmitted from the cytoplasmic membrane to the outer membrane by a complex that consists of the three proteins TonB, ExbB, and ExbD (which together form the Ton system) (Fig. (**4**)). With the exception of infection by phage T5, all FhuA activities depend on the Ton system. It has been proposed that TonB interacts with FhuA, suggesting that TonB assumes an energized conformation in response to the proton motive force and allosterically opens the FhuA channel. MccJ25 has been classified by Braun et al. [38] in the group of TonB-dependent microcins, along with MccV, MccE492, H47, MccM, and Mcc24. Colicins as well as the outer membrane transport proteins contain proximal to the N-terminus a short sequence, called TonB box, which interacts with TonB and in which point mutants impair uptake. No TonB box is found in microcins. Colicins are composed of functional modules which during evolution have been interchanged resulting in new colicins. The modules define sites of interaction with the outer membrane transport genes, TonB, the immunity proteins, and the activity regions. Six TonB-dependent microcins with different primary structures are processed and exported by highly homologous proteins.

A survey of the sensitivity of several *Salmonella enterica* serovars showed that MccJ25 was highly active against some serovars, while *S. Typhimurium*, *S. Derby*, and some *S. Enteritidis* strains were completely resistant. Resistant strains became hypersensitive to MccJ25 when given the *fhuA* gene of *E. coli*, indicating that insensitivity is due to the inability of the FhuA protein to mediate penetration of MccJ25 [39].

Recently, the role of the outer membrane iron-transporter FhuA as a potential receptor for MccJ25 was studied through a series of *in vivo* and *in vitro* experiments. The requirement for both FhuA and the inner membrane TonB/ExbB/ExbD complex was evidenced by antibacterial assays using complementation of an $fhuA^-$ strain, and by using isogenic strains mutated in the complex encoding genes, respectively [40]. In addition, MccJ25 was shown to block phage T5 infection of *E. coli, in vivo*, by inhibiting phage adhesion, which sug-

gested that MccJ25 prevents the interaction between the phage and its receptor FhuA [40]. This *in vivo* activity was confirmed *in vitro* as MccJ25 inhibited phage T5 DNA ejection triggered by purified FhuA. Direct interaction of MccJ25 with FhuA was demonstrated for the first time by size-exclusion chromatography and isothermal titration calorimetry. MccJ25 bound to FhuA with a 2:1 stoichiometry and a Kd of 1.2 µM. Altogether, the results demonstrate that FhuA is the receptor for MccJ25 and that the ligand-receptor interaction may occur in the absence of other components of the bacterial membrane [40]. Finally, differential scanning calorimetry and antimicrobial assays showed that MccJ25 binding involves FhuA external loops [40].

The conformational change caused by the proton motive force, transmitted via TonB to FhuA, opens the FhuA channel and bound MccJ25 is released into the periplasmic space, where upon it would be translocated across the cytoplasmic membrane by the SbmA protein (Fig. (**4**)). Mutants in sbmA were shown to be B17 resistant [41]. Later, Yorgey *et al.* [42] reported that sbmA mutants show an increased resistance to bleomycin, a non-ribosomally synthesized peptide antibiotic widely prescribed as an anticancer drug. One year later, it was demonstrated that they were also completely resistant to MccJ25 [33]. Analysis of the 406-amino-acid sequence of SbmA, deduced from the gene, predicts seven transmembrane domains, and fractionation experiments have shown that SbmA is located to the inner membrane [43]. It was therefore inferred that SbmA transports MccB17, MccJ25, and bleomycin into the cell cytoplasm. Recently, Mattiuzzo *et al*. demonstrated that SbmA is necessary for the transport of, and for susceptibility to, proline-rich antimicrobial peptides of eukaryotic origin [44].

Mutations in *sbmA*, even deletions of the entire gene, do not have any effect in cell growth, showing that the SbmA protein is dispensable for cell viability. The question then arises as to what is the biological role of SbmA. Insight into this question comes from the work of the Graham Walker group, at the Massachusetts Institute of Technology. They found that a *Rhizobium meliloti* gene, termed *bacA*, encodes a protein of 420 amino acids that is 64% identical to SbmA, and is also predicted to span the cytoplasmic membrane seven times [45]. Moreover, BacA and SbmA are functionally interchangeable in both *E. coli* and *R. meliloti* [46]. The BacA protein is essential for bacterial differentiation into bacteroids (hence the gene named bac) within plant cells in the symbiosis between *R. meliloti* and a leguminous plant such as alfalfa. Similar to *R. meliloti*, a *Brucella abortus* homolog of the *bacA* gene is critical for survival of this mammalian pathogen in macrophages [47]. On the basis of the known SbmA-mediated transport of MccB17, MccJ25, proline-rich peptides and bleomycin, these authors postulated that the symbiotic role of BacA might involve the uptake of a signal (possibly a peptide) from the eukaryotic cytoplasm into the bacterial cell that would be important for intracellular development [45, 46]. Homologs of the Bac/SbmA proteins were found in a wide variety of free-living bacteria, including plant and animal pathogens [45]. Thus, functions related to that of BacA/SbmA must confer an important advantage in diverse environments. Undoubtedly, elucidation of the role of these intriguing proteins will have far reaching implications.

Since MccJ25, MccB17, proline-rich peptides and bleomycin are substrates of SbmA, it is conceivable that the transporter recognizes some structural feature shared by these compounds. MccB17 contains thiazole and oxazole rings as posttranslational modifications, and bleomycin is a peptide antibiotic that contains a bithiazole moiety. This led Yorgey *et al.* [42] to speculate that thiazoles/oxazoles are part of the MccB17 structure recognized by SbmA, and that thiazole- and oxazole-containing compounds may serve as signaling molecules for a variety of bacteria. However, it would be worth reconsidering this hypothesis in light of the finding that MccJ25 does not contain any thiazole or oxazole ring. One possibility is that SbmA has a wider specificity and, as happens with FhuA, it would be able to recognize and transport structurally dissimilar compounds. Analysis of the substrate specificity of BacA- or SbmA-mediated transport should provide insights into the molecular nature of the *in vivo* substrates of these transporters.

## MECHANISM OF ACTION OF MccJ25

MccJ25 appears to have two intracellular targets: (i) RNA polymerase (RNAP), which has been described in *E. coli* and *Salmonella enterica* serovars [39, 48, 49], and (ii) the respiratory chain, reported first in *S. enterica* serovars [50] but recently also demonstrated in *E. coli* [51]. It was shown that the observed difference between the actions of microcin on the respiratory chain in *E. coli* and *S. enterica* is due to the relatively low microcin uptake via the chromosomally encoded FhuA. Higher expression by a plasmid-encoded FhuA allowed greater uptake of MccJ25 by *E. coli* strains and the consequent inhibition of oxygen consumption. The two mechanisms, inhibition of RNAP and oxygen consumption, are independent of each other. Further analysis revealed for the first time that MccJ25 stimulates the production of reactive oxygen species ($O_2^{\bullet -}$) in bacterial cells, which could be the main reason for the damage produced on the membrane respiratory chain. We will describe henceforth the most typical aspects of both targets.

## RNA POLYMERASE

MccJ25 is primarily active on gram-negative bacteria related to the producer strain, inducing cell filamentation in an SOS-independent way. A mutation in the conserved segment of the rpoC gene, which codes for the largest RNAP subunit, was found to make *E. coli* cells resistant to MccJ25 [48]. The mutant RNAP prepared from MccJ25-resistant cells, but not the wild-type RNAP, is resistant to MccJ25 in vitro, thus establishing that RNAP is a true cellular target of MccJ25. Genetic analysis indicated that the mutation reside in the *rpoC* gene, encoding the β' subunit of RNAP, at 90 min on the *E. coli* genetic map [48].

Bacterial RNAP is a large and complex enzyme made up of multiple polypeptide chains. The core enzyme consists of four subunits: two α, one β, one β', and one ω. The RNAP core is fully capable of catalyzing the synthesis of RNA, but only upon binding of a fifth subunit, σ, the core is converted to an holoenzyme that can bind specifically to promoters to initiate transcription.

To identify the putative alteration in *rpoC*, the chromosomal mutation was cloned by *in vivo* recombination into an *rpoC* plasmid. Nucleotide sequencing of the mutated *rpoC* gene revealed only an ACC-to-ATC change at codon 931. The C to T transition caused a substitution of an evolutionarily conserved $Thr^{931}$ for Ile (represented as T931I). $Thr^{931}$ is part of segment G, the seventh of eight stretches of amino acids (A through H) which share extensive sequence similarity with β' homologues from eubacteria, archaea, and eukaryotes [52]. These results suggested that *E. coli* RNAP might be the target of MccJ25, which was supported by the observation that MccJ25 had an inhibitory effect on *in vivo* transcription by microcin-sensitive cells, whereas in the mutant cells RNA synthesis remained unaffected [48]. No effect of MccJ25 was observed on protein and DNA synthesis. Most importantly, MccJ25 inhibited a steady-*state in vitro* transcription by wild-type *E. coli* RNAP (purchased from a commercial source), strongly implying that RNAP is a direct target of MccJ25.

Later it was demonstrated that RNAP purified from the mutant, MccJ25-resistant cells, is resistant to MccJ25 *in vitro*, while transcription by the RNAP prepared from the parent, MccJ25-sensitive strain, is sensitive to the antibiotic [49]. These results provided the final proof that RNAP is the target of MccJ25. It was also reported the isolation of additional *rpoC* mutations that lead to MccJ25 resistance *in vivo* and *in vitro*. The new mutations affect amino acids in evolutionarily conserved segments (G, G' and F) and are exposed into the RNAP secondary channel. It was also report that previously known rpoB (RNAP_subunit) mutations that lead to streptolydigin resistance cause resistance to MccJ25. It was hypothesized that MccJ25 inhibits transcription by binding in RNAP secondary channel and blocking substrate access to the catalytic center [49].

Biochemical results indicate that inhibition of transcription occurs at the level of NTP uptake or NTP binding by RNAP. Genetic results indicate that inhibition of transcription requires an extensive domain, comprising more than 50 amino acid residues, within the RNAP secondary channel. Molecular modeling indicates that binding of MccJ25 within the RNAP secondary channel obstructs the RNAP secondary channel [53]. The secondary channel also accepts transcript cleavage factors GreA and GreB. It was demonstrated that MccJ25 inhibits GreA/GreB-dependent transcript cleavage, impedes formation of backtracked complexes, and can be crosslinked to the 3'-end of the nascent RNA in elongation complexes [54]. It was concluded that MccJ25 inhibits transcription by binding within and obstructing the RNAP secondary channel, preventing the access of the substrates to its active sites as a "cork in a bottle." Obstruction of the RNAP secondary channel represents an attractive target for drug discovery.

These results point to the RNA polymerase as the target of MccJ25 action and favor the possibility that the filamentous phenotype induced by MccJ25 results from impaired transcription of genes coding for cell division proteins.

## EFFECT OF MccJ25 ON MEMBRANE

The first paper studying the effect of MccJ25 on biological membrane reported the effects of the peptide on the microviscosity and permeability of phospholipid vesicles of different compositions [55]. These results indicate that MccJ25 interacts with egg L-α-phosphatidylcholine (PC) vesicles as demonstrated by peptide intrinsic fluorescence determinations. The interaction depends on the lipid composition of the vesicles. MccJ25 interaction induces a significant fluidity increase of egg PC vesicles. This effect is time and concentration dependent. Both trimethyl ammonium 1,6-diphenyl-1,3,5-hexatriene and 1,6-diphenyl-1,3,5-hexatriene gave the same results. The microviscosity of L-α-phosphatidylcholine dipalmitoyl small unilamellar vesicles was affected while that of L-α-phosphatidylcholine dimyristoyl vesicles was not, indicating that the effect was strongly dependent on the chain length of fatty acids. On the other hand, negatively charged L-α-phosphatidyl-DL-glycerol (PG) vesicles remarkably inhibited the peptide effect. Nevertheless, vesicles composed of L-α-phosphatidylethanolamine:PG:cardiolipin (7:2:1), a composition resembling bacterial membrane, were sensitive to the MccJ25 effect. MccJ25 effectively dissipated the valinomycin-induced membrane potential, but induced only a modest leakage (5%) of the trapped $Tb^{+3}$/dipicolinic acid complex. These results indicate that the peptides interact and perturb the bilayer of SUVs.

Supporting these findings, it was also found that MccJ25 acts on the cytoplasmic membrane of *Salmonella newport* cells producing alteration of membrane permeability, and the subsequent gradient dissipation, that initiate the inhibition of process, such as oxygen consumption [50]. These results, taken together with the *in vitro* observations, strongly suggest that the disruption of the cytoplasmic membrane gradient is closely related to the bactericidal activity of MccJ25 in *S. newport*.

Recently, it was demonstrated that the effect of MccJ25 on cell membrane is also present in *E. coli*, and the effect becomes noticeable when the bacteria over-express the MccJ25-FhuA receptor [51]. This study indicates, as the most important finding, that MccJ25 induces an increase in the production of ROS which is responsible for the inhibition of oxygen consumption. *E. coli* strains harboring a MccJ25-resistant RNAP and MccJ25-GA (a chemically altered MccJ25 that does not inhibit RNAP) were employed to demonstrate that MccJ25 could inhibit the growth of *E. coli* in an RNAP-independent manner. The *in vivo* experiments showed that the inhibitory effect of MccJ25 on cell respiration depends on the expression and/or activity of the transporter protein FhuA, which is involved in peptide uptake and consequently is responsible for the cytoplasm peptide concentration. The growth-inhibitory effect of MccJ25-GA on the strain MC4100 transformed with pGC01 (carrying the fhuA gene), but not on MC4100 *sbmA*::Tn*5* (pGC01), led to conclude that the antibiotic must reach the cytoplasm to act on the cellular respiration. This interesting observation could explain the absence of an additional periplasmic immunity protein in the MccJ25 production system. Such immunity proteins were described by Braun *et al.* [38] as indispensable for microcins, which act from the periplasm space.

Normal aerobic metabolism gives rise to active oxygen species, such as superoxide radicals and hydrogen peroxide, formed by the partial reduction of molecular oxygen [56,

57]. Moreover, redox cycling of various chemical substances, including some antibiotics, affects the reactive species of oxygen produced by cells during oxidative processes. For example, plumbagin and paraquat were used for several years as models to study oxidative stress [58, 59]. Recently, a number of antibiotics, including ciprofloxacin, have been demonstrated to stimulate the production of ROS in bacterial cells [60, 61]. The involvement of superoxide anion in the antibacterial action of MccJ25 was analyzed *in vitro* with isolated bacterial membrane and also *in vivo* experiments. The action of MccJ25 on respiratory-chain enzymes was irrefutably demonstrated by a direct measurement of ROS formation and SOD damage protection. The loss of the growth inhibitory capacity of MccJ25 under anaerobic conditions simultaneously with the overexpression of the *katG* gene in the presence of the peptide confirms the results obtained *in vitro*. The lack of peptide effect on bacterial growth in the anaerobic state simultaneously with the overexpression of the *katG* gene in MccJ25 presence supports and decisively confirms the results obtained *in vitro*. Recently, Korshunov and Imlay [62] reported that substantial superoxide were released into the periplasmic compartment as an incidental by-product of respiration, apparently due to the adventitious oxidation of menaquinone in *E. coli* cultures. The rate of periplasmic superoxide formation is quite high, about 3 μM/s, when normalized to the estimated periplasmic volume. That value is comparable to the 5 μM/s that has been estimated for superoxide formation in the cytosol [62]. The superoxide produced by cytosolic enzymes cannot cross inner membranes at physiological pH [63, 64]. The simple hypothesis that MccJ25 increases the ROS concentration could be assumed, although the site where ROS is overproduced is unknown.

## STRUCTURE AND FUNCTION STUDIES

The antibiotic activity of MccJ25 depends on the peptide capacity to inhibit alternatively the RNAP and/or the oxygen consumption through the ROS generation. It is important to remark here the importance of the MccJ25 uptake requiring the outer membrane receptor FhuA and the inner membrane proteins TonB, ExbD, ExbB, and SbmA; and the extrusion through the ABC transporter TolC and YojI. It is possible to change the molecular structure either by chemical/enzymatic reactions or genetic mutations and determine the region that should be conserved in order to fulfill the requirement of each step responsible of the antibiotic activity.

MccJ25 could be cleaved by hydrolysis with thermolysin giving a peptide (MccJ25-Th19) composed of two inseparable amino acid chains of 10 and 9 residues (Fig. (**2**)). Data and structural analysis of intact and thermolysin-digested MccJ25 suggest that distinct regions of MccJ25 are involved in transcription inhibition and cell entry [65]. MccJ25-Th19 with deep modifications in β-hairpin region had no effect on *E. coli* growth, but still inhibited RNA polymerase in vitro and oxygen consumption in *Salmonella* strains. MccJ25-Th19 showed antibiotic activity on *E. coli* transformed with plasmids containing either *fhuA* or *sbmA* genes. These results suggest that an intact β-hairpin region is crucial for MccJ25 import but not for inhibition of *E. coli* RNA polymerase or oxygen consumption in *Salmonella* strains [65].

Afterward, these results were corroborated by Semenova *et al.* [66] working with two MccJ25 derivatives that were obtained by chemical degradation, h16-MccJ25 and h18-MccJ25, lacking β-hairpin region amino acids 13 to 17 and 15 to 17, respectively. Both MccJ25 derivatives efficiently inhibited transcription by wild-type RNAP. The results clearly show that MccJ25 derivatives with lesions in the β-hairpin region (see Fig. (**2**)) are at least as active in transcription inhibition as intact MccJ25. The result therefore excludes the role of the β-hairpin region in RNAP binding and suggests that it is the unusual ring-tail part of the MccJ25 molecule (see Fig. (**2**)) that interacts with RNAP and blocks the secondary channel. The lack of bactericidal activity of MccJ25-Th19 suggests that the loop region of MccJ25 is important for the uptake, or alternatively, the loss of structure in this region may make MccJ25 proteolytically labile inside the cell. The latter alternative is unlikely, since the report of Bellomio *et al.* indicates that cells in which MccJ25 uptake was increased become sensitive to MccJ25-Th19 [66]. The loop region shall thus be an attractive target for genetic and/or chemical modifications to obtain more permeable and hence more potent MccJ25 derivatives.

MccJ25 carries only two charged residues: a positively charged histidine ($His^5$) localized in the lariat ring and a negatively charged glycine ($Gly^{21}$) at the carboxyl terminus of the molecule. The two charged groups are close in the three-dimensional structure [11] and were shown to be important for MccJ25 activity [67]. C-terminal glycine amidation of the threaded segment localized in the MccJ25 lariat ring region specifically blocked the RNAP inhibition, but not the cell respiration inhibition and peptide uptake [68]. On the other hand, carbethoxylation of the imidazole ring of MccJ25 $His^5$ decreased the inhibitory effect of the antibiotic on both *E. coli* cell growth and *in vivo* RNA synthesis [67]. The biological activity of carbethoxylated MccJ25 was completely recovered after treatment with hydroxylamine, which gives the native MccJ25. Thus, it appeared that the polar histidyl residue is required for MccJ25 transport into the cell, its extrusion outside the cell, or RNAP inhibition [67]. The inhibitory effects on cellular growth and in vitro RNAP activity were also determined with MccJ25 mutants carrying a substitution of $His^5$ by Lys, Arg, or Ala [69]. The results show that all mutants inhibited RNAP in vitro. However, the mutants were defective in their ability to inhibit cellular growth. Experiments in which the FhuA protein was bypassed showed that substitutions of MccJ25 $His^5$ affected the SbmA-dependent transport. These results thus suggest that MccJ25 $His^5$ located in the lariat ring is involved, directly or indirectly, in specific interaction with SbmA and is not required for MccJ25 inhibition of RNAP.

Taken together these results let us suggest four important conclusions: (a) the determinants for respiration and RNAP inhibition are different, (b) they are localized away from the β-hairpin region which is important for the MccJ25 uptake, (c) $His^5$ placed in the lariat ring is implicated in specific interaction with SbmA and (d) the C-terminal region is an important determinant for RNAP inhibition. This property could be useful to design new analogues with a powerful antibiotic activity and additionally to elucidate the mechanism of action of MccJ25.

## POTENTIAL APPLICABILITY OF THE MICROCIN J25 AS A THERAPEUTIC AGENT AND IN FOOD PRESERVATION

Recent investigations tested the antimicrobial MccJ25 to define potential interactions between the peptide with biological matrices, using a conventional antibiotic (ampicillin) as a comparator [70]. The authors evaluated the durability of MccJ25 in complex fluid biomatrices such as human whole blood, plasma and serum compared with those in conventional laboratory media by an ex vivo assay. The antibiotic was shown to be as effective in biomatrices as in artificial media, indicating that its antimicrobial activity was not affected by any blood component. This constitutes an important advantage which distinguishes it from other peptides with therapeutic potential. Besides, the antimicrobial activity in biomatrices appeared to be significantly higher than that of a conventional antibiotic, ampicillin, at least for Salmonella Newport. Many antimicrobial peptides have the disadvantage of being cytotoxic and/or erythrolytic, and this could limit their potential therapeutic utility. It was shown that MccJ25 has no hemolytic activity, which suggests that it could be harmless for other mammalian cell types. Another interesting finding is that MccJ25 displays a prolonged systemic antimicrobial activity in a mouse infection model. Intraperitoneal administration of the antibiotic resulted in a significant reduction in the bacterial number in both the spleen and liver of mice infected with *Salmonella Newport* [70] .

The MccJ25 has advantageous properties that allow conjecture a potential human use as a therapeutic agent. These advantages could be summarized as follow: i) is not toxic to human since it was isolated from feces of healthy infant, ii) can withstand extreme temperatures, iii) is stable in a wide range of pH and iiii) is resistant to proteolytic digestion [9]. In spite of these advantages the question that remains to be answered is: would MccJ25 be active on RNAP from different bacterial strain? There are no data in the literature showing a systematic study about the inhibition of MccJ25 on RNAP coming from different sources. Taking account the phylogenic studies we can suppose that most of the RNAP are inhibited by the peptide. However an important aspect limits the MccJ25 usefulness, it is the relatively narrow antimicrobial spectrum. Only Gram-negative bacteria are sensitive to MccJ25. In line with its spectrum of activity *in vivo*, limited to gram-negative species, the MccJ25 inhibits transcription by RNAPs *in vitro* of the three Gram-negative bacteria tested, *E. coli*, *Pseudomonas aeruginosa* and *Xanthomonas oryzae* [49]. Nevertheless, the last two are not susceptible to the antibiotic *in vivo*. This is an example of insensitivity associated with the inability of the MccJ25 to reach its cytoplasmic target, indicating that the antibiotic resistance is a simple question of bacteria membrane permeability. Therefore, it would be very useful to obtain antibiotic derivatives preserving its antibiotic activity but able to go across the bacteria membrane nonspecifically. An interesting possibility would be to join the MccJ25 molecule with some hydrophobic polypeptide or to make fusion with another antibiotic peptide active against Gram-positive bacteria. Although additional studies are required to improve the therapeutic window and potency of MccJ25, the results suggest that this antibiotic has potential for systemic administration and treatment of otherwise antibiotic-resistant infections.

Another important application of MccJ25 is its use as food preservative. At present only the peptide nisin was approved by the Food and Drug Administration. The MccJ25 is active on pathogenic strains of *E. coli, Salmonella* and *Shigella*, including strain O157: H7 and non-O157 (Solomon and Farias, personal communication), which are the cause of outbreaks of foodborne diseases. In addition, MccJ25 result the most active microcin against 12 of 15 diarrheagenic *E. coli* tested [71]. These authors also showed that the MccJ25 inhibits *E. coli* O157: H7 in biological products such as milk, egg yolk, and meat extract. These findings suggest that MccJ25 seems to be an efficient alternative to nisin for the food preservation, but there are some limitations that must be overcome. The high resistance of MccJ25 to digestion by proteolytic enzymes of the stomach (pepsin) and the intestinal contents (trypsin, chymotrypsin, carboxypeptidase) could cause disturbance of the normal microbiota. It is therefore necessary to broaden the spectrum of antibiotic activity of the peptide as well as turn it into degradable by protease enzymes before their likely application.

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