Peptides antimicrobiens des entérobactériesEtude de la voie de maturation et du mécanisme d'import de la microcine J25, peptide antimicrobien inhibiteur de l'ARN polymérase

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Etude de la voie de maturation et du mécanisme d'import de la microcine J25, peptide antimicrobien inhibiteur de l'ARN polymérase

Antimicrobial peptides from enterobacteria

Maturation process of the antibacterial RNA polymerase inhibitor microcin J25, and study of its bacterial uptake mechanism

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Sommaire

Liste des Abréviations

ACN	Acétonitrile
ADN	Acide déoxyribonucléique
ADNase	Désoxyribonucléase
Amp^R	Résistant à l'ampicilline
ARN	Acide ribonucléique
ARNase	ribonucléase
ATP	Adénosine triphosphate
BCIP	Bromochlorylindolophosphate
BET	Bromure d'éthidium
Bq	Becquerel
BSA	Sérum albumine bovine
cfu	"Colony forming unit", unité formant colonie
CMB	Concentration minimale bactéricide
CMI	Concentration minimale inhibitrice
Chl^R	Résistant au chloramphénicol
cpm	Coups par minute
Da	Dalton
DC	Dichroïsme circulaire
dNTP	2'-déoxynucléoside 5'-triphosphate
DO	Densité optique
DSC	"Differential scanning calorimetry", calorimétrie différentielle à balayage
DTT	Dithiothréitol
EDTA	Acide éthylènediamine tétraacétique
ESI-QTOF	"Electrospray ionisation-quadripole-time of flight", ionisation par électronébulisation, avec analyseur quadripole-temps de vol
FA	Acide formique
FPLC	"Fast performance liquid chromatography", chromatographie liquide à performance rapide
GST	Gluthatione-S-Transférase
HPLC	"High performance liquid chromatography", chromatographie liquide à haute performance
IPTG	Isopropyl-β-D-thiogalactoside

ITC	calorimétrie de titration isotherme
Kan^R	Résistant à la kanamycine
kb	kilobases
LB	“Luria-Bertani Broth”, milieu de culture Luria-Bertani
MccJ25	Microcine J25
MALDI	“Matrix assisted light desorption ionisation”, ionisation par désorption de lumière assistée par matrice
MOI	Multiplicité d’infection
NBT	Nitrobleu de tétrazolium
NTA	Acide nitrilo-triacétique
OG	Octyl glucoside
pb	Paire de bases
PB	“Poor-Broth”, milieu pauvre
PBS	Tampon phosphate salin
PCR	“Polymerase chain reaction”, Amplification par réaction en chaîne
pfu	“Plaque forming unit”, unité formant plage
PVDF	Chlorure de polyvinylidène
psi	“Pound per square inch”, livre par pouce carré (1psi = 0.06894 bar)
RBS	“Ribosome binding site”, séquence de Shine-Dalgarno
SDS	Sodium dodecyl sulfate
TAE	Tampon Tris acétate EDTA
Tet^R	Résistant à la tétracycline
t-MccJ25	Variant de MccJ25 résultant du clivage à la thermolysine
Tm	Température de fusion
TOF	“Time of flight”, temps de vol
AF	Acide formique
TFA	Acide trifluoroacétique
u	Unité enzymatique
X-Gal	5-Bromo-4-chloro-3-indolyl-ß-D-galactoside

Code utilisé pour les acides aminés

Alanine	A	Ala	Leucine	L	Leu
Arginine	R	Arg	Lysine	K	Lys
Asparagine	N	Asn	Méthionine	M	Met
Aspartate	D	Asp	Phénylalanine	F	Phe
Cystéine	C	Cys	Proline	P	Pro
Glutamate	E	Glu	Sérine	S	Ser
Glutamine	Q	Gln	Thréonine	T	Thr
Glycine	G	Gly	Tryptophane	W	Trp
Histidine	H	His	Tyrosine	Y	Tyr
Isoleucine	I	Ile	Valine	V	Val

Code utilisé pour les bases de l'ADN

A	Adénine
T	Thymine
G	Guanine
C	Cytosine

Introduction

Introduction

Par leur nombre et leur diversité, les microorganismes occupent une place essentielle dans tous les écosystèmes. Par référence aux activités humaines, ils sont parfois considérés comme utiles ou nuisibles. Leur importance est à l'origine de nombreux travaux, en écologie et en agroalimentaire notamment, et les recherches sur la génétique des microorganismes sont de plus en plus pointues et riches d'applications.

Les diverses espèces cohabitant dans une niche écologique donnée et leurs propriétés métaboliques, peuvent être exploitées pour leur capacité de transformation et dégradation de composés chimiques (station d'épuration) ou de fermentation (production d'éthanol, de méthionine, …). La modification génétique des espèces permet alors d'optimiser la production ou la dégradation de tel ou tel composé. Parallèlement à ces métabolites de petites masses moléculaires, de nombreux peptides et protéines sont sécrétés par les microorganismes, et en particulier des peptides et des protéines aux activités antimicrobiennes qui présentent un intérêt important.

La majorité des êtres vivants, eucaryotes ou procaryotes sécrètent des peptides et protéines aux propriétés antimicrobiennes. Chez les eucaryotes, ils sont majoritairement impliqués dans l'immunité dite innée. Ils contribuent ainsi à la protection de la plante ou de l'animal vis-à-vis d'un environnement riche en microorganismes, parmi lesquels bon nombre sont pathogènes, en tant que barrière chimique d'une part, mais aussi en tant qu'activateurs des processus de réponse inflammatoire. Ils sont ainsi sécrétés par un grand nombre de tissus en contact direct avec les microorganismes, tels que l'épiderme, l'épithélium respiratoire, digestif et urinaire, ou les cellules circulantes comme les phagocytes (pour revues, consulter Gallo *et al.* 2002, Jenssen *et al.* 2006)). Chez les procaryotes, si le rôle de ces peptides et protéines se révèle multiple, leur fonction première semble malgré tout être une fonction de défense. En effet, comme chez les eucaryotes, ils empêchent, par leur caractère antibiotique, la prolifération de microorganismes externes ou internes à leur écosystème naturel et rivalisent pour les nutriments présents dans leur niche écologique. Ils permettent ainsi de stabiliser un équilibre de la flore microbienne dans un écosystème donné (Riley et Wertz 2002, Riley et Goldstone 2003).

Contrairement aux peptides d'organismes eucaryotes pluricellulaires, la plupart des peptides antimicrobiens d'origine procaryote sont synthétisés de manière non ribosomique.

Cette voie métabolique spécialisée, nommée voie des peptide-synthétases, utilise des enzymes multifonctionnelles de très grande taille (plusieurs millions de daltons), quelquefois appelées « méga-enzymes » ou plus souvent « non ribosomal peptide synthetases ». Ces NRPSs incorporent des acides aminés naturels, pouvant ensuite subir des modifications par différents domaines spécifiques de la synthétase, ou bien encore directement des acides aminés non codés (acides aminés de la série D, α,α-dialkylés, *N*-méthylés, ornithine, …). Cette voie de biosynthèse permet de produire des peptides comportant jusqu'à 20 monomères, de structures très diverses (pour revue, consulter Finking et Marahiel 2004, Sieber et Marahiel 2005). Ainsi, depuis de nombreuses années, un grand nombre de peptides d'origine non ribosomique possédant des activités variées ont été caractérisés. Parmi ceux-ci, on retrouve des composés utilisés couramment en thérapeutique non seulement pour leurs propriétés antibiotiques (gramicidine) mais aussi immunosupressives (cyclosporine A)(pour revue, consulter Finking et Marahiel 2004, Sieber et Marahiel 2005).

Cependant, de façon beaucoup plus limitée, des peptides ou protéines antimicrobiens produits par les entérobactéries (colicines et microcines) et les bactéries lactiques (bactériocines) sont synthétisés selon la voie ribosomique (pour revue, consulter Klaenhammer 1993). Contrairement à la précédente, cette voie de biosynthèse permet de générer des peptides de plus grande taille à partir seulement des 20 acides aminés naturels génétiquement codés. L'introduction de modifications post-traductionnelles à l'aide d'enzymes diverses est associée à des structures et des modes d'action originaux. Ainsi, parmi les peptides et protéines issus de cette voie de biosynthèse, nombreux sont ceux qui possèdent un caractère cationique ou hydrophobe qui leur permet d'interagir avec la membrane plasmique des bactéries et d'en perturber les propriétés. Cependant, les mécanismes d'action sont souvent bien plus complexes et la perturbation de la membrane plasmique n'est parfois qu'une composante de leur mécanisme d'action (Park et Hahm 2005).

L'apparition d'un nombre croissant de microorganismes résistants aux antibiotiques usuels a suscité, au cours de ces dernières années, un intérêt tout particulier pour la recherche de nouveaux composés antibiotiques possédant des structures originales et agissant par de nouveaux modes d'action. Dans ce contexte, la compréhension du fonctionnement de la synthèse non ribosomique, puis l'ingénierie de peptide synthétases ou l'exploitation de domaines enzymatiques isolés pour la biochimie combinatoire a déjà permis de générer des peptides « non naturels » aux propriétés biochimiques modifiées, et ainsi montré son potentiel biotechnologique (pour revue, consulter Sieber et Marahiel 2005). Les enzymes de modifications post-traductionnelles intervenant dans la voie ribosomique, moins faciles à

appréhender puisque moins systématiques a priori, représentent elles aussi un potentiel biotechnologique intéressant.

L'équipe « Molécules de Défense et de Communication dans les Ecosystèmes Microbiens» de l'unité de « Chimie et Biochimie des Substances Naturelles » du Muséum National d'Histoire Naturelle (UMR 5154 CNRS), s'intéresse aux voies de biosynthèse, aux mécanismes d'action et aux relations structure/activité des microcines.

Les microcines sont des peptides antibactériens génétiquement codés, de faibles masses moléculaires (<10 kDa), sécrétés par les entérobactéries. Leur activité est dirigée contre d'autres entérobactéries, dont certaines espèces de *Salmonella* ou d'*Escherichia* entéropathogènes ont été impliquées dans des épidémies majeures chez l'homme et dans les élevages. De plus, leur activité antibactérienne est particulièrement puissante (concentrations inhibitrices inférieures à 0,1 µM). Depuis une vingtaine d'années, de nombreuses études menées dans les différentes équipes internationales impliquées dans cette problématique ont permis de déterminer la structure et le mode d'action de plusieurs microcines. Ainsi, les structures tout a fait originales des microcines B17, C7/C51, E492 et J25 (MccB17, MccC7/C51, MccE492 et MccJ25), résultant de modifications post-traductionnelles diverses, ont été élucidées. De plus, différents travaux ont permis d'acquérir des informations concernant leurs modes d'action.

Parmi les microcines, MccJ25 a suscité un grand intérêt du fait de son originalité structurale et de son mécanisme d'action. De plus, il a récemment été montré que le traitement par injections intrapéritonéales de MccJ25 permettait de réduire très nettement l'infection de souris par une souche de *Salmonella* (Lopez *et al.* 2007). MccJ25 est donc aujourd'hui l'objet d'une recherche intensive et au cœur d'une forte compétition internationale. MccJ25 est un peptide de 21 acides aminés, naturellement produit par d'*E. coli* AY25. Elle a été isolée, puis caractérisée au laboratoire (Blond *et al.* 2001). Sa structure tridimensionnelle est particulièrement originale, puisqu'elle implique un cycle formé entre le groupement amine de la glycine *N*-terminale et le groupement carboxylique de la chaîne latérale de l'acide glutamique en position 8. De plus, la queue *C*-terminale passe à travers le cycle, où elle est stériquement maintenue du fait des deux résidus aromatiques, Phe19 et Tyr20, de part et d'autre du cycle (Rosengren *et al.* 2003). De telles structures en lasso n'ont pour le moment été décrites que pour de rares peptides naturels, isolés pour la plupart d'espèces de *Streptomyces* (Frechet *et al.* 1994, Katahira *et al.* 1995, Katahira *et al.* 1996). Par ailleurs, leur voie de biosynthèse reste à ce jour inconnue. Le système génétique de MccJ25 a été séquencé

et caractérisé (Solbiati *et al.* 1999), ce qui constitue un avantage majeur pour l'étude de la biosynthèse de ce peptide lasso. La structure de MccJ25, qui résulte de l'action d'enzymes de modification inconnues sur un précurseur linéaire de taille supérieure à celle de la microcine mature, est responsable de son activité antibactérienne puissante, ainsi que de sa grande stabilité à des conditions de pH, température, ou de protéolyse particulièrement rudes (Blond *et al.* 2002). Récemment, il a été montré que son activité reposait sur l'inhibition de l'ARN polymérase (Adelman *et al.* 2004, Mukhopadhyay *et al.* 2004), dont elle obstrue le canal d'entrée des nucléosides triphosphates. De la même manière, les autres peptides lasso présentent une stabilité hors du commun et leur activité consiste souvent en l'inhibition d'une fonction enzymatique vitale. La compréhension du mécanisme et des conditions de maturation de MccJ25, ainsi que de son mode d'import dans les bactéries, représente donc un défi en vue de futures applications biotechnologiques.

La première partie de cette thèse, **Rappels Bibliographiques,** consiste en une étude bibliographique détaillée portant sur les microcines. Un premier article, sous presse dans *Natural Product Reports*, donne une vision globale des mécanismes de production des microcines par les entérobactéries, de leurs structures et des différents mécanismes qui leurs sont associées. Un second article, sous presse dans *Journal of Molecular Microbiology and Biotechnology*, insiste plus particulièrement sur la diversité structurale et fonctionnelle des microcines, au travers de trois exemples, MccE492, MccC7/C51 et MccJ25.

Après une description des protocoles expérimentaux utilisés (**Matériels et Méthodes**), les résultats obtenus au cours de cette thèse sont présentés dans les chapitres I et II.

Le **Chapitre I** traite du mécanisme d'import de MccJ25 dans les bactéries. Les résultats sont présentés dans un article paru dans *Biochemical Journal*.

Le **Chapitre II** décrit l'étude de la voie de maturation de MccJ25. L'isolement des enzymes responsables de ce processus de maturation, ainsi que la reconstitution *in vitro* de cette réaction enzymatique sont présentés dans un article soumis à *Chemistry and Biology*.

Enfin, la conclusion générale présente la synthèse et une discussion des résultats, pour conduire aux perspectives envisagées.

Introduction

Because of their high diversity and distribution, microorganisms are of considerable importance in ecosystems. Referring to human activities, they can be considered either useful or harmful. Their importance resulted in many studies, in ecology or food industry for examples. Research on their genetics is increasingly powerful in terms of applications.

Various bacterial species from a given ecosystem, as well as their metabolic pathways, can be exploited for their ability to degrade (wastewater purification basin) or ferment desired compounds (production of ethanol, methionine, …). Genetic manipulations of species then enabled to optimize the production or degradation of chemical compounds. Besides these low molecular mass metabolites, various peptides and proteins are also secreted by microorganisms. Amongst them are peptides and proteins displaying antimicrobial activities, and which thus serve another kind of interest.

Most eukaryotes and prokaryotes from the living kingdom synthesize peptides and proteins with antimicrobial activities. In eukaryotes, they are mainly involved in the so-called innate immunity. They thus contribute to the protection of plants and animals against surrounding microorganisms, among which many are potential pathogens. Not only they act as chemical barriers, but they also activate the inflammatory response. They are found mostly secreted by epithelia in direct contact with microorganisms, such as epidermal tissues or respiratory, digestive and urinary tracts (for a review, see Gallo *et al.* 2002). In prokaryotes, albeit the role of these secreted antimicrobial peptides and proteins appears to be multiple, one of their main function seems to consist in the protection of their producer. Indeed, as in eukaryotes, they prevent the proliferation of both internal to the ecosystem or external microorganisms, thus acting as regulators of the microbial flora within a given ecosystem (Riley and Wertz 2002, Riley and Goldstone 2003).

Contrary to antimicrobial peptides and proteins from pluricellular eukaryotes, most peptides from prokaryotic origin are synthesized via the non ribosomal pathway. This metabolic pathway, termed the non ribosomal peptide synthetase (NRPS) pathway, uses multifunctional, large enzymes (million daltons) that assemble peptide units to lead to the final product. This pathway enables the synthesis of small peptides (< 20 peptidic units) displaying a large structural diversity. Indeed, peptide units can be natural amino acids, further modified by specific tailoring domains from the synthetase, as well as non-

proteinogenic amino acids (α,α-dialkylated, *N*-methylated, D-amino acids, ornithine, …) (for a review, see Finking and Marahiel 2004, Sieber and Marahiel 2005). Since the past decade, the screening of microbial extracts has afforded a very large number of new non ribosomal peptides exhibiting antimicrobial (gramicidin) or immunosuppressive (cyclosporin A) properties (for a review, see Finking and Marahiel 2004, Sieber and Marahiel 2005).

Nevertheless, various antimicrobial peptides and proteins secreted by enterobacteria (colicins and microcins) and lactic acid bacteria (bacteriocins) are gene-encoded and produced *via* the ribosomal pathway (for a review, see Klaenhammer 1993). This biosynthesis route generates large peptides and proteins from the 20 natural amino acids. A great diversity, both in the structures and mechanisms of action of these compounds is still observed, due to post-translational modifications. Whereas many of the ribosomally synthesized peptides and proteins act through the disruption of the bacterial membrane, their mechanisms of action are often far more complex (Park and Hahm 2005).

During the last few years, the emergence of an increasing number of microbial species resistant to conventional antibiotics has required to intensify the search for new antibiotics with original structures and innovating mechanisms of action. The understanding of non ribosomal biosynthesis and further engineering of peptide synthetases and use of isolated domains for chemoenzymatic applications have already shown the biotechnological potential of this biosynthesis pathway (for a review, see Sieber and Marahiel 2005). Nevertheless, enzymes involved in the post-translational modifications of ribosomally synthesized peptides and proteins, which are less easily studied due to a less a priori systematic character, should also represent a non negligible potential.

The “Defense and Commmunication Molecules in Microbial Ecosystems” team housed at the laboratory of “Chemistry and Biochemistry of Natural Substances” of the National Museum of Natural History (UMR 5154 CNRS), studies microcins, and in particular, their biosynthesis, mechanisms of action and structure/activity relationships.

Microcins are low molecular mass (< 10 kDa) gene-encoded antibacterial peptides produced by enterobacteria. Their highly potent activity (minimal inhibitory concentrations in the nanomolar range) is directed against phylogenetically-related species, among which several pathogen species of *Salmonella* and *Escherichia* have been involved in major epidemic diseases in humans and animals. Since the last twenty years, the structures and

mechanisms of action of various microcins were determined. Thus, microcins B17, C7/C51, E492 and J25 (MccB17, MccC7/C51, MccE492 and MccJ25) display original structures resulting from post-translational modifications, which are correlated to diverse mechanisms of action.

Among microcins, MccJ25 was the subject of an intensive international research because of its atypical three-dimensional structure and mechanism of action. Moreover, peritoneal injections of MccJ25 were recently shown to decrease the infection by *Salmonella* strains in mouse (Lopez *et al.* 2007). MccJ25 is a 21-residue peptide naturally secreted by *E. coli* AY25. It was first isolated and characterized in the laboratory (Blond *et al.* 2001). Its three-dimensional structure consists of a side-chain to backbone cyclisation between the amino group of the *N*-terminal glycine and the carboxyl group of the glutamic acid in position 8. The *C*-terminal tail is firmly entrapped in the ring, due to the two bulky aromatic side chains from residues Phe19 and Tyr20 on each side of the ring (Rosengren *et al.* 2003). Such naturally occurring lasso-type structures have only been rarely encountered, mostly in peptides isolated from *Streptomyces* species (Frechet *et al.* 1996, Katahira *et al.* 1995, Katahira *et al.* 1996). Nevertheless, nothing is known yet about their maturation process. The genetic system responsible for the production of mature MccJ25 has been sequenced and characterized (Solbiati *et al.* 1999). This thus appeared to constitute an inestimable advantage for the study of the biosynthesis of lasso peptides. Furthermore, the structure of MccJ25, which results from the action of unknown post-translational modification enzymes on a longer linear precursor, is responsible for both its highly potent antibacterial activity and its peculiar stability to chaotropic agents, temperature and proteolytic cleavage (Blond *et al.* 2002). Recently, MccJ25 was shown to act as an inhibitor of the RNA polymerase of phylogenetically related bacteria, by obtruding the NTP-uptake channel (Adelman *et al.* 2004, Mukhopadhyay *et al.* 2004). Similarly, other naturally occurring lasso peptides display an uncommon stability to harsh conditions and their activity often consists in the inhibition of vital enzymes. Therefore, the understanding of MccJ25 mechanism of action and maturation process, as well as its uptake into target bacteria is a challenge in terms of future biotechnological applications.

Bibliographic Notes consists of an exhaustive bibliographic study of the present knowledge on microcins. The first article, in press in *Natural Product Reports*, gives an overview of the general organization of microcin genetic determinants, of their maturation

processes, export and uptake mechanisms in bacterial targets, to further relate their mechanisms of action. The second article, in press in *Journal of Molecular Microbiology and Biotechnology*, focuses on the structural and functional diversity observed among microcins, through three peculiar examples, MccE492, MccC7/C51 and MccJ25.

The experimental protocols used for this PhD work are described first (**Materials and Methods**), and the results obtained are then reported through two chapters (Chapter II and III).

Chapter I reports the study of MccJ25 uptake into target bacteria. Results are presented through an article published in *Biochemical Journal*.

Chapter II relates the study of MccJ25 maturation process. The isolation of the two enzymes responsible for MccJ25 post-translational modification process, as well as the *in vitro* reconstitution of the enzymatic reaction are presented through an article submitted to *Chemistry and Biology*.

Finally, the general conclusion gives an overview of the results obtained during this PhD work and opens the way to prospects.

Rappels Bibliographiques

Les microcines

Tout comme les eucaryotes, les procaryotes sont une source importante de peptides et protéines aux propriétés antimicrobiennes. Parmi eux, nous nous intéresserons ici aux peptides et protéines synthétisés par les bactéries selon la voie de biosynthèse ribosomique, et n'aborderons pas le cas, cependant très répandu, des peptides résultant de la voie des NRPS.

Ces substances ont longtemps été regroupées sous le terme générique de bactériocines (Tagg *et al.* 1976), qui s'appliquait à toute « substance protéique présentant une activité bactéricide dirigée contre des espèces phylogéniquement proches ». Le terme est devenu beaucoup plus restrictif, puisque les bactériocines sont aujourd'hui généralement scindées en trois classes : i) les bactériocines, qui désignent les peptides antimicrobiens produits selon la voie ribosomique par les bactéries à gram positif, et plus particulièrement les bactéries lactiques, ii) les colicines, qui sont des protéines antimicrobiennes thermolabiles de hautes masse moléculaire produites par les bactéries à gram négatif, et plus précisément des souches d'*Escherichia coli*, et iii) les microcines, qui sont définies, par opposition aux colicines, comme des substances protéiques de plus faibles masses moléculaires, produites par des bactéries à gram négatif.

Dans ce chapitre, nous présenterons, au travers de deux articles, une revue bibliographique exhaustive sur les microcines, qui font directement l'objet de cette thèse.

I. Les Microcines : peptides antibactériens des entérobacteries synthétisés selon la voie ribosomique

Ce premier article, sous presse dans *Natural Product Reports*, présente de manière détaillée les systèmes génétiques nécessaires à la biosynthèse des microcines, leurs voies de maturation, d'export et d'import dans les bactéries, et leurs mécanismes d'action. Elle montre à quel point les stratégies utilisées par les microcines pour tuer les bactéries sont à la fois fascinantes et complexes.

Publication n°1 :

Microcins, gene-encoded antibacterial peptides from enterobacteria

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Microcins, gene-encoded antibacterial peptides from enterobacteria

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Covering 1982 to 2006

Microcins are gene-encoded antibacterial peptides, with molecular masses below 10 kDa, produced by enterobacteria. They are secreted under conditions of nutrient depletion and exert potent antibacterial activity against closely related species. Typical gene clusters encoding the microcin precursor, the self-immunity factor, the secretion proteins and frequently the post-translational modification enzymes are located either on plasmids or on the chromosome. In contrast to most of the antibiotics of microbial origin, which are non-ribosomally synthesized by multimodular enzymes termed peptide synthetases, microcins are ribosomally synthesized as precursors, which are further modified enzymatically. They form a restricted class of potent antibacterial peptides. Fourteen microcins have been reported so far, among which only seven have been isolated and characterized. Despite the low number of known representatives, microcins exhibit a diversity of structures and antibacterial mechanisms. This review provides an updated overview of microcin structures, antibacterial activities, genetic systems and biosyntheses, as well as of their mechanisms of action.

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1 Introduction

Together with colicins, microcins are toxic peptides secreted by enterobacteria (mostly *Escherichia coli*) that belong to the large class of bacteriocins. The name microcin was introduced[1] to distinguish this class of antibacterial peptides, with molecular masses below 10 kDa, from the higher molecular mass colicins.[2–4] Microcins are generally hydrophobic and show a high stability to heat, extreme pH and proteases. Produced under conditions of stress, such as nutrient depletion, they have potent antibacterial activity against closely related bacteria, with minimum inhibitory concentrations (MICs) in the nanomolar range. They are therefore believed to be efficient weapons of the intestinal microbiota, contributing to the control of possible takeover by competing enterobacteria. The potent activity exerted by microcins, associated with a narrow spectrum of bacterial targets, make them particularly attractive tools for food preservation applications or for the replacement of conventional antibiotics.

Whereas many antimicrobial peptides from microbial origin are produced by large multidomain enzyme complexes termed peptide synthetases, microcins are typically produced as ribosomally synthesized precursors, similar to the bacteriocins from Gram-positive bacteria (for reviews, see Jack *et al.*[5] and Drider *et al.*[6]). Microcins are encoded by gene clusters carried either by plasmids or by the chromosome. Their gene clusters, which typically include open reading frames (ORFs) encoding the microcin precursor, self-immunity factors, secretion proteins and in general modification enzymes, give rise to an amazing diversity of microcin structures and mechanisms of action.

Microcins have been studied to a much lesser extent compared to other antibacterials such as colicins from Gram-negative bacteria, and bacteriocins from Gram-positive bacteria. However,

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Delphine Destoumieux-Garzón studied biochemistry and microbiology at the National Institute for Applied Sciences and at the University Paul Sabatier in Toulouse (France). During her PhD, obtained from the University of Montpellier in 1998, she focused on antimicrobial peptides in invertebrate immunity. As a post-doctoral fellow in the Ganz lab, at the University of California, Los Angeles, she studied the role of defensins in skin immunity. In 2000, she obtained a permanent research position at the CNRS and joined the Rebuffat group in Paris. There, she studied the molecular basis of microbial competitions, with particular interest in microcin recognition and uptake pathways. In 2006, she returned to the University of Montpellier, where she is studying the resistance of Vibrio *species to marine invertebrate antibacterials.*

Jean Peduzzi studied biochemistry, molecular and cellular pharmacology at the University Paris VI, Pierre and Marie Curie, from which he obtained his PhD in 1979. His PhD research focused on the isolation and inhibition of β*-lactamases by clavulanic acid. He was hired by the CNRS in 1980 and obtained a permanent research position in 1983 after a post-doctorate in the laboratory of Professor J. Rosa, at the hospital Henri Mondor in Créteil (France), where he studied diphosphoglycerate mutase, a minor protein from red blood cells. Since 1985 he has been a member of the Laboratory of Chemistry and Biochemistry of Natural Substances at the National Museum of Natural History, in Paris (France). Together with Sylvie Rebuffat, he is currently heading the Biochemistry team, in which he is studying the structures, biosynthetic pathways and mechanisms of action of microcins.*

Sylvie Rebuffat studied chemistry and biochemistry at the University Paris VI, Pierre and Marie Curie, and then completed a PhD in chemistry and spectroscopy in 1982, carrying out her research at the National Museum of Natural History. Subsequently she got a Maitre de Conferences position at the same Institution. She was a CNRS fellow in 1987 at the Gesellshaft für Biotechnologische Forschung (GBF) in Germany in the group of Professor G. Höfle. She is now Professor in the Laboratory of Chemistry and Biochemistry of Natural Substances at the National Museum of Natural History. Her present research focuses on the molecular aspects of the adaptative processes and defence mechanisms developed by microorganisms in specific ecosystems, such as the intestinal microflora and the symbiotic bacteria associated with marine invertebrates.

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numerous articles have been produced on the subject over the last few years. Among the fourteen microcins identified so far, only seven have been structurally characterized. Those are microcin B17 (MccB17), MccC7/C51, MccE492, MccJ25, MccL, MccM, and MccV (also known as ColV). Other microcins (MccH47, MccI47, Mcc24) had their structures predicted by genetic studies only. Finally, MccD93, Mcc140, Mcc15m and Mcc15n, which would be microcins of low molecular mass (below 1000 Da),[7–10] were only evidenced by few partial biochemical studies, and will not be described further in this review article.

In contrast with the very large number of bacteriocins from Gram-positive bacteria and colicins (for reviews, see Sablon *et al.*[11] and Braun *et al.*[4]), which have been assembled into classes according to common structural features and mechanisms of action, it appears to be more difficult to define sub-groups inside a family that is as restricted and diverse as the microcins. The first classification was attempted by Pons and collaborators,[12,13] who proposed to define two classes of microcins according to the occurrence of post-translational modifications. However, our recent finding that MccE492, initially described as an unmodified 84 amino acid peptide, was also secreted in a modified form[14] changed this vision. Therefore, we propose, in this review, a novel classification of microcins (Table 1) that agrees with most of the following criteria: (i) the presence, nature and localization of the post-translational modifications, (ii) the gene cluster organization, and (iii) the leader peptide sequences. In this classification, class I microcins are peptides with a molecular mass below 5 kDa, which are subject to extensive backbone post-translational modifications (MccB17, MccC7/C51, MccJ25). Class II includes higher molecular mass peptides (in the 5–10 kDa range). Class II is further subdivided into two subclasses: class IIa, some of which contain disulfide bonds but no further post-translational modification (MccL, MccV, Mcc24), and class IIb, which gathers together those linear microcins that may carry a *C*-terminal post-translational modification (MccE492, MccM and presumably MccH47 and MccI47).

Table 1 Overview of the main structural characteristics of the secreted microcins

Microcin	Class	Precursor/Promicrocin[a]	Leader peptide/Propeptide[a]	Microcin					
		Size (number of residues)	Size (number of residues)	Size (number of residues)	Molecular mass (Da)	Gly content (%)	Ser content (%)	Cys content (number of residues)	Net charge
MccB17	I	69	26	43	3093	60.5[b]	13.9[b]	4[b]	1+[b]
MccC7/C51	I	7	0	7	1177	0	0	0	0
MccJ25	I	58	37	21	2107	28.6	4.8	0	0
MccV	IIa	103	15	88	8733	16.9	9.1	2	1+
MccL	IIa	105	15	90	8884	15.6	6.6	4	3–
Mcc24[c,d]	IIa	90 (88)	17 (15)	73	7457	13.5	5.4	0	3+
MccE492[e]	IIb	103 (99)	19 (15)	84	8717	22.6	11.9	0	3–
u-MccE492[e]	IIb	103 (99)	19 (15)	84	7886	22.6	11.9[f]	0	3–
MccM	IIb	92	15	77	7283	19.5	23.4	0	1–
MccH47[c,d]	IIb	75	15	60	4865	26.6	15	0	0
MccI47[c,d]	IIb	77	15	62	6276	10.8	16.2	1	2–

[a] Numbers in parentheses refer to the length of the precursor and leader peptide, assuming transcription starts at the second of the two neighboring AUG codons. [b] Values from the theoretical amino acid composition determined for the precursor after cleavage of the leader peptide and before modification of Gly-Cys and Gly-Ser sequences to thiazole and oxazole rings. [c] This microcin has not been isolated; the sizes of the precursor, of the leader peptide and of the mature microcin have been hypothesized from the amino acid sequence deduced from the nucleotide sequence of the structural gene and from leader peptide alignments. [d] The biochemical characteristics (molecular mass, Gly/Ser/Cys content, and net charge) have been calculated from the putative sequence. [e] MccE492 and u-MccE492 were formerly termed MccE492m and MccE492, respectively. [f] The three extra serines from the siderophore-type post-translational modification are not included.

This review provides an updated overview of microcin structures and antibacterial activities, of their genetic systems and biosyntheses, as well as of their mechanisms of action.

2 Genetic system organization

The organization of microcin gene clusters is partially conserved and involves at least four clustered genes grouped in a single or several operons. The minimal structure is composed of (i) the structural gene encoding the microcin precursor, (ii) the self-immunity gene generally adjacent to the former, which encodes the self-immunity factor that protects the producing strain from its own antibacterial substance, and (iii) genes encoding the microcin export system necessary for the external secretion of the microcin. Additionally, genes encoding post-translational modification enzymes can be found. The content of microcin gene clusters and their overall organization are summarized in Table 2 and Fig. 1, respectively. The reader should be aware that the name given to each gene is not standardized throughout the different microcin gene clusters. For instance, genes encoding microcin precursors were often termed A (class I microcins as well as MccE492 and MccM), but some genes encoding microcin export proteins were also termed A (class IIa microcins). Moreover, with the exception of MccE492 genetic system, all genes termed I encode a self-immunity protein, but not all self-immunity proteins are encoded by a gene termed I. Two strategies have been used to identify the role of the different genes in microcin gene clusters. The first was based on genetics (mutagenesis, functional complementation, subcloning, gene fusion, *etc.*), and the second resulted from sequence homologies. The detailed roles of gene products are specified in Sections 4, 5 and 6.

2.1 Class I microcins: MccB17, MccC7/C51 and MccJ25

Class I microcins are encoded by gene clusters in which the self-immunity gene is not located near to the microcin structural gene. Two or three genes involved in post-translational modifications of the amino acid backbone are located adjacent to the structural gene. Furthermore, at least one gene is involved in both self-immunity and export.

MccB17 is produced by various *E. coli* strains harbouring the 70-kb single-copy, conjugative pMccB17 plasmid (formerly pRYC17).[15,16] The MccB17 gene cluster is composed of seven genes[17–19] (Fig. 1A). The gene *mcbA* encodes the 69-aa MccB17 precursor,[20] while *mcbB*, *mcbC* and *mcbD* encode the three components of the MccB17 synthetase[19] involved in post-translational modifications of McbA.[21] The genes *mcbE* and *mcbF* encode two proteins mainly involved in MccB17 secretion, which also contribute to self-immunity towards MccB17.[18] Full self-immunity requires the product of a last gene, *mcbG*.[18] The *mcb* genes probably form a single transcriptional unit[22,23] under the control of a stationary-phase promoter P_{mcb},[19,24] located upstream of *mcbA*. Two additional promoters were identified within the MccB17 gene cluster:[19] P_2, located within *mcbC*, can direct a weak transcription of *mcbD*, whereas the role of P_3, located within *mcbD*, and which directs transcription in the opposite direction, remains unclear.

MccC7/C51 is the smallest microcin hitherto characterized. MccC7 was first isolated by Moreno and collaborators from culture supernatants of *E. coli* strains harbouring the 43-kb

Table 2 Characteristics of microcin gene clusters. The length of each gene (bp) is given in parentheses

Microcin	Structural gene	Self-immunity genes	Export genes	Post-translational modification genes	Genes of unknown function	EMBL database accession number	G + C content (%)
MccB17	*mcbA* (210)	*mcbE* (726), *mcbF* (744), *mcbG* (564)	*mcbE* (726), *mcbF* (744)	*mcbB* (888), *mcbC* (819), *mcbD* (1191)	—	M24253, X07875	38.0
MccC7/C51	*mccA* (24)	*mccC* (1215), *mccE* (1566), *mccF*[a] (1035)	*mccC* (1215)	*mccB* (1053), *mccD* (804), *mccE* (1566)	—	X57583, AJ487788	34.4
MccJ25	*mcjA* (177)	*mcjD* (1743)	*mcjD* (1743)	*mcjB* (627), *mcjC* (1542)	—	AF061787, AM116873	33.1
MccV	*cvaC* (312)	*cvi* (237)	*cvaA* (1242), *cvaB* (2097)	—	—	X57524, X57525	42.0
MccL	*mclC* (318)	*mclI* (156)	*mclA* (1242), *mclB* (2097)	—	—	AY237108	40.1
Mcc24	*mtfS* (273)	*mtfI* (282)	*mtfA* (1245), *mtfB* (2124)	—	—	U47048	43.2
MccE492	*mceA* (312)	*mceB* (288)	*mceH* (1242), *mceG* (2097), *mceF* (540)	*mceC* (1113), *mceD* (1245), *mceI* (492)	*mceE* (345), *mceJ*[b] (1575)	AF063590	40.0
MccH47/MccI47	*mchB* (228), *mchS2*[c] (234)	*mchI* (210), *mchS3*[c] (435)	*mchE* (1242), *mchF* (2097)	*mchA* (1119), *mchS1* (1266), *mchD* (453)	*mchX* (120), *mchC*[b] (1551), *mchS4*[c] (246)	AJ009631	40.3
MccM/MccH47	*mcmA*[d] (279), *mchB* (228)	*mcmI*[d] (222), *mchI* (210)	*mchE* (1242), *mchF* (2097)	*mcmL*[d,e] (1119), *mcmK*[d,e] (1275), *mchD* (453)	*mcmM*[d] (687), *mchX* (120), *mchC* (1551)	AJ515251, AJ515252, AJ586887	40.3–41.4

[a] Gene absent from MccC51 gene cluster. [b] Genes of unknown function that are necessary for microcin production. [c] Genes specific for MccI47. [d] Genes specific for MccM. [e] Genes absent from *E. coli* Nissle 1917 strain.

single-copy pMccC7 plasmid (formerly pRYC7).[25,26] The authors termed it MccC7 after the name of the plasmid. Later, the same molecule was isolated by Khmel and collaborators from *E. coli* strains harbouring the 38-kb low-copy number pMccC51 plasmid (formerly pC51),[27] and the peptide was termed MccC51. A 6.5-kb and a 5.7-kb DNA fragment containing the microcin gene clusters were cloned from pMccC7 and pMccC51, respectively.[27–29] The nucleotide sequences of the two microcin gene clusters (Fig. 1A) display 98–100% sequence identity for *mccA* to *mccE.*[29] The 24 bp structural gene, *mccA* has been described as the smallest known gene.[30] It encodes the 7-aa precursor of MccC7/C51, MccA. The genes *mccB*, *mccD* and *mccE* are involved in the post-translational modifications of MccA, whereas *mccC* and *mccE* are required for self-immunity towards MccC7/C51. MccC, which exhibits similarity to multidrug efflux transporters, is also probably involved in the MccC7/C51 export. The product of the last gene on the cluster, *mccF*, which is transcribed from the opposite strand, contributes weakly to the self-immunity towards MccC7[28] but not to self-immunity towards MccC51, since only a truncated *mccF* gene is present on MccC51 genetic system.[29] One promoter was identified in the MccC7/C51 gene clusters.[30,31] Located upstream of *mccA*, P_{mcc} directs transcription from *mccA* to *mccE.* Therefore, this region most probably forms an operon.[23]

MccJ25 is encoded by the 60-kb low-copy number pTUC100 plasmid, found in the *E. coli* AY25 faecal strain.[32] A 4.8-kb DNA fragment of pTUC100 plasmid, containing all the MccJ25 determinants, has been cloned and sequenced.[33,34] Four genes, arranged in two divergent operons, are required for MccJ25 production, export and self-immunity (Fig. 1A). The gene *mcjA* encodes the 58-aa MccJ25 precursor, while *mcjB* and *mcjC* encode proteins probably involved in the post-translational modifications of McjA. The sequence of *mcjC*, which was recently reinvestigated, is 213 bp longer than that previously described (Duquesne *et al.*, unpublished work, GenBank, accession no. AM116873). The last gene, *mcjD*, is required for both MccJ25 secretion and self-immunity towards MccJ25. Similar to an ABC (ATP-binding cassette) transporter, McjD is responsible for the secretion of endogenous MccJ25 outside the cell,[34] but also for the export of exogenous MccJ25 that may enter in the producing bacteria.[33] Two promoters were found in the *mcjA–mcjB* intergenic region. The first one, P_{mcjA}, directs the transcription of the structural gene, whereas P_{mcjB} directs the transcription of the three other genes, in the opposite direction.[23,35]

2.2 Class II microcins

In class II microcin gene clusters, at least two genes are involved in export. This set of genes, which are homologous among class II microcins, requires the chromosomally located *tolC* to be functional.[4,36–39]

2.2.1 Class IIa microcins: MccV, MccL and Mcc24. Class IIa microcin gene clusters are composed of only four plasmid-borne genes, which are organized in a similar fashion.

MccV was the first antibiotic substance reported to be produced by *E. coli.*[40] This antibacterial agent was initially named colicin V (ColV).[41] However, on account of several characteristics (low molecular mass, non-inducible production, and dedicated export system), it became obvious that ColV should be classified within

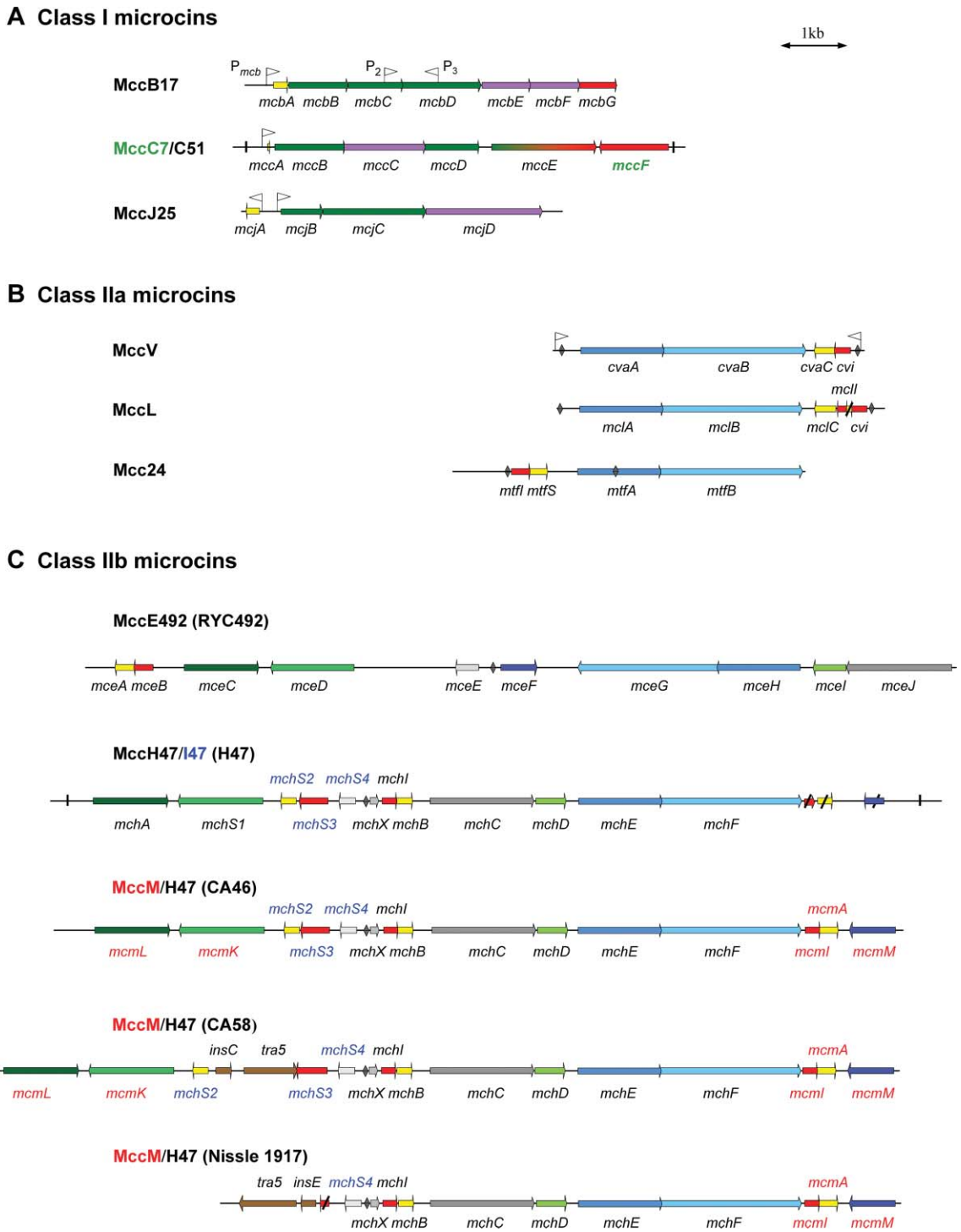

Fig. 1 Genetic organization of microcin gene clusters. Genes are indicated by arrows whose direction refers to gene transcription. An overview of the gene functions is given in Table 2. Genes encoding microcin precursors are shown in yellow. Genes required for self-immunity, microcin export and post-translational modifications are shown in red, blue, and green, respectively. Direct repeats flanking the MccC7/C51 and MccH47 gene clusters are indicated by vertical lines. Promoters are indicated by flags. Sequences with the most significant homology to the *fur* (ferric uptake regulation) boxes are shown by diamonds. The name of genes is indicated below or above each gene. A colour code is used for genes specific for one microcin within the gene cluster. Thus, names in green, blue or red are specific for microcins whose names are labelled with the same colour. Class I microcins are shown in (**A**). Genes required for both immunity and export are shown in purple. The gene *mccE*, whose product is involved both in post-translational modification (*N*-terminal region) and immunity (*C*-terminal region) towards MccC7/C51 is shown in green and red gradations. Class IIa and class IIb microcins are shown in (**B**) and (**C**), respectively. For class IIb microcins the name of the *E. coli* strain is indicated in parentheses. Genes encoding proteins of unknown function are indicated in grey. Genes encoding homologous or identical proteins in different clusters are coloured by different shades of the same colour. The genes *tra5*, *insC*, and *insE*, coloured in brown, encode transposases for insertion sequences IS2 and IS3. Truncated genes in MccL, MccH47/I47 and MccM/H47 (Nissle 1917) gene clusters are crossed through.

the microcins.[36,42] In this review, we therefore propose to name it MccV, but the reader should keep in mind that most of the literature on this microcin uses the ColV terminology. MccV is secreted by various *E. coli* strains harbouring large (>80 kb), low-copy number pColV plasmids.[43] A 4.2-kb DNA fragment from the 144-kb pColV-K30 plasmid is required for MccV production, export and self-immunity. Four genes distributed in two converging operons have been identified (Fig. 1B).[36,44,45] The structural gene *cvaC*, encoding the 103-aa MccV precursor, and the self-immunity gene *cvi* form the first operon. The dedicated export system of MccV has been well characterized[46–48] and involves two genes that form the second operon.[36] The gene *cvaA* encodes a protein anchored at the inner membrane with a *C*-terminal region extending into the perisplamic space.[49] The gene *cvaB* encodes an inner membrane ABC transporter. Two promoters were identified upstream of *cvaA* and downstream of *cvi*.[50] The nucleotide sequence analysis of 12 MccV-producing plasmids isolated from natural *E. coli* strains revealed a low level of polymorphism in the 683 bp *cvaC–cvi* region,[51] which suggests a strong stability of the MccV gene cluster.

MccL is produced by the *E. coli* LR05 strain isolated from poultry intestine.[12] This isolate also expresses MccB17, MccD93 and MccJ25.[52] Sequencing of pL102, which results from the cloning of the DNA conjugative plasmids of *E. coli* LR05, showed that the MccL gene cluster (Fig. 1B) consists of four genes encoding the 105-aa MccL precursor (*mclC*), the microcin self-immunity protein (*mclI*), and the microcin export proteins (*mclA* and *mclB*). The genes *mclA* and *mclB* are highly homologous (99% and 96% identity) to *cvaA* and *cvaB* encoding the MccV export system. The concomitant expression of MccV self-immunity and precursor genes inferred that the two genes are grouped in an operon.[39] Furthermore, *mclA* and *mclB* are translated from the opposite strand and probably form a second operon. Downstream of *mclI*, two ORFs were identified. Surprisingly, the first encodes a 27-aa peptide whose first 15 amino acids are identical to MccV leader peptide. The second exhibits 98% identity with the MccV self-immunity gene *cvi*, and makes the MccL-producing strain resistant to MccV.[52]

Mcc24 (formerly colicin 24) is secreted by the uropathogenic *E. coli* strain 2424, and its genetic determinants are located on the 43.5-kb conjugative plasmid p24-2.[53] A 5.3-kb DNA fragment from the pGOB18 recombinant plasmid was sequenced (O'Brien and Mahanty, 1996, unpublished work). Analysis of the nucleotide sequence (EMBL database accession no U47048) revealed that Mcc24 gene cluster (Fig. 1B) contains the following genes: *mtfS*, which encodes the probable 90-aa Mcc24 precursor; *mtfI*, which encodes the self-immunity protein; *mtfA* and *mtfB*, which encode proteins similar to the MccV export proteins, CvaA and CvaB. In contrast with MccV and MccL, the four genes apparently form a single operon.

2.2.2 Class IIb microcins: MccE492, MccH47, MccI47 and MccM. Unlike the previously described microcins, which are all plasmid-encoded, the class IIb microcins are chromosomally encoded. In addition, their gene clusters show a complex transcriptional organization.

MccE492 is secreted by *Klebsiella pneumoniae* RYC492, a strain isolated from human faeces.[54] A 13-kb DNA fragment containing the entire MccE492 gene cluster was cloned to raise the pJAM434 recombinant plasmid.[55] Ten genes (*mceA* to *mceJ*) are necessary for MccE492 production, export and self-immunity (Fig. 1C).[38] The structural gene *mceA* encodes the 103-aa MccE492 precursor, and *mceB* is involved in the self-immunity towards MccE492.[56] The genes *mceC*, *mceD* and *mceI*, which encode proteins homologous to a glycosyltransferase, a ferric enterobactin esterase, and an acyltransferase, respectively, are required for MccE492 post-translational modifications.[57] The gene *mceJ* would also be involved in the maturation process but its exact role remains to be elucidated.[38,58] Two other genes, *mceG* and *mceH*, are required for the export of MccE492. They encode an ABC transporter and an accessory protein, respectively. The gene *mceF* would also be involved in export.[38] The role of the last gene, *mceE*, remains unknown. The ten genes are organized in at least six transcriptional units, which is unusual for a bacterial gene cluster.[38] The gene *mceA* is transcribed with the self-immunity gene *mceB*, while *mceC*, *mceD*, *mceE*, and *mceF* are all transcribed as monocistronic single units. Finally, *mceGHIJ* are organized in a polycistronic operon, but *mceGH* may also be transcribed as a bicistronic unit.[38] Amazingly, the orientation of *mceGHIJ* on the chromosome of *K. pneumoniae* RYC492 is opposite to that of the homologous *mchCDEF* from MccH47 and MccM gene clusters (see below). MccE492 is also produced by *E. coli* harbouring the pJAM229 recombinant plasmid. This plasmid differs from pJAM434 by an inverted orientation of the 6.9-kb *Xho*I fragment that contains *mceGHIJ.* However, both plasmids are reported to express MccE492.[55]

Since MccH47, MccI47 and MccM gene clusters are closely interwoven, they are described simultaneously. MccH47 was initially detected in culture supernatants of *E. coli* H47 strain, isolated from human faeces.[59] MccM is secreted by the nonpathogenic *E. coli* Nissle 1917 (DSM 6601) isolated from human faeces. This strain, also named Mutaflor,[60] is used as a probiotic agent for the treatment of various intestinal diseases.[61–63] Initially described as colicin X,[64] the antibacterial substance was recently renamed MccM after the name Mutaflor.[65] MccM and MccH47 are both secreted by *E. coli* CA46 and CA58 strains,[65] which were initially described as producers of colicins G and H.[66,67]

The genetic determinants required for MccH47 production, export and self-immunity are all located within a 10.5-kb DNA fragment on *E. coli* H47 chromosome[59] (Fig. 1C), and eight genes were first identified. These include *mchA–mchF*, *mchI*, and *mchS1*, which is located in a 3-kb silent region neither involved in MccH47 production nor in self-immunity.[37] The gene *mchB* encodes the 75-aa MccH47 precursor[68] and *mchI* confers the self-immunity towards MccH47.[69] The genes *mchE* and *mchF*, which exhibit high homology (96.3% and 90% identity) to *cvaA* and *cvaB*, respectively, are probably involved in MccH47 secretion.[37,70] The genes *mchA*, *mchS1* and *mchD*, which are homologous to *mceC*, *mceD* and *mceI*, respectively, are presumably involved in MccH47 post-translational modifications. The gene *mchC*, which is homologous to *mceJ*, is necessary for the activity of the microcin, but its precise function is unknown. Upstream of *mchI*, *mchX* was later identified. It encodes a 39-aa peptide that may be involved in the regulation of *mchI* and *mchB* expression.[69] More recently, three other genes were identified in the silent region.[71] The gene *mchS2* encodes the 77-aa precursor of a new microcin termed MccI47, and *mchS3* confers the specific self-immunity towards MccI47.[72] Additionally, *mchS4*, which was found to be responsible for

the overproduction of the catecholate siderophore enterobactin,[71] encodes an 81-aa protein whose role is unclear. Downstream of *mchF*, three truncated genes (*mcmI*, *mcmA*, *mcmM*) were also found (see below).[4,72]

Analysis of microcin gene clusters on genomic island I from *E. coli* Nissle 1917[73] and *E. coli* CA46 and CA58 genomes[4,65] showed that these gene clusters direct the synthesis of both MccM and MccH47. The three gene clusters share a common organization except for the 5′ region located upstream of *mchX* (Fig. 1C). Three MccM-specific genes were identified in the 3′ region. The gene *mcmI* encodes the MccM self-immunity protein, *mcmA* (formerly *mcmC*),[4] encodes the 92-aa MccM precursor, whereas *mcmM*, which is transcribed in the opposite direction, encodes a protein similar to MceF (62% identity over 176 residues).[65] The MccM secretion does not involve specific genes and is probably carried out by *mchE* and *mchF* gene products. The minimal region necessary for the MccM production[65] is carried by *mchDEF* and *mcmIA*. In *E. coli* CA46 and CA58, two additional genes, *mcmL*/*mcmK*, were identified upstream of *mchX*[4] (Fig. 1C). They are homologous to *mchA*/*mchS1* and *mceC*/*mceD*, and are probably involved, together with *mchD*, in MccM post-translational modifications. Moreover, in the MccM/MccH47 gene cluster from *E. coli* CA46 (Fig. 1C), three genes, *mchS2S3S4*, are present between *mcmK* and *mchX*. The gene *mchS2* encodes a 77-aa protein that differs from the MccI47 precursor by only one amino acid substitution (glycine for alanine in position 22). Thus, the *E. coli* CA46 strain could secrete a third microcin. Similar features are observed in the MccM/MccH47 gene cluster from *E. coli* CA58 (Fig. 1C). Downstream of *mcmK*, a putative *mchS2* is also found when the undetermined nucleotide N, located at position 3452, is deleted. Nevertheless, an additional 1.3-kb DNA fragment, composed of genes encoding transposase and insertion sequences,[4] is inserted upstream of *mchS3*. The MccM/MccH47 gene cluster from *E. coli* Nissle 1917 differs from those of *E. coli* CA46 and CA58 mostly by the absence of *mcmL* and *mcmK*[65] (Fig. 1C). A MccM/MccH47 gene cluster identical to that of *E. coli* Nissle 1917 is also encountered with 100% nucleotide sequence identity in the *serX* pathogenicity island of the uropathogenic *E. coli* strain CFT073.[74] Moreover, a partial MccM gene cluster including the 3′ region of *mchF* and *mcmIAM* is also encountered in the pathogenicity island II from *E. coli* strain 4787[75] and in the pathogenicity island III from *E. coli* strain 536.[76] The presence of genes encoding transposase and insertion sequences, which are known to be involved in genetic recombination, strongly supports the hypothesis of a MccM/MccH47 gene cluster exchange between bacteria.

The G+C contents of all Mcc gene clusters, which range from 33.1% to 43.2% (Table 2), are lower than those of their bacterial host genomes (about 51% for various *E. coli* and 57.5% for *K. pneumoniae* MGH78578 strain). Thus, these bacteria would appear not to be the original hosts of microcin gene clusters. Moreover, partial or complete MccM gene clusters are encountered in genomic or pathogenicity islands. Such structures represent a large group of mobile elements that contribute to microbial evolution (for reviews, see Hacker *et al.*[77] and Dobrindt *et al.*[78]). Altogether, the identification of short direct repeats flanking the MccC51 and MccH47/I47 gene clusters[29,72] (Fig. 1), the location of some gene cluster in genomic islands, and the G+C content strongly suggest the possibility of a horizontal transfer of genes responsible for the microcin biosynthesis.

3 Purification, structures and antibacterial activity

Elucidation of microcin structures, which include in many cases complex and unusual post-translational modifications, requires the optimization of culture conditions and purification protocols in order to isolate substantial amounts of highly purified microcins. These time-consuming and difficult (but necessary) steps have often hampered the elucidation of many microcin structures and the reliable determination of their antibacterial activities. Indeed, while all microcins show a potent antibacterial activity specifically directed against enterobacteria, the literature often reports the activity of the producing strains instead of the purified microcins. Since a number of microcinogenic strains were described as producing several microcins[52,65,72] or other antibacterials such as colicins, old published data should be interpreted with the greatest care. Because all microcins have not been purified to homogeneity nor accurately quantified prior to antimicrobial assays, quantitative measurements leading to MICs and minimal bactericidal concentrations (MBCs) are rarely available. Finally, MICs and MBCs are rarely comparable due to differences in the experimental protocols used.

3.1 Class I microcins: MccB17, MccC7/C51, MccJ25

Class I microcins have the lowest molecular masses, ranging from 1 to 3 kDa, and display extensive post-translational modifications of their peptide backbone. Thus, MccB17 is a 43-residue peptide characterized by the presence of thiazole and oxazole rings, MccC7/C51 is a nucleotide–heptapeptide, and MccJ25 is a 21-residue cyclic peptide that adopts a particular lasso three-dimensional structure.

MccB17, as purified for structure determination, was produced by *E. coli* BM21 cells harbouring the pMM39 plasmid, and grown in tryptone–yeast extract medium. The purification protocol included an initial hot acid extraction step of the harvested cells at pH 2.9 and 100 °C for about 10 min to ensure complete extraction from the cells.[79] The following steps consisted of size-exclusion chromatography and reversed-phase high performance liquid chromatography (RP-HPLC). MccB17 was reported to display potent bactericidal activity against a wide range of Gram-negative bacteria including *Escherichia*, *Citrobacter*, *Klebsiella*, *Salmonella*, *Shigella* and *Pseudomonas*.[1,80] MccB17 was shown to derive from a 69-aa precursor, McbA (Fig. 2), endowed with an atypical 26-residue leader peptide. Mature MccB17 carries four oxazole and four thiazole rings that derive from the unusual post-translational modification of six glycines, four serines, and four cysteines spanning residues 39–66 of the MccB17 precursor. Those rings are formed by the reaction of serine and cysteine side-chains with the carbonyl groups of the preceding glycine in the peptide chain[79,81] (Fig. 2). This complex structure was elucidated by Jung and collaborators in 1995, through the combined use of UV spectroscopy, mass spectrometry (MS), amino acid analysis, Edman sequencing and, above all, multi-dimensional nuclear magnetic resonance (NMR) applied to unlabelled and stable isotope-labelled samples.[79] The complete structure of the microcin was indeed deduced from a detailed analysis of the data arising from homo- and heteronuclear multi-dimensional NMR and triple resonance experiments performed on the $^{13}C/^{15}N$ doubly

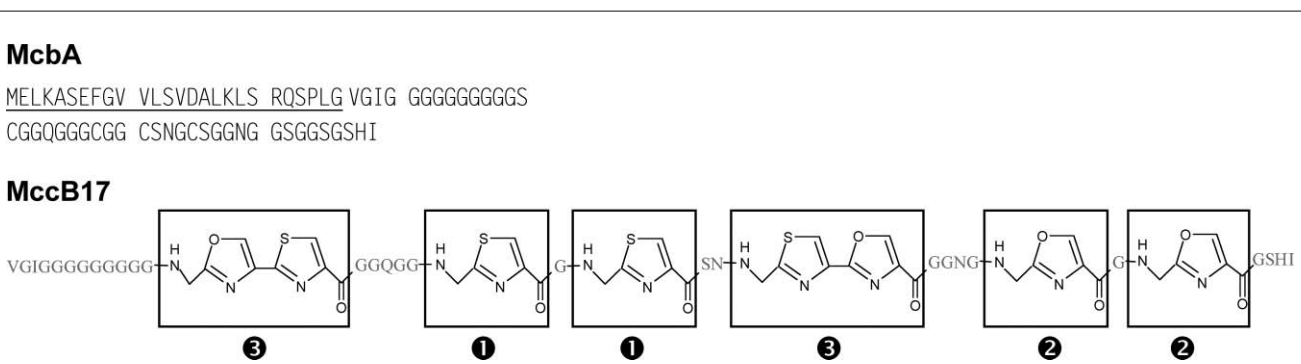

Fig. 2 Sequence of the MccB17 precursor (McbA) and structure of mature MccB17. The 26-residue leader peptide is underlined in McbA. The amino acids are figured in grey for mature MccB17. In MccB17, thiazoles (**1**), oxazoles (**2**), and bis-heterocycles (**3**) are boxed.

labelled MccB17. Two isolated thiazole and oxazole rings and two adjacent bis-heterocyclic systems consisting of directly linked oxazole–thiazole and thiazole–oxazole entities were characterized. They were found to result from two Gly-Cys and two Gly-Ser dipeptides on the one hand, and from Gly-Ser-Cys and Gly-Cys-Ser tripeptides, respectively, on the other hand. This unique structure was fully confirmed through the total synthesis of MccB17.[82] The three-dimensional structure of MccB17 has never been described until now.

MccC7 was isolated from *E. coli* MC4100 harbouring the pMM550 plasmid and cultivated in M63 medium, using a protocol associating size-exclusion and RP chromatographies.[83] MccC51 was purified from culture supernatants of *E. coli* TG1 harbouring either the pUHAB or pBM43 plasmid and grown in M63 minimal medium. The purification protocol involved solid-phase extraction and subsequent RP-HPLC.[84,85] The spectrum of activity of MccC7 and MccC51 was reported to cover several genera of enterobacteria, such as *Escherichia*, *Enterobacter*, *Klebsiella*, *Salmonella*, *Shigella*, *Proteus* and *Yersinia*.[27,86,87] MccC7 and MccC51 were shown to have an identical structure (see below). They were characterized as an *N*-formylated heptapeptide, which also contains a modified adenosine monophosphate (AMP) covalently attached to the *C*-terminal Asp through a phosphoramide bond. Conversely to *mccA*, which encodes an Asn as the seventh amino acid, MccC7/C51 heptapeptide ends with an Asp (Fig. 3). The phosphoramidate group, substituted by an *n*-aminopropanol chain, subsequently contains a chiral phosphorus atom. This is the only microcin known to carry a nucleotide as post-translational modification. The structure was identified for MccC7 by Delepierre and collaborators in 1995.[83] The same year, the structure of MccC51 was published.[88] The structures of MccC7 and MccC51, based on NMR studies, differed in both the linkage between the peptide and nucleotide parts and the nucleotide structure itself. MccC51 was described as a nebularin 5′-monophosphate *C*-terminal entity linked to the Asp7 side-chain through three methylene bonds. In 2000, the structure of MccC51 was re-investigated in our group. By a combined hetero- and homonuclear NMR study, we determined that the structure of MccC51 was actually identical to that of MccC7.[29,84] In particular, the presence of a phosphoramide bond acting as a linker between the heptapeptide and the nucleotide and the location of the *n*-aminopropanol chain, which were the two critical points of the structure, were unambiguously assigned in MccC51 through typical cross-peaks in two-dimensional ^{1}H–^{31}P NMR heteronuclear single quantum coherence spectra.[84] Therefore, MccC7 and MccC51, which arise from two distinct *E. coli* strains, share a common nucleotide–peptide structure (Fig. 3), disclosing the first observation of two closely related microcins. At the present time, the three-dimensional structure of MccC7 and MccC51 remains unknown. It is worth noting that because of their common structure, MccC7 and MccC51 have been occasionally termed MccC.[23,85] However, this terminology is also used for the *mccC* gene product. We therefore prefer to use the MccC7/C51 terminology, which avoids this confusion. Interestingly, the secreted microcin undergoes activation by proteolytic cleavage inside the susceptible bacteria (Fig. 3).[85] To distinguish these two forms of microcin, we propose to term MccC7/C51* the intracellularly processed MccC7/C51.

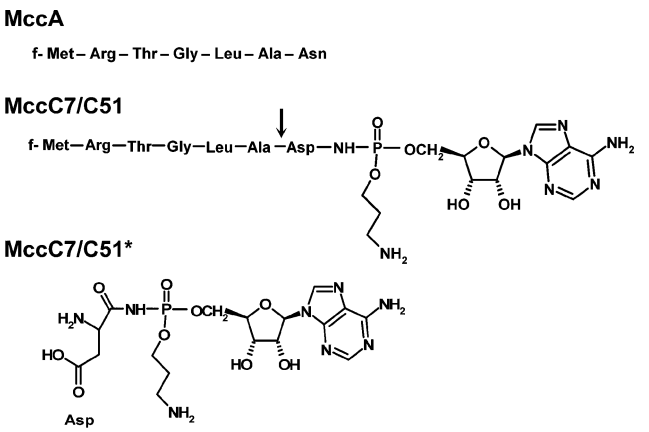

Fig. 3 Sequence of MccA and structures of MccC7/C51 and MccC7/C51*. Formylation of Met1 is indicated by an 'f' in the sequences. MccC7/C51 is the antibacterial peptide secreted by the producer, whereas MccC7/C51* is the translation inhibitor generated by cleavage of MccC7/C51 within susceptible cells. The arrow indicates the cleavage site of MccC7/C51.

MccJ25 was efficiently purified from culture supernatants of *E. coli* MC4100 harbouring the pTUC202 plasmid, and grown in M9 or M63 minimal medium. Thus, as MccC7/C51, MccJ25 was isolated by solid-phase extraction and further RP-HPLC.[89] The spectrum of MccJ25 antibacterial activity was found to be restricted to few genera of enterobacteria, mainly *Escherichia* and *Salmonella*, with MICs in the 2–5 nM range.[89,90] MccJ25 was shown to be bactericidal against strains of *E. coli* and *S. enterica*,

serovars Enteritidis and Paratyphi.[90] MccJ25 is generated from a 58-aa precursor, McjA (Fig. 4A). Mature MccJ25 is a 21-residue hydrophobic peptide that displays a three-dimensional lasso-type structure (Fig. 4B). MccJ25 cyclization results from the linkage between the *N*-terminal Gly1 amino group and the Glu8 side-chain carboxylate, leading to a small ring (Fig. 4B). The resulting 13-residue linear *C*-terminal tail is entrapped into this ring through (i) non-covalent interactions, and (ii) steric hindrance by the two bulky aromatic side-chains from Phe19 and Tyr20, straddling each side of the ring (Fig. 4B). The tail can only be released by cleavage of the ring. This structure was identified simultaneously by three groups using combined MS and NMR studies.[91–93] However, similar to MccC7/C51, the structure of MccJ25 has been a subject of debate in the literature.[94] MccJ25 was first isolated in 1992 by Salomón *et al.*,[32] and characterized as a 20-residue hydrophobic peptide with a blocked *N*-terminal end. It was further shown in our group to be a 21-residue head-to-tail macrocyclic peptide.[89,95] Re-investigation of the structure in 2003 showed that the cycle actually engaged Glu8 side-chain carboxylate instead of Gly21 one.[91–93] The MccJ25 lasso structure was shown to be required for optimal antibacterial activity[96] and to be responsible for MccJ25 high stability. Indeed, MccJ25 retained both its three-dimensional structure and its antibacterial activity at 165 °C, as well as up to 95 °C in the presence of potent denaturing agents.[96] MccJ25 structure is also resistant to proteolysis. The loop can be enzymatically opened by thermolysin at the Phe10–Val11 amide bond (Fig. 5), or can be targeted by a strong acidic medium (Fig. 5).[96,97] However, the initial lasso structure is not destroyed during these processes and the resultant entities are two-chain peptides, the tail (or a shortened tail) remaining firmly anchored to the ring, both in solution and in gas phase, as shown by NMR and MS studies.[97] In fact, ring cleavage can only be accomplished by partial hydrolysis in basic medium.[93] Such an original structure had never been identified previously among antibacterial peptides. Nevertheless, either additionally stabilized or not by disulfide bond(s), similar lasso-type structures had already been encountered among enzyme inhibitors synthesized by *Streptomyces* species.[94,98–100] Recently, such a structure was also found in lariatins from *Rhodococcus* sp.[101]

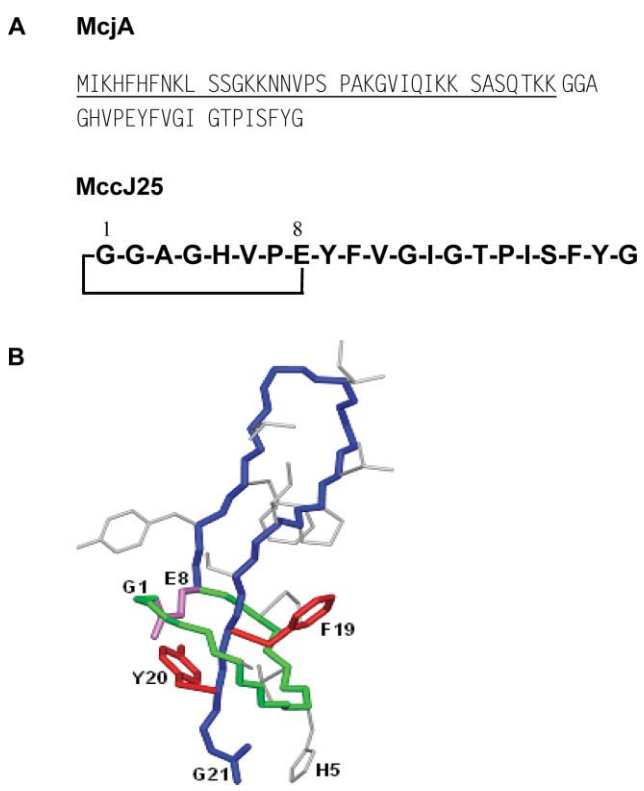

Fig. 4 (**A**) Sequence of the MccJ25 precursor (McjA) and structure of mature MccJ25. The 37-residue leader peptide is underlined. (**B**) Three-dimensional structure of MccJ25. Note the steric hindrance imposed by Phe19 and Tyr20, which strongly contribute to the blocking of the *C*-terminal tail into the ring.

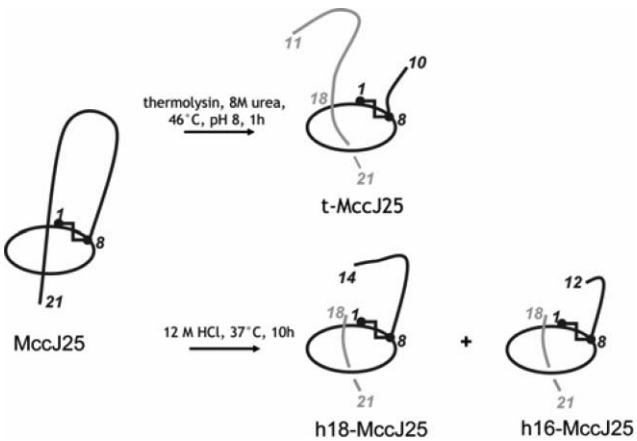

Fig. 5 Structures of the two-chain peptides generated from MccJ25. t-MccJ25 is obtained by thermolysin cleavage of MccJ25, which breaks the lasso structure between Phe10 and Val11. h18-MccJ25 and h16-MccJ25, which contain 18 and 16 amino acids, respectively, are obtained by hydrochloric acid cleavage of MccJ25. The cleaved fragments (in grey) remain tightly attached to the main peptide chain (in black), which contains the cycle.

3.2 Class II microcins

Class II microcins are higher molecular mass microcins (4.9 to 8.9 kDa). Their peptide backbones do not undergo extensive modifications. Besides disulfide bonds, they may carry a siderophore-type post-translational modification.

3.2.1 Class IIa microcins: MccV, MccL and Mcc24. MccV was isolated, purified and characterized in 1994 by Kolter and collaborators[102] simultaneously to Håvarstein and collaborators.[103] From these studies, MccV can be isolated from culture supernatants of *E. coli* MC4100 harbouring the pHK11 or pHK22 plasmid. Since the MccV gene expression is repressed by excess iron, the strains were grown on tryptone broth or Luria broth containing the iron chelator 2,2′-dipyridyl. MccV purification used a four-step procedure involving trichloroacetic acid or ammonium sulfate precipitation, amberlite XAD16 absorption, cation exchange chromatography and RP-HPLC. MccV showed an antibacterial activity directed against related Gram-negative bacteria with an MIC of about 0.1 nM against *E. coli*.[103] Expressed as a 103-aa precursor, the mature MccV is an 88-aa peptide (Table 1), without post-translational

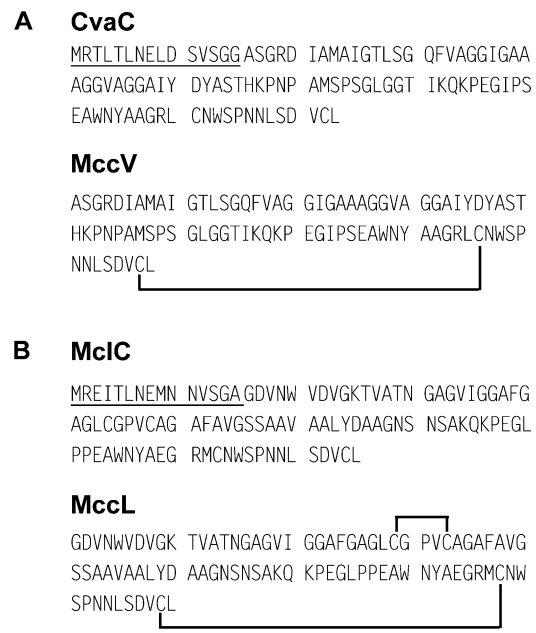

Fig. 6 (**A**) Sequences of the MccV precursor (CvaC) and of mature MccV. (**B**) Sequences of the MccL precursor (MclC) and of mature MccL. The 15-residue leader peptides are underlined. Disulfide bonds are shown as black lines.

modification, which possesses a single disulfide bond connecting Cys76 to Cys87 (Fig. 6A).[103]

MccL was isolated from the supernatant of the wild-type producer *E. coli* LR05 grown in M63 medium, and its purification mainly used RP-chromatography.[12] MccL was reported to be active against *Shigella* sp., several *E. coli* including diarrheagenic strains, *Pseudomonas* sp. and several *Salmonella enterica* strains, including serovars Enteritidis and Typhimurium, with MICs in the nanomolar range.[39] MccL, which is generated from a 105-aa precursor, is composed of 90 unmodified amino acids.[39] It is a glycine-rich, anionic, and highly hydrophobic peptide (46.7% non-polar amino acids) (Table 1, Fig. 6B). MccL shares an identical 13-aa *C*-terminal sequence with MccV, which is believed to be folded by a disulfide bond connecting Cys78 and Cys89 in MccL.[39] However, MccL possesses an additional disulfide bond connecting Cys29 to Cys33. Homology searches show a strong similarity between the 32 *C*-terminal amino acids of MccL and MccV (87.5% identity). Moreover, in the region surrounding the first disulfide bond (Ile20–Ala38), MccL exhibits significant similarity (43–52% identity) with lafA subunit from lactacin F[104] and gassericin T,[105] two non-lantibiotic bacteriocins from *Lactobacillus*.

Mcc24 has neither been isolated nor characterized. Although its precursor sequence makes it undoubtedly a class II microcin (Table 1, Fig. 7), it is difficult to classify it within class IIa/IIb. Indeed, it does not contain any cysteine and lacks the *C*-terminal sequence found in other class IIa microcins (Fig. 6). Similarly, the Mcc24 precursor displays major homologies (50% identity) with MccE492 precursor (Section 5.1.1), but lacks the 10-aa *C*-terminal sequence typical of class IIb microcins. Therefore, Mcc24 appears atypical among class II microcins. Because the structure of Mcc24 does not allow its classification, we have considered that its gene cluster, which contains four genes only, makes it belong to class IIa.

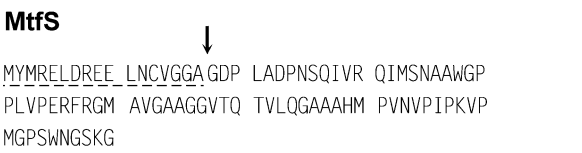

Fig. 7 Sequence of the Mcc24 precursor (MtfS). The putative leader peptide is underlined with a dashed line. The putative cleavage site of Mcc24 precursor, whose location is based on multiple alignment of class II microcin precursors deduced from their DNA sequences (Section 5.1.1; Fig. 10), is indicated by an arrow.

3.2.2 Class IIb microcins: MccE492, MccM, MccH47 and MccI47. Class IIb microcins are devoid of disulfide bonds. All of them have a conserved serine-rich *C*-terminal region and they may carry a siderophore-type post-translational modification.

MccE492 was purified from culture supernatants of *E. coli* strains harbouring the recombinant pJAM229 plasmid.[55] Culture conditions were found to be critical to obtain fully mature MccE492.[14] Basically, MccE492 should be expressed under iron-poor conditions, M63 minimal medium being appropriate, and in the absence of free aromatic amino acids (unpublished work). Indeed, the use of casamino acids should be prevented, since it led to unmodified MccE492 (termed here u-MccE492), which we have recently shown to be an incompletely processed microcin (unpublished work). Efficient purification of both MccE492 and u-MccE492 can be achieved by solid-phase extraction followed by RP-HPLC.[14,106] Both MccE492 and u-MccE492 were found to be bactericidal, mainly against *E. coli* strains. However, upon complete maturation, MccE492 activity increased by 4–8 fold (MICs in the 40–80 nM range) and its spectrum of activity extended to *K. pneumoniae* and *Enterobacter cloacae*.[14] MccE492 is generated from a 103-aa precursor, MceA, by elimination of a 19-aa leader peptide.[56] This cleavage results in the release of u-MccE492, the unmodified form initially characterized by Pons *et al.*[107] (Table 1, Fig. 8). Fully mature MccE492 (formerly termed MccE492m) is a siderophore-peptide that carries a linear trimer of *N*-(2,3-dihydroxybenzoyl)-L-serine (DHBS) anchored at the *C*-terminus (Ser84) through a β-D-glucose.[14] This structure was determined in our group by subjecting the 11-residue *C*-terminal fragment of MccE492 to ion trap MS and high field two-dimensional ^{1}H–^{13}C NMR. The β-D-glucose was shown to be linked to the Ser84 carboxylate through an *O*-glycosidic bond at C6, and to the first DHBS entity through a *C*-glycosidic bond at C1 (Fig. 8).[14] The amino acids composing MccE492 are mainly uncharged and hydrophobic, with the exception of one histidine, three aspartic acids and one glutamic acid (Table 1) that give an anionic character to this microcin. MccE492 post-translational modification mimics siderophores (*i.e.* molecules designed by bacteria to chelate ferric iron, enabling its uptake across the bacterial outer membrane *via* specific receptors (Section 7.1.1), and particularly salmochelins.[108] Indeed, we showed by MS that mature MccE492 selectively binds ferric iron through its catecholate moieties. This makes MccE492 the first natural siderophore-peptide to be described.[14]

E. coli Nissle 1917 was shown to produce two bactericidal activities attributed to MccH47 and MccM,[65] but none of these microcins had been isolated until now. Very recently, MccM was isolated and purified in our group (unpublished work) from

MceA

MELRMREISQ KDLNLAFGAG ETDPNTQLLN DLGNNMAWGA
ALGAPGGLGS AALGAAGGAL QTVGQGLIDH GPVNVPIPVL
IGPSWNGSGS GYNSATSSSG SGS

MccE492

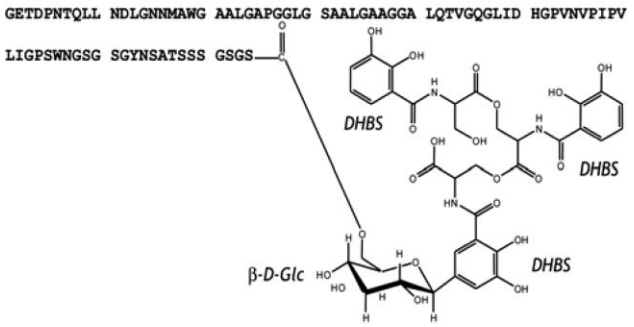

Fig. 8 Sequence of the MccE492 precursor (MceA) and structure of mature MccE492 carrying the siderophore post-translational modification. The leader peptide is underlined. *Glc* and *DHBS* stand for glucose and *N*-(2,3-dihydroxybenzoyl)-L-serine, respectively.

E. coli MC4100 transformed with the pMM75 plasmid (Moreno and collaborators, unpublished work). MccM was produced in M63 medium and purified by a protocol similar to that used for MccE492 purification.[14] No spectrum of activity is available at this time. MccM is a 77-aa peptide generated from a 92-aa precursor, McmA (Table 1, Fig. 9A). This was recently shown in our group (unpublished work) by MS, Edman sequencing and analysis of MccM trypsin digest. Matrix-assisted laser desorption/ionization time-of-flight MS data also suggested that MccM secreted by *E. coli* harbouring the pMM75 plasmid carries a post-translational modification similar to that characterized for MccE492. Interestingly, although MccM appears to belong to class IIb microcins, it exhibits 34% identity with MccV.

A **McmA**

MRKLSENEIK QISGGDGNDG QAELIAIGSL AGTFISPGFG
SIAGAYIGDK VHSWATTATV SPSMSPSGIG LSSQFGSGRG
TSSASSSAGS GS

B **MchB**

MREITESQLR YISGA↓GGAPA TSANAAGAAA IVGALAGIPG
GPLGVVVGAV SAGLTTAIGS TVGSGSASSS AGGGS

MchS2

MREISDNMLD SVKGG↓MNLNG LPASTNVIDL RGKDMGTYID
ANGACWAPDT PSIIMYPGGS GPSYSMSSST SSANSGS

Fig. 9 (**A**) Sequence of the MccM precursor (McmA). The leader peptide is underlined. Similar to mature MccE492, MccM can be modified by a siderophore moiety linked to the *C*-terminal serine. (**B**) Sequences of the precursors of MccH47 (MchB) and MccI47 (MchS2). The putative leader peptides are underlined with dashed lines. The putative cleavage sites of MccH47 and MccI47 precursors, whose location is based on multiple alignment of class II microcin precursors (Section 5.1.1; Fig. 10), are indicated by an arrow.

Difficulties in isolating MccH47 have hampered the characterization of its primary structure. The recently discovered MccI47 has never been isolated either. The structures of both microcins were predicted by the genetic studies of Laviña and collaborators.[37,59,72] On the basis of their deduced amino acid sequences (Fig. 9B), both microcins are believed to display the highly conserved *C*-terminus found in MccE492 and MccM (Fig. 10, Section 5.1.1). Because MccE492, and probably MccM (see above), carry a catechol-type siderophore on this conserved *C*-terminal region, a similar post-translational modification could occur in MccH47 and MccI47. As discussed earlier,[57] this hypothesis is reinforced by (i) highly conserved genes believed to encode modification enzymes in MccE492, MccM/MccH47, and MccH47/MccI47 gene clusters (Fig. 1C, Section 2.2.2), and (ii) the requirement of all four microcins (MccE492, MccM, MccH47, MccI47) for catechol-type siderophore receptors at the outer membrane of *E. coli* (Section 7.1.1).

It thus appears that mature microcins share common structural features, such as high hydrophobicity and high content in glycine and serine (Table 1). Most of the isolated microcins are devoid of cysteine residues, except the class IIa microcins MccV and MccL, which show 1 and 2 disulfide bonds, respectively. All 4 cysteine residues present in MccB17 precursor are modified to heterocyclic rings in the mature microcin. Most microcins are anionic peptides, except MccB17, MccV and putatively Mcc24, which are slightly cationic. Within class I microcins, no similarity can be highlighted, but low molecular mass and extensive backbone modification. Alignment of class II microcins (Section 5.1.1, Fig. 10) shows strong similarities in the *C*-terminal region of the class IIa MccL and MccV on the one hand, and of the class IIb MccE492, MccM, MccH47 and MccI47 on the other hand. However, similarities between members of class IIa and IIb are also underlined.

4 Export machinery

The machinery in charge of microcin secretion into the extracellular medium was identified either by genetic or homology analysis. In many cases, this machinery appears to be associated either to self-immunity or to proteolytic cleavage of the promicrocin.

There is no standard export machinery for class I microcins. MccB17 export has been shown to be driven by McbF and McbE,[18] which are related to an ABC transporter and its accessory protein, respectively. McbF is predicted to contain a nucleotide-binding domain and McbE to span the inner membrane. For this reason, McbF and McbE could serve as a pump to export MccB17 to the perisplasmic space.[18] However, the presumed ABC transporter involved in MccB17 export does not show any proteolytic domain, as class II microcins ABC transporters do. Moreover, the outer membrane component required for MccB17 export across the outer membrane remains unidentified. The mechanism of MccC7/C51 secretion is unclear. It involves MccC, a hydrophobic protein that shows significant similarity to multidrug efflux transporters belonging to the major facilitator superfamily (MFS).[28,29] MFS transporters export small solutes only, such as sugars and secondary metabolites (for a review, see Pao *et al.*[109]). This efflux mechanism would be consistent with the low molecular mass of MccC7/C51. To date, three proteins involved in MccJ25 export have been identified. McjD,

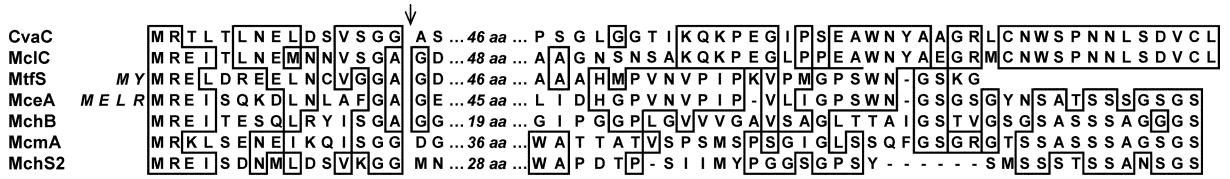

Fig. 10 Multiple alignment of *N*- and *C*-terminal regions of class II microcin precursors. The sequences of the central regions are not aligned. Their lengths in amino acids (aa) are given in italics. Alignments were performed with Multalin[122] and improved manually. Dashes indicate gaps. All the amino acid sequences are from the Swiss-Prot Database. CvaC (accession no P22522), MclC (accession no Q841V4), MtfS (accession no Q46971), MceA (accession no Q9Z4N4), MchB (accession no P62530), McmA (accession no Q83TS1) and MchS2 (accession no Q712Q0) correspond to MccV, MccL, Mcc24, MccE492, McH47, MccM and MccI47 precursors, respectively. The arrow indicates the known or putative cleavage site of microcin precursors. The extra 4 and 2 residues in the *N*-terminal region of MceA and MtfS, respectively, are in italics (see Section 5.1.1).

whose sequence contains a putative transmembrane domain and a *C*-terminal domain similar to nucleotide binding domain, was classified as an ABC transporter.[34] It would be responsible for MccJ25 efflux, a mechanism by which it would also confer self-immunity to the producing strain. McjD would work together with the chromosomally encoded TolC.[110] This outer membrane protein forms a trimeric channel in the outer membrane, extending from the extracellular side of the outer membrane, through most of the periplasmic space, to finally end close to the periplasmic side of the inner membrane.[111] Another chromosomally encoded protein of *E. coli*, YojI, which is also similar to ABC transporters, was found to protect TolC-expressing bacteria against MccJ25.[112] McjD and YojI display the same ABC transporter features, and could play the same role in MccJ25 export, *i.e.* form a complex with TolC, with or without a supplementary accessory protein.

Class II microcin export machinery displays a canonical structure. It consists of three components, two of which, the ABC transporter and the accessory protein, are encoded by the microcin gene cluster. Table 3 summarizes the percentage identities of class II microcin export machineries. The CvaB protein from MccV export machinery and the highly homologous MclB, MtfB, MceG and MchF proteins responsible for the export of MccL, Mcc24, MccE492 and MccH47/I47/M, respectively, are all similar to ABC transporters (for reviews, see Jones and George[113] and Holland *et al.*[114]) responsible for the export of other antibacterials such as class II bacteriocins from Gram-positive bacteria[115] and RTX toxins.[116] These ABC transporters possess three domains. Besides a central, poorly conserved, transmembrane domain, they contain (i) an *N*-terminal domain (about 130 amino acids), which has a protease activity and would be located in the cytoplasm of bacteria,[117,118] and (ii) a *C*-terminal domain, which contains a highly conserved nucleotide-binding cassette required for ATP binding.[119] A model in which binding of the microcin promotes the transition of the ABC transporter from an inactive dimer bound to nucleotide diphosphate to an active high-energy dimer bound to nucleotide triphosphate has been proposed for MccV.[48] This energized state would enable the transmembrane domain of the protein to form a channel in the inner membrane. The second component of the export system is referred to as the accessory protein. It is predicted to be a periplasmic protein anchored at the inner membrane by an *N*-terminal transmembrane helix.[49,120] In class II microcins, accessory proteins are CvaA from the MccV export machinery, as well as MclA, MtfA, MceH and MchE for MccL, Mcc24, MccE492 and MccH47/I47/M, respectively. As ABC transporters, the accessory proteins are also highly conserved (Table 3). Although their role in secretion is still unclear, they may serve as connectors to the outer membrane protein TolC, which is the third component of the class II microcin export machinery.[36–39] TolC is believed to enable the secretion of the microcins by forming a continuous channel from the cytoplasm to the extracellular medium. The MccE492 export machinery seems to require another protein encoded by the microcin gene cluster, MceF. This putative inner membrane protein could interact with MceGH for processing or export.[38] Similarly, McmM which is

Table 3 Percentages identity between export proteins of class II microcins. The corresponding microcin is given in parentheses

	MceG (MccE492)	MchF[a] (MccH47)	MtfB (Mcc24)	MclB (MccL)	CvaB (MccV)
MceG	100	92.7	77.3	88.5	87.2
MchF[a]	—	100	75.7	92.1	89.5
MtfB	—	—	100	74.3	73.7
MclB	—	—	—	100	95.6
CvaB	—	—	—	—	100
	MceH (MccE492)	MchE[a] (MccH47)	MtfA (Mcc24)	MclA (MccL)	CvaA (MccV)
MceH	100	91.8	72.9	92.0	91.3
MchE[a]	—	100	71.4	99.8	98.1
MtfA	—	—	100	71.7	71.2
MclA	—	—	—	100	97.8
CvaA	—	—	—	—	100

[a] MccH47 gene cluster from *E. coli* H47.

homologous to MceF (61.6% identity), would be involved in MccM processing or export.

5 From genes to structures: biosynthesis and regulation

5.1 Microcin precursors, the promicrocins

Similar to bacteriocins from Gram-positive bacteria,[5,121] microcins generally derive from a precursor, the promicrocin (Table 1). This latter consists of (i) a *C*-terminal structural region and (ii) an *N*-terminal leader peptide comprising 15 to 37 residues.[22] As previously mentioned, MccC7/C51 is the only one to be secreted by the producing strain without previous cleavage of a longer precursor.[25,27]

5.1.1 Biochemical characteristics and conserved domains. Class I microcin precursors do not display common features. MccB17 and MccJ25 have long leader peptides (26 and 37 amino acids) relative to the size of their precursor (69 and 58 amino acids, respectively). The processing site of both microcins was accurately determined by isolation of the mature peptide.[20,89] As with several other microcins from class II (see below), processing of the MccB17 precursor occurs *C*-terminal to a glycine (Fig. 2). However, this residue is not involved in a double-glycine or glycine–alanine motif, and the leader has not the typical sequence of double-glycine-type leader peptides, as do class II microcin precursors (see below). In MccJ25, cleavage occurs *C*-terminal to a lysine and *N*-terminal to a double-glycine motif (Fig. 4A).

Class II microcins are generated from large precursors carrying small conserved leader peptides. Indeed, alignment of class II microcin precursors using the Multalin program[122] reveals that these microcins exhibit highly conserved leader peptides (Fig. 10). All seven microcin precursors, whose sizes range from 75 to 105 residues, have a 15–19-residue leader peptide. Because Mcc24 and MccE492 precursors derive from genes with two neighboring AUG codons, transcription may actually begin at a second AUG encoding Met3 and Met5 in Mcc24 and MccE492, respectively. Mcc24 and Mcc492 precursors would then be lacking the extra 2 and 4 residues in the *N*-terminal position, respectively. It is therefore likely that all class II microcin leader peptides, including those of Mcc24 and Mcc492, are 15 residues long. Leader peptides from class II microcins contain an M-R-X-[I/L]-X_9-G-[A/G] (X denotes any amino acid) conserved sequence (Fig. 10), with a typical double-glycine motif (MccV, MccM and MccI47) or a glycine–alanine motif (MccL, Mcc24, MccE492, and MccH47), found as an alternative to the double-glycine motif in proteins exported through ABC transporters.[115,123] Based on the *N*-terminal sequences of mature MccV,[102] MccL,[12] MccE492,[107] and MccM (unpublished work), it is likely that Mcc24, MccI47, and MccH47 precursors are also processed after the double-glycine or the glycine–alanine motif.

5.1.2 Role of the leader peptide. A wide variety of functions have been proposed for *N*-terminal leader peptides from antimicrobial peptide precursors. The leader peptide could alternatively (i) ensure stabilization of the antibacterial peptide by preventing intracellular degradation of the produced peptide or its encoding DNA/RNA,[124] (ii) act as a chaperone folding the molecule so that it is recognized by the maturation machinery,[125] or (iii) serve as a recognition sequence for the maturation and/or export machineries.[126] Microcin leader peptides appear to achieve function(s) that differ from one microcin to another.

Several class I microcins do not require their leader peptide for export. Instead, it can be used for recognition by post-translational modification enzymes,[127] as shown for MccB17. Indeed, following the formation of the oxazole and thiazole rings of various fusion peptides, Kolter and collaborators demonstrated that MccB17 leader peptide is essential for the post-translational modifications of MccB17, and serves as a prime determinant for the recognition and recruitment of the precursor by MccB17 synthetase.[21,127] In addition, because exogenous MccB17 (lacking the leader peptide) is pumped out by McbEF-expressing strains,[18] it was proposed that the MccB17 leader peptide was involved in MccB17 recognition by the post-translational modification enzymes rather than by the export machinery. Although the MccJ25 precursor has been poorly studied until now, it is possible that the MccJ25 leader peptide would serve an identical function. Indeed, the McjD export protein confers resistance to exogenous MccJ25.[33] This indicates that the MccJ25 leader peptide is not required for recognition by the export machinery.

Based on MccV studies, leader peptides from class II microcins could be involved in export. Indeed, the MccV leader peptide was shown to be an *N*-terminal export signal. Thus, while the 29 *N*-terminal residues of the MccV precursor are sufficient to promote translocation across the inner membrane, amino acids 30–38 contain the information affecting the efficiency of recognition/export of the protein.[36] Conversely, deletion of 21 residues at MccV *C*-terminal end does not modify its secretion.[102] Because of the sequence similarities described above, leader peptides of class II microcins, which do not resemble the typical *N*-terminal signal sequence-specific for Sec-dependent translocation,[128,129] could nonetheless exhibit a common export recognition signal.

5.1.3 Antibacterial activity of promicrocins. Due to their self-immunity and export genes, microcin-producing bacteria are protected from the toxicity of their own microcin. However, several microcin precursors do not display antibacterial activity until processed. This is the case for class I microcin precursors.

Studies on the MccB17 precursor (McbA) revealed that compounds with six out of the eight heterocycles found in mature MccB17 are active, in contrast to McbA.[130] This indicates that at least partial modification is required for antibacterial activity. Similarly, the synthetic heptapeptide moieties of both MccC7/C51 and MccA lack antibacterial activity.[83] Moreover, it was recently shown that the secreted nucleotide–peptide remains inactive until proteolysis inside target cells.[85] As with MccB17 and MccC7/C51, MccJ25 precursor (McjA) is devoid of antibacterial activity. This was recently demonstrated using an *E. coli* expressing recombinant McjA (Duquesne *et al.*, unpublished work). Interestingly, the chemically synthesized 21-residue linear MccJ25 was not active either.[96] This suggests that in the case of MccJ25, the antibacterial activity depends on the acquisition of the three-dimensional structure, rather than on the elimination of the leader peptide.

In contrast, class II microcin precursors could display some antibacterial activity. Indeed, bacteria only harbouring MccV structural (*cvaC*) and self-immunity (*cvi*) genes showed antibacterial activity in the lysates,[44,131] suggesting that CvaC, the MccV

precursor, possesses antibacterial activity. Interestingly, antibacterial activity was completely abolished upon alanine replacement of the two cysteines involved in the MccV disulfide bond.[46] This indicates that, as with MccJ25, folding of MccV is required for antibacterial activity. Similar to MccV, lysates from bacteria only harbouring MccL structural (*mclL*) and self-immunity genes (*mclI*) were found to possess antibacterial activity,[39] suggesting that unprocessed MccL is active. However, in contrast to MccV, the folding imposed by the two disulfide bridges seems not to be involved in MccL activity, since addition of high amounts of dithiothreitol does not abolish the antibacterial activity.[39] Disulfide bonds were therefore proposed to be exclusively responsible for the high stability of mature MccL.[39] Since production of the MccH47 precursor (MchB) was deleterious to the producing strain, MchB was proposed to display antibacterial activity.[68] However, lysates from bacteria only harbouring the structural (*mchB*) and self-immunity (*mchI*) genes were inactive against MccH47-susceptible cells. Lack of activity was also observed in lysates of bacteria that do not express *mchACD*,[68] three genes putatively involved in MccH47 production. This might be due to a dramatic decrease in MccH47 production, as also observed with u-MccE492 when *mceC* (homologous to *mchA*) is disrupted (unpublished work). The same lowered microcin production could explain why no antibiotic activity was detected for bacteria harbouring *mchS2* (MccI47 structural gene) but disrupted *mchA*, *mchC* or *mchD*, the genes thought to be responsible for MccI47 production.[72] The unprocessed MccE492 precursor (MceA) has never been isolated to date. However, the presence of the MccE492 siderophore post-translational modification increased the peptide potency against all tested *E. coli* and *Salmonella* strains and broadened its spectrum of activity.[14]

Altogether, these studies conclude that the class I microcin precursors are devoid of antibacterial activity. To gain their activity, extensive backbone post-translational modifications are required. In contrast, class II microcin precursors are thought to have some antibacterial activity. The gain of activity associated with the processing step was not directly measured. However, for MccE492 and MccV, antibacterial activity is significantly improved by subsequent post-translational modifications and/or folding.

5.2 Maturation of microcins

Maturation of microcins requires proteolytic enzymes that cleave the leader peptides from the promicrocins, and enzymes that ensure post-translational modifications.

5.2.1 Proteolytic cleavage of promicrocins. Although all promicrocins except MccC7/C51 require proteolytic removal of a leader peptide before secretion, little is known about the proteases involved. Nevertheless, it seems that proteolytic cleavage of class II microcin leader peptides occurs during export, and that it would result from the action of the export machinery. However, this does not apply to class I microcins, for which precursor processing appears to rely on a broader variety of mechanisms.

The best-studied class I microcin is MccB17. Its processing is carried out by chromosomally encoded protease(s). Indeed, *mcbA–lacZ* fusions expressed in *mcbBCDEF*-deficient strains were found to be cleaved from their 26-residue *N*-terminal leader peptide.[132] A chromosomal gene called *pmbA* (or *tldE*) was shown to be involved in MccB17 maturation[133] (Fig. 11). Subsequently, studies of *tldE* and *tldD* mutant strains harbouring *mcbABCDEF* demonstrated that modified McbA accumulated in those bacteria, indicating that MccB17 export machinery was not responsible for the cleavage of the leader peptide.[134] MccC7/C51 is secreted as an uncleaved nucleotide–heptapeptide. However, it was recently shown to be cleaved after entry into the target bacteria to generate MccC7/C51*, a modified aspartyl adenylate (Fig. 3), which is the actual intracellularly active form of the microcin[85] (Section 7.2.2). As for MccB17, cleavage of the last peptide bond in MccC7/C51 does not depend on any of the genes encountered on the microcin gene cluster. Indeed, in this case, the processing is performed in susceptible bacteria, and amazingly, it can also be carried out by peptidases from eukaryotic extracts.[85] MccJ25 processing enzymes remain unidentified to date. Nevertheless, bacteria harbouring disrupted *mcjB* or *mcjC* genes are impaired in the production of mature MccJ25 (Duquesne *et al.*, unpublished work). Therefore, and although neither McjB nor McjC are similar to known proteases, they are believed to be involved in MccJ25 processing rather than the self-immunity/export protein McjD or any other chromosomally encoded enzyme.[34] However, which of these two proteins displays a proteolytic activity, and at what stage of the maturation this step happens, remain to be elucidated.

Maturation of class II microcins is concomitant with export. Indeed, processing of MccV precursor involves the CvaA/CvaB/TolC export machinery described above.[135] Because MccV processing could be inhibited by *N*-ethylmaleimide and antipain, the protease was proposed to be a cysteine protease.[135] Since CvaA is devoid of cysteine residues, and the *N*-terminal cytoplasmic domain of CvaB contains a proteolytic domain, the protease activity may be accomplished by CvaB. Similar to MccV, MccH47 precursor is believed to be cleaved from its leader peptide during export.[70] This is consistent with the identification of a glycine–alanine motif in MchB,[68] while the alternative double-glycine motif is found in MccV precursor, CvaC (Fig. 10). MchF, which is homologous to CvaB, would then be responsible for the cleavage of MccH47 leader peptide. Consistently, MccV export system was actually shown to be competent for recognizing and exporting mature MccH47 into the extracellular medium.[70] Moreover, since MccM and MccI47 (i) share the conserved leader sequence displayed by MccH47 and MccV (Fig. 10), and (ii) are encoded on the same gene cluster as MccH47 in *E. coli* CA46/CA58 and *E. coli* H47, respectively (Fig. 1), they are likely to share the processing/export machinery of MccH47. As both the leader peptides of MccE492, MccL and Mcc24 precursors (Fig. 10) and their export machineries are highly similar to those of MccV (Table 3), processing is likely to be carried out by the same mechanism involving the ABC transporter of their dedicated export machinery, namely MceG, MclB and MtfB for MccE492, MccL, and Mcc24, respectively.

5.2.2 Post-translational modifications. Biosynthesis of post-translationally modified microcins involves enzymes encoded on the microcin gene clusters. They are believed to be responsible for the large panel of post-translational modifications displayed by microcins. However, only a few reports on *in vitro* synthesis of microcins have been published.

Consistent with the heterogeneity of structures they display, class I microcins use various enzyme machineries to achieve post-translational modifications. MccB17 post-translational modification has undoubtedly been the most extensively studied, and at least three gene products are required for MccB17 cyclization process. In 1996, Walsh and collaborators reported the first *in vitro* reconstitution of MccB17 biosynthesis. The purified synthetase, consisting of McbB, McbC, and McbD, was used to synthesize oxazole and thiazole rings within a recombinant His-tagged McbA.[21] The three-step model proposed at that time could be further verified.[136,137] Thus, the zinc-dependent McbB performs the initial cyclodehydration step, leading to oxazoline and thiazoline rings, which are further desaturated by the flavine-dependent dehydrogenase, McbC. Photo-labelling of McbA showed that within the complex, the ATPase McbD is responsible for the initial recognition and interaction with McbA[137] (Fig. 11). The enzymes responsible for MccB17 biosynthesis were shown to be chemoselective, processing cysteine residues faster than serine ones, and regioselective, only one ring being made before nascent product is released. In addition, the post-translational modification process was shown to be carried out directionally, ring formation taking place from the *N*- to the *C*-terminal extremity.[138] NMR analysis of MccB17 leader peptide showed it consists of an amphipathic α-helix spanning residues 5–21.[139] Ser6, Ser13 and Ser20 on one face of the helix form a polar stretch, while the side-chains of Phe8, Leu12 and in a lesser extent Val11 form a hydrophobic patch. Mutagenesis analysis demonstrated the stringent role of Phe8 and Leu12 in the recognition events by the MccB17 synthetase.[139] Moreover, the polyglycine linker (Gly30 to Gly39), whose length influences the synthethase turnover, was proposed to act as a spacer allowing the correct positioning of the heterocyclization site (Fig. 11).[140] The moderately polar face of the helical leader peptide including the serine array would interact with the inner membrane in order to target the modified McbA for cleavage of the leader peptide, and subsequent export. Finally, substitution of the glycine located immediately upstream of the cyclizable sequence, as well as substitution of cysteine and serine residues involved in the cyclization process, inhibited ring formation.[140]

MccC7/C51 post-translational modification would be carried out by MccB, MccD and MccE.[28,29] MccB exhibits similarity to proteins from the ThiF/MoeB/HesA family. These proteins catalyze the *C*-terminal adenylation of the ThiS and MoaD subunits from the thiamine and molybdopterin synthase, respectively.[141,142] MccB, as all of these proteins, contains a nucleotide-binding domain and a repeated cysteine metal-binding motif. The latter motif may be important for the activity of MccB, since a *Tn5* insertion in this repeat abolishes MccC7/C51 production.[28] The MccD sequence was found to be similar to proteins of the methyltransferase family.[29] Finally MccE possesses two putative domains that consist of an *N*-terminal region resembling pyridoxal phosphate-dependent amino acid decarboxylase and a *C*-terminal region displaying homologies with proteins catalysing the acetylation of ribosomal proteins.[29] This would account for a dual role of MccE in post-translational modification and self-immunity towards MccC7/C51. According to these similarities, three steps could be suggested for MccC7/C51 maturation. The inner membrane anchored MccB would adenylate the *C*-terminal aspartate of MccA. Moreover, MccE might be responsible for the formation of the *n*-aminopropanol through homoserine decarboxylation,[29] whereas MccD would be involved in the transfer of the

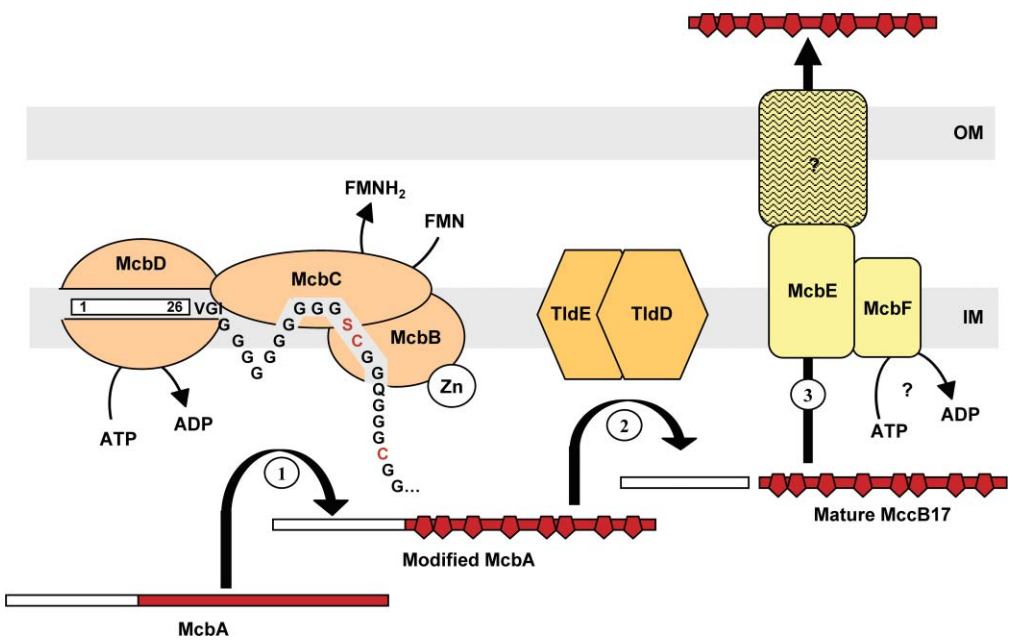

Fig. 11 Biosynthesis of MccB17. (**1**) McbA is modified by the MccB17 synthetase, consisting of McbB, McbC and McbD. The ATP-dependent McbD subunit first binds the McbA leader peptide, the polyglycine linker enabling the correct positioning of the substrate. The zinc-dependent cyclodehydratase McbB subunit then cyclizes Cys and Ser residue side chains at the upstream peptide carbonyl groups. The resulting oxazolines and thiazolines are finally desaturated by the dehydrogenase flavine-dependent McbC subunit. (**2**) The leader peptide is cleaved off from modified McbA by the chromosomally encoded TldE and TldD. (**3**) Mature MccB17 is secreted by the export machinery, consisting of McbE/McbF located in the inner membrane (IM), and an unknown component of the outer membrane (OM).

n-aminopropanol to the AMP group.[28,29] This role was supported by synthesis of a MccC7/C51 related product missing one amino group when MccC51 gene cluster was mutated on *mccD*.[29]

MccJ25 post-translational modification consists in the formation of a β-lactam bond between Gly1 and Glu8 side chain of the *C*-terminal 21-residue peptide, resulting in a lasso structure.[91–93] Although MccJ25 genetic system is one of the smallest among microcins, little is known about the genes involved in its post-translational modification. As discussed above, this process is thought to involve McjB and McjC. Whereas McjB does not share homologies with other known enzymes, McjC contains an ATP-binding motif and could thus be responsible for the activation of the glutamic acid, before β-lactam bond formation. Future studies should help identifying the functions of McjB and McjC, and whether or not they are sufficient to convert McjA into MccJ25. Whether maturation of MccJ25 occurs in one or two steps is unknown. Nevertheless, given the MccJ25 lasso structure (Fig. 4C), the ring closure involving Gly1 and Glu8 should occur after acquisition of the spatial structure of the molecule and an almost correct positioning of both Glu8 carboxylate and Gly1 amino group on the one hand, and Phe19 and Tyr20 aromatic side-chains on the other hand. Two hypotheses have thus to be considered: (i) initial cleavage from the leader sequence followed by cyclization by McjB and McjC separately, or (ii) concomitant cleavage from the leader sequence and subsequent cyclization by a McjBC complex.

Because class IIa microcins display little to no post-translational modification, their maturation process, limited to proteolytic cleavage and cysteine oxidation, has been little-studied. Conversely, an increasing interest is devoted to class IIb microcin post-translational modification enzymes. The recent finding in our group (i) that MccE492 is synthesized in a post-translationally modified form[14] and (ii) that the siderophore–peptide is the mature microcin (unpublished work), enabled the attribution of putative functions to some enzymes encoded by the MccE492 gene cluster. Recent studies on salmochelin biosynthesis[143–145] were very helpful to understand the MccE492 biosynthetic pathway. Indeed, MccE492 post-translational modification resembles salmochelins. On the basis of sequence similarity with *iroB* and *iroD*, two genes originally identified in *Salmonella*,[146] an enzymatic activity was postulated for *mceC* and *mceD* gene products.[38,57] Indeed, MceC is highly similar to IroB (76% identity), a glycosyl transferase involved in *C*-glycosylation of enterobactin,[143] while MceD resembles the enterobactin esterase IroD (57% identity).[144,145] It is therefore likely that MceC performs *C*-glycosylation of enterobactin, while MceD could break down the glycosylated enterobactin into its linear form. Because MceI shares homologies with acyltranferases involved in activation of RTX toxins such as hemolysin,[38,57] it could catalyse the acylation of β-D-glucose by the *C*-terminal serine residue of MccE492 precursor. This process is likely to also involve *mceJ*, which is co-transcribed with *mceI*, and is required for the detectable production/secretion of MccE492.[58] As discussed previously, the structures of class IIb microcins other than MccE492 remain either uncharacterized or partially characterized. However, depending on the producer strain, genes homologous to *mceC*, *mceD*, *mceI* and *mceJ* found in the MccE492 gene cluster can be encountered in these microcin gene clusters (Fig. 1C; Table 4). Moreover, heterologous complementation of *mceAB* by the MccH47 genetic system, which carries the *iroB* and *D* orthologues *mchA* and *mchS1*, produces MccE492.[72] It was therefore proposed that, similar to MccE492,[14] (i) other class IIb microcins can be modified by addition of a catechol-type siderophore (Glc-DHBS$_3$),[57] and (ii) that the conserved *C*-terminal sequence present in MccE492, MccH47, MccI47, and MccM (Fig. 10) serves as a recognition signal for modification enzymes.[72] Altogether, these data indicate that class IIb microcins are most likely post-translationally modified by similar enzymes with rather related substrate specificity, one system being able to complement the other.

Table 4 Percentages identity between putative post-translational enzymes of class IIb microcins. The corresponding microcin is given in parentheses

	MceD (MccE492)	MchS1[a] (MccH47)	McmK[b] (MccM)
MceD	100	74.9	75.4
MchS1[a]	—	100	98.8
McmK[b]	—	—	100
	MceC (MccE492)	MchA[a] (MccH47)	McmL[b] (MccM)
MceC	100	85.5	85.2
MchA[a]	—	100	99.1
McmL[b]	—	—	100

[a] MccH47 gene cluster from *E. coli* H47. [b] MccM gene cluster from *E. coli* CA46.

5.3 Regulation of microcin biosynthesis

The regulatory mechanisms underlying the production of microcins have been studied in detail for class I microcins, with much less attention being paid to class II microcins.

Like most of the antibiotics, toxins and secondary metabolites, it was claimed that microcins were produced when bacteria enter the stationary phase.[25,32,147] Gene expression studies, such as those performed with MccE492,[58] showed this statement is not general to all microcins. However, the need for survival within a microbial community when stress conditions appear may stimulate most microcin production. Thus, nutrient depletion, which occurs at the approach of the stationary phase, provides a first level of regulation for most microcins. Another stress that was considered to regulate the production of microcins is oxygen starvation. Nevertheless, not all microcins are controlled by identical stress stimuli.

Expression of *mcjA*, which encodes the MccJ25 precursor, was shown to be controlled neither by the pH variation that occurs in exponential-stationary phase transition, nor by cell density.[35] Because *mcjA* expression was immediately induced when cultures in mid-exponential phase were exposed to spent medium,[35] its induction at the onset of the stationary phase was supposed to rely on nutrient depletion. In agreement with this hypothesis, most microcins were shown to be overproduced in minimal medium compared to rich medium,[35,59,148] MccC7/C51 being the only exception reported to date.[31] As many conventional antibiotics, class I microcins production is repressed by glucose.[23,35,149] As glucose is a fast-used carbon source, it favours a high bacterial growth rate. Such culture conditions could then be related to a lowered production of stationary phase-produced microcins. Conversely, neither glucose depletion nor substitution of glucose by glycerol enhanced MccE492 production,[150] in agreement with

an exponential phase production of MccE492. The same difference was observed when examining the influence of the nitrogen source. Indeed, nitrogen starvation induced MccB17 production,[24] whereas de Lorenzo showed that the easier the utilization of the nitrogen source, the higher the amount of secreted MccE492.[150] As with glucose, nitrogen assimilation rate, on which the bacterial growth rate depends, was related to production of microcins. Mild air limitation, as well as shear stress could enhance MccB17 production.[149,151] In contrast, MccJ25 production decreased under anaerobic conditions.[35]

The subtle role of growth conditions in microcin production was further related to the expression of transcriptional regulators. Given that the expression of most of the regulators also depends on growth conditions, nutrients cited above might have both a direct and an indirect role in the control of microcin gene transcription. Several regulators of the growth-phase-dependent transcription of class I microcin structural genes were shown to be involved in complex and interplaying mechanisms. Transcription of P_{mcb} regulated genes from MccB17 gene cluster (Fig. 1A) was shown to increase as cells enter the stationary phase of growth.[147] This activation was dependent on the OmpR transcriptional factor, which is known to positively regulate the production of the outer membrane porins believed to be involved in the import of nutrients from nutritionally poor media (see Section 7.1.2). On the other hand, MccB17 production was found independent on RpoS,[152] the RNA polymerase sigma S factor (σ^S), which controls transcription of several stationary-phase-induced genes. Instead, the alternative sigma 70 (σ^{70}) factor seemed to be involved in this increased expression. In contrast, RpoS appeared to be involved in regulation of *mcjA* and *mccA* expression. Indeed, whereas the typical growth phase induction of *mcjA* was still observed in strains mutated on *rpoS*, these strains were reported to produce lower amounts of MccJ25 than wild-type strains.[35] The same phenomenon was noticed for *mccA*, whose basal expression decreased in both exponential and stationary phase of growth upon inactivation of *rpoS*, but was still stimulated during transition to the stationary phase.[31,153] Moreover, expression of *mcjA* was found positively regulated by a complex network at least consisting of the leucine responsive protein, the integration host factor and two unusual nucleotides (guanosine tetraphosphate or pentaphosphate, also termed (p)ppGpp).[35] Taking into account that these regulators are themselves growth-phase-responsive, and that induction of *mcjA* is practically abolished in strains deficient in any of them, their concerted action would stimulate the expression of *mcjA* at the onset of stationary phase. Several regulators that would negatively control microcin gene expression in the exponential phase were also described. These include the histone-like protein H-NS, which acts as a repressor of the genes encoding MccC7/C51 or MccB17.[23,31] Another repressor likely to affect transcription of *mcb* genes during exponential phase is encoded by the microcin production regulator gene *mprA*, which was further assimilated to the first gene of the *emrRAB* operon, and renamed *emrR* accordingly.[154] This gene was also found to repress MccC7/C51 and MccV production when a high-copy number of this gene was expressed.[155]

Another factor controlling the production of some microcins is the iron availability in the culture media. Iron-regulated gene expression in *E. coli* is largely mediated by Fur (ferric uptake regulation), a ferrous iron-binding protein that binds to the so-called *fur* boxes (the 19-bp 5′-GATAATGATAATCATTATC-3′ inverted repeat consensus sequence) and blocks iron-regulated promoters in a metal-dependent fashion (for a review, see Hantke[156]). Gene clusters from class I microcins are devoid of *fur* boxes (Fig. 1A). Consistently, iron availability was reported not to significantly affect MccB17 and MccC7/C51 production.[157] However, MccJ25 production dropped by 95% when iron was added to the culture medium. Accordingly, the use of chelating agents restored MccJ25 production. However, the iron-control of MccJ25 production is Fur-independent.[157] Some significant *fur* boxes are found in all class II microcin gene clusters (Fig. 1B and 1C). However, iron regulation of microcin production has not been shown for all class II microcins. The production of MccV was shown to be induced under iron-limiting conditions. For this reason, the iron chelator 2,2′-dipyridyl is used in culture media to increase the production of MccV.[36] Consistent with the presence of *fur* boxes ahead of *cvi* and *cvaA* (Fig. 1B), *cvaC* expression could be de-repressed upon mutation of the *fur* gene.[45] Contradictory results have been published about iron-regulation of MccE492 production.[45,157,158] This discrepancy is mostly due to the use of antibacterial assays for quantifying MccE492 production, since both MccE492 and an antagonist[158] are found in culture supernatants of MccE492-producing strains. No *fur* box could be found in an extensive region (240 bp) upstream of *mceB*[56] (Fig. 1C). Therefore, *mceB* is likely to be expressed independently of the iron concentration. Conversely, MccE492 post-translational modification is repressed by high iron concentration (unpublished work). Despite the presence of a *fur* box ahead of *mchX* (Fig. 1C), MccH47 production was reported not to be regulated by iron either.[71] However, synthesis of both MccE492 and MccH47 have been shown to be dependent on enterobactin synthesis.[14,71] Therefore, if MccH47 and MccM are secreted as siderophore–peptides similarly to MccE492, their putative post-translational modification is likely to be iron-dependent.

6 Self-immunity of the producing strains

Bacteria producing antimicrobial compounds must protect themselves from their toxic products. In contrast to colicin- and bacteriocin-producing strains, the mechanisms by which microcinogenic bacteria acquire self-immunity towards their own microcin remain largely unexplained. Nevertheless, the characterization of microcin gene clusters showed that at least one resistance-conferring gene is associated with the production of a given microcin.

As previously mentioned (Sections 2.1 and 4), self-immunity and export of class I microcins are tightly associated, at least one gene being involved in both mechanisms. Self-immunity towards MccB17 involves three genes from the microcin gene cluster. Indeed, bacteria harbouring either *mcbEF* or *mcbG* showed partial self-immunity, whereas those harbouring all three genes were fully resistant to MccB17.[18] McbE and McbF presumably mediate the export of mature MccB17 from the cytoplasm to the periplasmic space, but the exact role of McbG is still unknown. Besides McbE, McbF and McbG, Baquero *et al.* suggested that during the stationary phase, SbmC may contribute to bacterial cell protection by binding and sequestering MccB17, thus preventing the microcin interaction with its intracellular target, the DNA gyrase.[159] Self-immunity towards MccC7/C51 seems to involve

at least MccC and MccE. MccC would function as an efflux pump, decreasing the intracellular microcin concentration. The *C*-terminal region of MccE is similar to RimL and RimJ, two bacterial enzymes that catalyze the acetylation of the *N*-terminal residue of ribosomal proteins.[28,29] Thus, it was originally proposed that MccE could modify the putative intracellular target of the microcin.[28] However, it was recently shown[85] that secreted MccC7/C51 requires activation by an unknown peptidase to generate the intracellularly active MccC7/C51* (Section 7.2.2). Thus, MccE could prevent MccC7/C51 activation by either downregulating the expression of the gene encoding the peptidase or by interacting with the peptidase or its substrate, MccC7/C51, in the producing strain. A third protein, MccF, seems to contribute, although to a lesser extent, to self-immunity towards MccC7.[28] However, *mccF* is not functional in the MccC51 genetic system.[29] Because of its similarity to numerous hypothetical proteins from non-microcinogenic bacteria, MccF is thought to have a widely distributed uncharacterized function.[29] Uniquely, MccJ25 gene cluster (Fig. 1A) possesses a single gene, *mcjD*, putatively involved in self-immunity. On the basis of sequence homologies and mutational assays, McjD was proposed to be responsible for both the export and self-immunity towards MccJ25.[33] The mechanism of protection would then be an efflux of the antibiotic that enables keeping intracellular MccJ25 below the inhibitory concentration.

In contrast to class I microcins, all class II microcins are characterized by a dedicated self-immunity protein. This is also true when several microcins are encoded by a same gene cluster (*e.g. E. coli* H47, CA46, CA58 and Nissle 1917). Thus, MccH47, MccI47 and MccM, which have a common export system consisting of MchE and MchF, have specific self-immunity proteins. Self-immunity proteins of class II microcins likely range in size from 51 to 144 amino acids. Except for MchS3, the MccI47 self-immunity protein, class II microcin self-immunity proteins are probably membrane-bound, with two or three transmembrane helices. Cloning of MccL self-immunity and precursor genes only, led to an intracellular antibacterial activity and to full immunity of the bacteria to exogenous MccL.[39] This suggests that the self-immunity protein MclI protects the bacteria from the MccL precursor as well as from MccL itself. As previously mentioned (Sections 5.1.1 and 5.2.1), the leader peptides of class II microcins are highly similar and are cleaved by the export machinery. Moreover, it was proposed that the similar *C*-terminal region of class IIb microcins could be involved in receptor recognition (Section 7.1.1). Thus, the *N*-terminal region of mature class II microcins, which is highly variable, could be involved in a specific interaction with the self-immunity protein. No significant similarity is observed between self-immunity proteins of class II microcins, except for MceB and MtfI, which are involved in MccE492 and Mcc24 self-immunity, respectively, and exhibit 38.7% identity. Such a similarity is also found between MccE492 and Mcc24 precursors (Section 3.2.1) and between their export proteins (Section 4). Therefore, it is tempting to speculate that either the short Mcc24 gene cluster derives from that of MccE492, or that both microcin gene clusters have a common ancestor.

The data available show that self-immunity towards microcins may arise from different mechanisms that confer either partial or full immunity to the producers. Further studies will be required to elucidate the delineated mechanisms in greater details.

7 Mechanisms of action

The broad variety of microcin structures correlates with diverse mechanisms of action, such as inhibition of vital enzymatic functions and damages to the inner membrane. While microcins display a broad diversity of cellular targets, the initial recognition/uptake pathways may be common to several microcins.

7.1 Recognition/uptake: role of the stress response-regulated machineries

Evocative of receptor-mediated mechanisms of action, mature microcins were shown to display narrow spectra of activity, limited to few genera of enterobacteria, and low MICs (often below 0.1 μM).[14,90] The existence of microcin receptors in enterobacteria was also strongly supported by the isolation of microcin-resistant mutants impaired in outer membrane proteins normally involved in nutrient uptake.[160–164] As these proteins are also exploited by bacteriophages, antibiotics, and bacterial toxins for cell entry, they constitute an "Achilles' heel" for the bacterium. Their utilization by microcins is illustrated below.

7.1.1 Role of TonB-dependent iron-uptake machineries. Over recent years, it has become obvious that the iron uptake machineries and their associated energy-transduction system, the TonB/ExbB/ExbD inner membrane complex, are required for recognition of various microcins. Iron is imported into enterobacteria through three pathways that involve dedicated outer membrane receptors: (i) FhuA, which binds hydroxamate siderophores (*e.g.* ferrichrome), (ii) FepA, Cir and Fiu, which bind catecholate siderophores (*e.g.* enterobactin), and (iii) FecA, which is involved in the uptake of hydroxycarboxylates (*e.g.* citrate) (for reviews, see Ferguson and Deisenhofer[165] and Letellier and Santamaria[166]). FepA and FhuA show similar three-dimensional structures.[167–169] Both receptors are composed of a β-barrel embedded in the outer membrane with an *N*-terminal globular domain, either called the plug or the cork domain, folded inside the barrel. This domain spans most of the interior of the barrel and occludes it. It is connected to the β-barrel and to the external hydrophilic loops by numerous hydrogen bonds and salt bridges. The external loops contain the binding sites for iron–siderophore complexes.[170,171] Interestingly, FhuA and FepA are multifunctional proteins which, besides their physiological function, also transport antibiotics and serve as receptors for colicins and bacteriophages (for a review, see Letellier and Santamaria[166]), which bind to diverse external loops on the receptors.[171] As iron–siderophore complexes, most of these ligands require the TonB/ExbB/ExbD complex to be anchored at the inner membrane for uptake. This complex is responsible for the transduction of the proton-motive force energy from the inner membrane, where it is generated, to the outer membrane (for a review, see Postle and Kadner[172]).

The ferrichrome receptor FhuA. Early studies showed that the iron–siderophore receptor FhuA, as well as the TonB and SbmA inner membrane proteins, were most likely involved in MccJ25 uptake.[162,163] The MccJ25 requirement for both FhuA and the TonB/ExbB/ExbD complex (Fig. 12A) was ascertained by homologous complementation assays in *E. coli* strains impaired in one of these proteins.[90] Heterologous complementation in *Salmonella* species, whose FhuA genes were sequenced,[173]

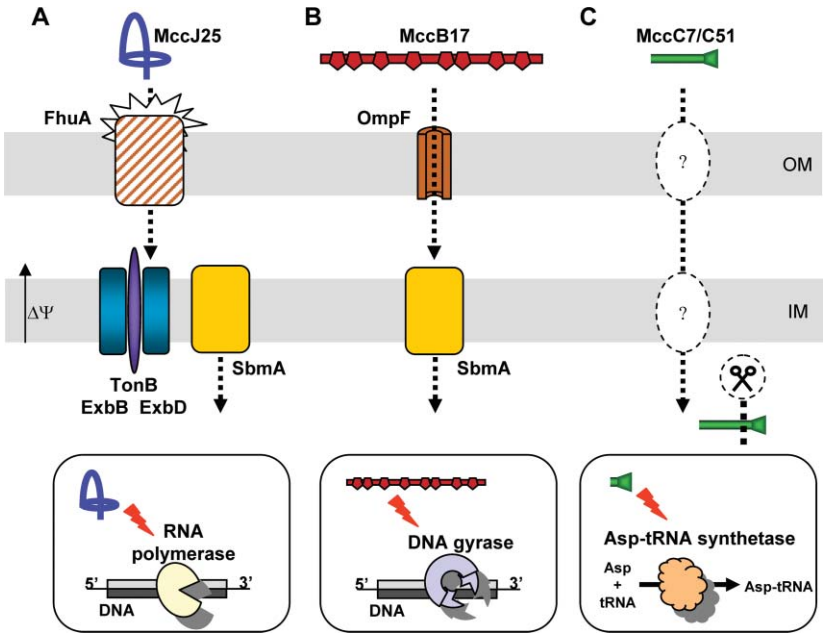

Fig. 12 Uptake and mechanism of action of class I microcins. (**A**) MccJ25 is recognized by the high affinity receptor FhuA at the outer membrane (OM). The recognized structural motif is the MccJ25 β-hairpin region. Translocation of MccJ25 across the OM requires the inner membrane (IM) potential $\Delta\psi$ and needs the TonB/ExbB/ExbD complex as well as the SbmA protein at the IM. Once into the cytoplasm, MccJ25 inhibits transcription by obstructing the RNA polymerase secondary channel. The ring-tail part of MccJ25 is proposed to be involved in this obstruction. (**B**) MccB17 passes across the OM through the OmpF porin. The IM protein SbmA is involved in MccB17 further uptake into the cytoplasm, whereupon it inhibits DNA supercoiling by the DNA gyrase. The binding site of MccB17 is likely to be the *C*-terminal domain of GyrB. (**C**) The components enabling the translocation of MccC7/C51 across bacterial membranes are unknown. MccC7/C51*, which is generated by proteolytic cleavage of MccC7/C51 after uptake, is a modified aspartyl-adenylate that inhibits translation by targeting the aspartyl-tRNA synthetase.

indicated that resistance of *S. enterica* serovars such as Typhimurium is due to variations in the FhuA sequence.[174] Besides genetic evidence, the role of FhuA in MccJ25 recognition was demonstrated functionally. Indeed, MccJ25 was shown to inhibit phage T5 adhesion to its receptor FhuA both *in vivo* and *in vitro*.[90] Moreover, MccJ25/FhuA interaction was demonstrated by size-exclusion chromatography and isothermal titration calorimetry. MccJ25 binds to FhuA with a 2 : 1 stoichiometry and a K_d of 1.2 μM. Both differential scanning calorimetry and antibacterial assays showed that MccJ25 binding involves FhuA external loops. By using the thermolysin-cleaved variant of MccJ25 (Fig. 5; Section 3.1), it was also demonstrated that the MccJ25 Val11-Pro16 β-hairpin region, which is disrupted upon thermolysin cleavage, is required for microcin recognition by FhuA.[90]

The catecholate siderophore receptors FepA, Cir and Fiu. MccE492 was shown to require the catecholate siderophore receptors for recognition at the outer membrane (Fig. 13). Indeed, MccE492, which inhibited the growth of *E. coli* H1443 at 40 nM, was inactive against the isogenic *fepA cir fiu* triple mutant (MIC >10 μM).[14] Besides the need for catecholate siderophore receptors, MccE492 was found to be dependent on both energy and TonB for antibacterial activity and translocation across the outer membrane[14,57] (Fig. 13). The need for FepA, Cir and Fiu as well as for TonB was also demonstrated for u-MccE492, which lacks the siderophore post-translational modification.[57,90] As with MccE492, MccM- and MccH47-producing strains failed to inhibit the growth of a *tonB* mutant and a *fepA cir fiu* triple mutant, where both strains were derived from a susceptible *E. coli* with an identical genetic background.[65] This suggests that not only MccE492, but also MccM and MccH47, require the catecholate siderophore receptors and the associated TonB for antibacterial activity. Moreover, it was recently shown that the *C*-terminal region, which is conserved among class IIb microcins (Fig. 10; Section 5.1.1), is essential for the activity of extracellular but not intracellular MccE492.[175] This strongly suggests that the *C*-terminal sequence from class IIb microcins is required for receptor recognition and/or translocation accross the outer membrane. As discussed previously,[57] MccM and MccH47, as well as the recently discovered MccI47, are able to carry a catecholate siderophore as a post-translational modification on their *C*-terminal serine (Section 3.2.2). We showed that the modification increases the antibacterial activity,[14] probably by providing the microcin with a higher affinity for its receptors.[57] Thus, as with MccE492, it is tempting to speculate that all class IIb microcins use structural mimicry (*i.e.* a siderophore post-translational modification) to improve their recognition by the catecholate siderophore receptors. Furthermore, while the *C*-terminal region is thought to be required for optimal uptake, the remaining part of the protein is likely to endow the specificity of the mechanism of action, which greatly differs among these microcins (see below). While the three receptors FepA, Cir and Fiu would be needed for class IIb microcin antibacterial activity, genetic evidence strongly supports the idea that Cir alone is involved in MccV recognition at the outer membrane. Indeed, contrary to what was shown for the former microcins, mutations in *cir* were found to be sufficient to confer resistance to MccV.[45] Evocative of an energy-dependent uptake similar to that of MccE492, MccV was also shown to require *tonB* and *exbB* for antibacterial activity.[45] As for MccV, the sole receptor

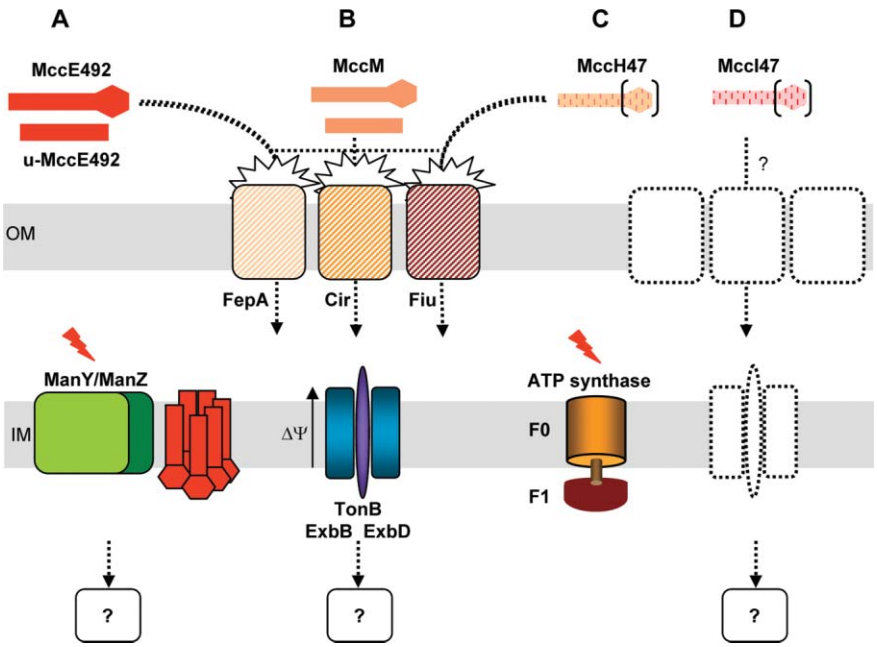

Fig. 13 Uptake and mechanism of action of class IIb microcins. (**A**) MccE492 and its incompletely processed form, u-MccE492, are recognized by the high affinity receptors FepA, and to a lesser extent Cir and Fiu, at the outer membrane (OM) of *E. coli.* MccE492 is then translocated across the OM *via* an uncharacterized but TonB- and energy-dependent mechanism. Once into the periplasmic space, MccE492 inserts into the inner membrane (IM), whereupon it induces proton leakage and subsequent drop of the IM potential ($\Delta\psi$). Membrane insertion and antibacterial activity are dependent on ManY and ManZ, two membrane components of the mannose permease. The *C*-terminal sequence of MccE492 is not required for interaction with the mannose permease. The orientation of MccE492 monomers in the IM remains hypothetical. The occurrence of a cytoplasmic target remains unknown. (**B**) MccM in both modified and unmodified forms is also recognized by FepA, Cir and Fiu, and further translocated into the periplasmic space by a TonB-dependent process. Its mechanism of action remains unknown. (**C**) MccH47 has never been isolated to date but it is hypothesized to bear a *C*-terminal siderophore modification similar to MccE492 and MccM. It utilizes the same receptors for recognition and is translocated in a TonB-dependent process. It then inhibits the membrane component F0 of the ATP synthase in the IM. (**D**) MccI47 has not been purified either, and its uptake and mechanism of action have not been investigated. Nevertheless, MccI47 bears significant *C*-terminal sequence homologies to other class IIb microcins, which could account for the same uptake mechanism. Siderophore-type post-translational modifications are depicted as hexagons.

Cir and the TonB/ExbB/ExbD complex were also shown to be involved in MccL recognition/translocation (Sable *et al.*, personal communication).

In a recent study, by screening a total of 49 Gram-negative clinical isolates from urine, Laviña and collaborators demonstrated that 71% of the strains generating an antibacterial activity were inactive against a *tonB*-deficient mutant and that 37% were both inactive against the *tonB*-deficient mutant and a *fepA cir fiu* triple mutant.[72] Among these, only 2 strains produced an activity attributable to MccE492 or MccH47. This strongly suggests that urinary tract bacteria synthesize other microcins, colicins, or other bacterial toxins that use the TonB-dependent catecholate siderophore uptake pathway.

7.1.2 Role of the outer membrane protein OmpF. Early studies on MccB17 mode of action led to the isolation of MccB17-insensitive mutants, most of which were uptake-deficient.[161] They contained mutations in *ompF* and *ompR*, two genes encoding outer membrane proteins (Fig. 12B). It was shown that OmpF, which serves as a passive diffusion pore across the outer membrane, is also required for the uptake of various group A colicins in association with the Tol/Pal translocation system (for a review, see Cao and Klebba[176]). It was proposed that OmpF is important for efficient nutrient uptake from nutritionally poor media.[177] The transcriptional upregulation of *ompF* expression under conditions of nutrient depletion is reminiscent of the upregulation of *fhuA*, *fepA*, *cir* and *fiu* under iron-poor conditions.[178,179] Since MccB17, MccJ25, MccE492, MccH47 and MccM are known and/or believed to use these porins or high affinity receptors for recognition, it is likely that nutritionally poor media enhance susceptibility to microcins[23,45,157] (Section 5.3).

7.1.3 Role of the inner membrane proteins SdaC and SbmA. We showed above that inner membrane proteins, such as TonB, and in some cases ExbB and ExbD, are required for the activity of MccJ25 and class IIb microcins. MccV bactericidal activity was shown to be dependent on another inner membrane protein, SdaC, also termed DcrA, which is involved in serine uptake.[180] SdaC was previously known as being required for infection by bacteriophages together with the outer membrane receptor FhuA (phage C6) or BtuB (phage C1).[181,182] It was recently proposed that SdaC also serves as a specific inner membrane receptor for MccV, thus helping it locate the inner membrane, a step required for channel formation and disruption of membrane potential[180] (Section 7.2.1).

Another inner membrane protein, SbmA, was proposed to be required for the activity of the class I microcins MccB17 and MccJ25 (Fig. 12A and B). Indeed, *E. coli* achieved high and specific resistance to MccB17 upon inactivation of *sbmA*.[161] Given that SbmA is also involved in the uptake of the antitumoral

antibiotic bleomycin, whose backbone displays thiazole rings, it was proposed that heterocycles could be a structural feature necessary for the recognition by SbmA, and further uptake into the cytoplasm.[183] The finding that SbmA is required for MccJ25 antibacterial activity[163] indicates that structural features different from the heterocycles may also be recognized by SbmA. Recently, de Cristóbal *et al.* showed that osmotic shock-treated bacteria, in which the FhuA-dependent outer membrane recognition step is bypassed, were resistant to His5 mutants of MccJ25 but not to wild-type MccJ25. Overexpression of SbmA sensitized these strains equally to both MccJ25 and its His5 mutant.[184] It was therefore inferred that MccJ25 interacts with SbmA, and that the interaction may involve the His5 located in the ring-tail part of MccJ25.

7.2 Cellular targets: from inner membrane to cytoplasmic targets

7.2.1 Inner membrane targets. While most of the gene-encoded antimicrobial peptides are believed to inhibit the growth of microorganisms by targeting the cell phospholipid bilayers,[185–187] such a mechanism of action was only reported for few microcins.

Modification of membrane permeability was shown to be induced by three microcins. Thus, MccV was reported to abolish *E. coli* membrane potential *in vivo.* However, pore formation could not be observed with liposomes,[42] and difficulties in isolating sufficient amounts of the peptide have hampered further studies on the MccV mechanism of action. MccJ25 was also reported to disrupt membrane integrity in *S. enterica* Newport,[188] in liposomes[189] and uncharged phospholipid monolayers.[190] However, these properties were reported to be specific to *S. enterica* serovars and were observed at concentrations much higher than the MICs. The best-studied microcin with regard to membrane-permeabilization properties is undoubtedly MccE492. Most of the work has been done prior to the elucidation of the structure of mature MccE492, by using either pre-purified culture supernatants (containing both MccE492 and u-MccE492) or u-MccE492 homogenous preparations. *In vitro*, the microcin was able to form ion-channels in planar lipid bilayers.[191] The u-MccE492 pore-forming activity could be observed at concentrations as low as 2×10^{-10} M, and the insertion was shown to be voltage-independent.[106] *In vivo*, the microcin depolarized the inner membrane of *E. coli*[192] in an energy- and TonB-dependent manner[106] and made the inner membrane permeable to chromogenic substrates, such as *o*-nitrophenyl-β-D-galactopyranoside.[106] Studies in our group also showed that mature MccE492 damages the inner membrane[57] (Fig. 13). Interestingly, we found (i) that membrane integrity (as observed by electron microscopy) was preserved in MccE492-killed bacteria, indicating that the observed leakage does not result from membrane lysis, and (ii) that interference with the inner membrane is not responsible by itself for the lethal effect of either u-MccE492 or MccE492.[57,106] It was therefore proposed that the inner membrane is not the sole target of the microcin.

In a recent study, Bieler *et al.* have further investigated the MccE492 mechanism of action. The authors have evidenced that, as MccH47, MccE492 targets inner membrane proteins. These belong to the mannose permease.[175] Indeed, by *Tn10* transposon insertion mutagenesis, *manY* and *manZ* were identified as critical for MccE492 antibacterial activity against *E. coli*[175] (Fig. 13). ManYZ is an inner membrane complex that functions together with the cytoplasmic ManX to form the mannose permease involved in the uptake of mannose and related hexoses.[193,194] It was shown that all the *manYZ* mutants resistant to MccE492 were unable to metabolize mannose. In addition, they became insensitive to the inner membrane depolarization mediated by periplasmic MccE492.[175] At this stage, the molecular basis of MccE492 bactericidal activity thus remains to be established. As Mcc24 is similar to MccE492, Bieler *et al.* proposed that Mcc24 antibacterial activity could also require ManYZ at the inner membrane.[175]

The MccH47 mechanism of action has been fairly well documented by Laviña and collaborators, although the microcin has never been purified. In their study of MccH47 precursor (MchB), the authors found that strains expressing *mchB* only were not viable, but that the mutants exhibiting an Atp^- phenotype resisted.[68] Confirming this first observation, the authors characterized mutants resistant to MccH47, obtained by *Tn5* transposon insertion mutagenesis, as impaired in the *atp* operon. It was therefore proposed that F_0F_1 ATP synthase was necessary for MccH47 antibacterial activity[164] (Fig. 13). Since then, the authors have shown that all *Tn5* insertions mapped to genetic determinants encoding the F_0 membrane component of ATP synthase.[195] In the same study, they used a complementation approach to confirm that the minimal structure of ATP synthase needed for MccH47 antibacterial activity was the F_0 proton channel, while the F_1 catalytic unit was dispensable.

7.2.2 Cytoplasmic targets. Class I microcins were shown to target intracellular enzymes responsible for DNA/RNA structure or synthesis.

MccB17 is a DNA gyrase inhibitor. A significant number of studies have been devoted to the elucidation of the mechanism of action of MccB17 over the past 20 years. MccB17 was shown to induce the SOS response and to block DNA replication.[196] Consistent with this last finding, besides uptake-deficient mutants, bacteria resistant to MccB17 displayed mutations on DNA gyrase,[161,197] a bacterial type II topoisomerase involved in DNA topology and essential in DNA replication. Point mutations in the DNA gyrase B subunit (encoded by *gyrB*) were actually sufficient to lower or abolish susceptibility to MccB17.[198] By studying the effect of semipurified MccB17 on *gyrB* mutants and on replicative cell-free extracts prepared from these cells, MccB17 was shown to induce the irreversible trapping of DNA–DNA gyrase complexes, leading to the accumulation of double-stranded DNA breaks and replication inhibition[197] (Fig. 12B). Confirming earlier finding that bisheterocycles are necessary for MccB17 antibacterial activity,[199] Zamble *et al.* showed the activity of MccB17 on DNA gyrase extends to supercoiling inhibition.[200] MccB17 was actually shown to slow down but not to completely inhibit the supercoiling and relaxation reactions of DNA gyrase and to stabilize the cleavage complex.[201] Later, by establishing the proteolytic signature of the gyrase in the presence of MccB17, the same authors showed that the binding site of MccB17 was likely to be the *C*-terminal domain of GyrB.[202] They also demonstrated that DNA strand passage was involved in the MccB17 mechanism of action. Based on this knowledge, it was inferred that MccB17 traps a transient intermediate state of gyrase reaction only present during DNA

passage. At this stage, the MccB17 mechanism of action remains incompletely elucidated. In particular, the molecular details of the inhibition are not characterized.

MccC7/C51 targets the aspartyl-tRNA synthetase. First studies showed that MccC7/C51, used at MIC, blocked the *in vivo* incorporation of radiolabelled leucine to proteins, while transcription remained unchanged.[86] This was the first indication that MccC7/C51 is a translation inhibitor. Afterwards, MccC7/C51 was shown to inhibit protein synthesis in a cell-free coupled transcription–translation assay. Based on the compared activities of MccC7/C51 and the synthetic heptapeptide part, it was suggested that the peptide backbone was required for translation inhibition, while the *C*-terminal post-translational modification was needed for recognition/uptake.[83] Nevertheless, the exactly opposite conclusions were recently found, since highly pure MccC7/C51 was unable to inhibit translation.[85] Actually, MccC7/C51 is processed by an uncharacterized intracellular peptidase present in crude bacterial extracts, and the resulting product, MccC7/C51* (Fig. 3B; Section 3.1), strongly inhibits translation. The mechanism underlying this process was found to be an inhibition of aminoacylated $tRNA^{Asp}$ synthesis by aspartyl tRNA-synthetase (Fig. 12C). Since MccC7/C51* is devoid of antibacterial activity, the authors proposed that the peptide backbone is required for recognition, and that following uptake, MccC7/C51 is the subject of cleavage, which renders it active in translation inhibition. Thus MccC7/C51 develops a clever "Trojan horse" strategy that involves the cleavage of the microcin inside the target bacteria to generate a potent inhibitor of bacterial cell growth, MccC7/C51*. Such cleavage of the promicrocin in the target cell rather than in the producer is unique among microcins.

MccJ25 targets RNA polymerase. While most of the MccJ25-resistant mutants were found to carry mutations in genes encoding membrane transporters, one mutant was isolated that carried a point mutation in *rpoC*.[203] Consistent with *rpoC* encoding the RNA polymerase β′ subunit, MccJ25 was able to inhibit the RNA polymerase of Gram-negative bacteria *in vitro*[203,204] (Fig. 12A), with an apparent K_i ranging from 1.2 to 20 μM.[205,206] Crosslinking, as well as fluorescence resonance energy transfer experiments, clearly showed that MccJ25 binds within the secondary channel of *E. coli* RNA polymerase.[205,206] The MccJ25/RNA polymerase interaction was characterized by a K_d of 0.5 μM.[206] Since MccJ25 was unable to bind RNA polymerases carrying mutations on the secondary channel, it is very likely that resistance to MccJ25 is conferred by inhibition of MccJ25 binding.[205] Therefore, it is believed that MccJ25 inhibits transcription by binding within and obstructing the RNA polymerase secondary channel, whereupon it prevents incoming nucleoside triphosphates from trafficking through the channel.[205,206] Structure–activity analyses were also performed. The thermolysin-cleaved variant of MccJ25, lacking the Val11–Pro16 β-hairpin region (Fig. 5; Section 3.1), was as efficient as the uncleaved microcin in inhibiting transcription.[207] This implies that the β-hairpin, which is involved in FhuA receptor-recognition,[90] is not involved in RNA polymerase binding.[207] It was therefore proposed that the ring-tail part of MccJ25, which is preserved in all variants, is responsible for the interaction with the RNA polymerase,[207] as well as with SbmA (Section 7.1.3), while the Val11-Pro16 β-hairpin region is required for recognition by FhuA (Section 7.1.1).

8 Comparison with other gene-encoded antibacterial peptides from bacteria

As mentioned in introduction, besides microcins, gene-encoded antibacterials from bacteria include colicins and bacteriocins secreted by Gram-negative (enterobacteria) and Gram-positive bacteria (lactic acid bacteria, LAB), respectively. Colicins have higher molecular masses (30–90 kDa) than microcins, while bacteriocins have molecular masses below 10 kDa, similar to microcins. In this section, microcins are compared to colicins and bacteriocins.

8.1 Colicins from Gram-negative bacteria

Microcins and colicins are both encoded by dedicated gene clusters that contain most of the information required for their production, export and self-immunity. To date, all colicins have been found to be plasmid-encoded, in contrast to microcins, among which class IIb microcins are chromosome-encoded. Colicin gene clusters appear to be highly conserved, but amazingly simple compared to microcin gene clusters (Section 2). Indeed, they include two to three genes only, the minimum requisite being a structural gene and a self-immunity gene. A third gene encoding a lysis protein is required for colicin secretion (for a review, see Van der Wal *et al.*[208]). In contrast to microcins, the production of colicins is mainly induced *via* the DNA repair network, called the SOS response (for a review, see Janion[209]). It can be activated by an environmental stress, such as UV irradiation, exposure to DNA-damaging agents, or starvation of cells.[209–211]

One of the major differences between microcins and colicins, besides an evident molecular mass difference, is their structure. Indeed, whereas most microcins were shown or proposed to bear post-translational modifications (Sections 3.1 and 3.2.2), the simple and conserved organization of colicin gene clusters leads to non-post-translationally modified proteins. Colicins are organized in three functional domains including a central receptor binding domain, an *N*-terminal translocation domain and a *C*-terminal catalytic domain. These domains, which are common to all colicins, ensure every common step of the colicin mechanisms of action, *i.e.* (i) recognition by a specific receptor at the outer membrane, (ii) translocation across the outer membrane and (iii) lethal interaction with a specific cellular target.[212] Another noticeable difference between microcins and colicins is their mechanism of export. Contrary to microcins, which utilize ABC transporters or efflux pumps (Section 4), the release of colicin results from the sole presence of lysis factors.[213,214] These small and highly similar lipoproteins, predominantly located in the outer membrane of colicinogenic strains, are first synthesized as precursor polypeptides. While the stable signal peptide would accumulate in the inner membrane, the mature lysis protein would activate the phospholipase A, both phenomena being responsible for the loss of membrane integrity and cell lysis.[2,208,215]

Both microcins and colicins have a narrow spectrum of activity, being active against bacterial strains phylogenetically related to the producer. This specificity relies in part on the use of outer membrane receptors specifically expressed by enterobacteria. Similar to microcins, most colicins parasitize multi-protein systems involved in important biological functions to enter bacteria, *i.e.* the vitamin

B_{12} receptor BtuB, the siderophore receptors FepA, Cir, Fiu or FhuA, or the nucleoside receptor Tsx (Section 7.1).

We have shown in this review that all studied microcins have in common the use of outer membrane proteins for recognition/translocation, but differ in their cellular targets. Colicins are also distinguished by their bacterial killing mechanisms. Indeed, some possess nuclease-activity (for a review, see James *et al.*[216]), pore-forming activity (for reviews, see Cramer *et al.*[217] and Duche[218]), or in rare cases, they target the peptidoglycan. This is evocative of microcin mechanisms of action. Nevertheless, whereas the mechanism of action of nuclease–colicins consists in hydrolysing DNA or RNA strands, microcin cytoplasmic targets are the enzymes responsible for the DNA/RNA structure or synthesis. Moreover, the third kind of activity, inhibition of peptidoglycan synthesis, which was reported for pesticin[219] and colicin M,[220] has never been described among microcins.

8.2 Bacteriocins from Gram-positive bacteria

As microcins and colicins, bacteriocins from Gram-positive bacteria are encoded by dedicated gene clusters, where the structural gene is accompanied by a self-immunity gene. Similar to microcins, bacteriocins from Gram-positive bacteria display a high stability to temperature, pH and most often proteases. They are assembled in four main classes according to their structural characteristics (for reviews, see Garneau *et al.*[221] and Drider *et al.*[6]). Surprisingly, microcins, which form a much more restricted group, have more diverse structures. Indeed, most of the bacteriocins from Gram-positive bacteria, except the highly modified lantibiotics (for a review, see Jack and Sahl[222]), also referred to as class I bacteriocins, are unmodified peptides. The latter contain unusual amino acids such as lanthionine and dehydrated amino acids (for reviews, see Patton and van der Donk[223] and Chatterjee *et al.*[121]). Precursors of bacteriocins from Gram-positive bacteria and microcins are similarly processed through elimination of a leader peptide. They also display similarities in their leader peptide sequence. In addition, bacteriocins from Gram-positive bacteria and microcins have in common the involvement of ABC transporters for their export into the extracellular medium.

Compared to microcins, bacteriocins from Gram-positive bacteria may exhibit a broader spectrum of antibacterial activity. This is the case for nisin, a lantibiotic that uses lipid II (the crucial precursor of peptidoglycan biosynthesis) as a docking molecule,[224,225] inhibits peptidoglycan synthesis and forms heteromolecular pores in bacterial membranes. Such a dual mode of action is responsible for the potent antibacterial activity of nisin. Nevertheless, bacteriocins from Gram-positive bacteria may also have mechanisms of action that are similar to those of microcins. Indeed, membrane permeabilization has been reported for nisin and for class II bacteriocins, such as leucocin A or mesentericin Y105 (for a review, see Fimland *et al.*[226]), which induce disruption of the proton-motive force at the inner membrane. This is reminiscent of the MccV and MccE492 mechanisms of action (see Section 7.2.1). Interestingly, similar to MccE492, class IIa bacteriocins target both the inner membrane and require the ManYZ components of mannose permease.[175,227,228] However, other mechanisms, such as the inhibition of cell wall synthesis described for nisin,[224,225] have not been described for microcins.

This rapid comparison with bacteriocins from Gram-positive bacteria and colicins illustrates how microcins combine features and strategies exemplified by these two classes of antibacterials. Indeed, they assemble the typical leader peptides, self-immunity, and maturation mechanisms of bacteriocins from Gram-positive bacteria with the uptake strategy of colicins. Such an efficient combination, which would be either at the root or at the top of evolution, constitutes an amazing model for the design and engineering of new antibacterials.

9 Current challenges in microcin research

We have shown in the previous sections that great progress has been made on microcin research over the last years, especially with the identification of several structures and mechanisms of action. However, some fundamental questions remain unresolved.

9.1 Unresolved questions

Self-immunity of microcinogenic strains. The most poorly studied topic in microcin research is certainly self-immunity. As with colicins,[229,230] multidisciplinary studies should aim at isolating and collecting structural information on microcin self-immunity proteins. This would clarify the basis of the self-immunity specificity, an essential point to determine how producers protect themselves from their own toxic substances.

Pheromone activity of microcins. Bacterial communication such as that involved in quorum sensing, which regulates many bacterial behaviours including symbiosis or virulence,[231] uses signalling molecules (for reviews, see Miller and Bassler[232] and Reading and Sperandio[233]). In Gram-positive bacteria, these are peptide pheromones, which display similarities with microcins. Indeed, they are concomitantly cleaved for maturation and exported *via* ABC transporters. Since bacteriocins from Gram-positive bacteria were reported to play the role of inducing agents,[234,235] one may speculate that microcins contribute to cell-to-cell signalling in Gram-negative bacteria. Future research should help determining whether, similar to eukaryotic defensins, prokaryotic antibacterial peptides are multifunctional, being involved in chemotaxis, signalling, and antibacterial defence.

Ecological role of microcins. Several surveys on enteric bacteria reveal that an average 10–50% of the strains sampled produce antibacterials including bacteriocins from Gram-positive bacteria, colicins and microcins.[236] Early studies on the ecological role of microcins were often contradictory or inconclusive.[8,237–239] However, recent studies emphasized the role of colicins and microcins as regulators of bacterial populations.[236,240–242] Indeed, the resulting rock–paper–scissors model[243] provides evidence that colicins, and potentially other bacteriocins, may promote rather than eliminate microbial diversity in their ecosystems.[243,244] Further studies should firmly assess the specific roles of microcins as antibacterial weapons or signalling molecules.

9.2 Miscellaneous applications of microcins

Based on our current knowledge of microcin biological activities, several applications may be considered for these peptides as

well as for their associated modification enzymes. Here we list the properties that may be valuable in terms of potential developments.

Probiotic agents. Based on their potential ecological role, microcinogenic strains were tested as probiotics. On the one hand, the role of microcins in preventing *Salmonella* invasion in humans by *E. coli* Nissle 1917, a microcinogenic probiotic commercially available under the name Mutaflor®, could not be demonstrated clearly.[245] Long term colonization and transmission of this strain, as well as the presence of microcins, was reported in swine herds.[246] On the other hand, inhibition of *Shigella flexneri* by an *E. coli* H22 strain was shown to be mediated by the production of MccC7/C51.[247] MccJ25-producing strains were observed to be widely distributed in poultry intestinal habitats.[248] Moreover, an Mcc24-producing *E. coli* was shown to inhibit the growth of pathogenic *Salmonella* and *Escherichia* O157:H7 in the intestinal tract of chickens.[238] Further studies should help defining how far microcins contribute to the prevention of intestinal infections.

Antitumoral agents. MccE492 was shown to induce biochemical and morphological changes typical of apoptosis in human cell lines,[249] opening a new research field for potential applications of microcins as antitumoral agents. Indeed, inducers of apoptosis are of great interest in the search for novel antitumoral agents, since apoptosis was observed as a response of eukaryotic cells to infection by a wide range of pathogens and is mediated by an array of pathogen-encoded virulence determinants (for a review, see Weinrauch and Zychlinsky[250]). Some microcins are encountered on genomic islands together with virulence factors including siderophores,[77] and could therefore promote virulence/pathogenicity.

Antimicrobials for health and food preservation. The worldwide emergence of antibiotic-resistant pathogens has led to an increasing demand for new antimicrobial agents. Since microcins differ from conventional antibiotics by their diverse mechanisms of action and a highly potent activity on a restricted bacterial spectrum, they may help in the design of novel drugs or substitutes. Colicin-engineered antibiotics obtained by fusing channel-forming colicins and pheromones from Gram-positive bacteria proved to be efficient and specific against pathogenic Gram-positive strains, without toxicity in mammal cells.[251,252] Inspired by the lantibiotic nisin, which is commonly used as a preservative against food spoilage, and because bacteriocins from LAB do not kill Gram-negative bacteria, heterologous production of MccV in LAB was performed. This engineered microcin, which has a LAB peptide leader and displays MccV activity,[253] should find applications to prevent food poisoning by Gram-negative bacteria. The design of chimeric peptides active against specific bacterial infections could thus be efficiently applied to microcins.

Enzymes for the design of more stable/specific antimicrobials. The large panel of microcin structures results from post-translational modifications. Some of the enzymes involved show interesting activity in terms of possible development. Thus, enzymes responsible for the lasso structure of MccJ25 would be of great biotechnological interest given the increased potency and stability of cyclic antimicrobial peptides. Similarly, several years before the isolation of the first natural siderophore–peptide,[14] conjugation with hydroxamate or catecholate siderophores provided vancomycin[254] or cephalosporins[255] with enhanced antibacterial activity compared to unsubstituted antibiotics. Thus, the enzymes involved in MccE492 siderophore modification would also be worth isolating to improve antibacterial activities.

Taking into account these different aspects and the potential applications, there is no doubt that for many years, microcins will continue to be an active area of fundamental and applied research.

10 References

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II. Les microcines E492, J25 et C7/C51 : diversités structurelle et fonctionnelle des microcines

Ce deuxième article, sous presse dans *Journal of Molecular Microbiology and Biotechnology*, présente plus particulièrement la diversité des microcines en matière de structures et de modes d'action. Trois exemples particulièrement représentatifs sont détaillés : MccE492, qui présente une modification post-traductionnelle mimant un sidérophore à son extrémité *C*-terminale, MccJ25, qui est un peptide cyclique en forme de lasso et MccC7/C51, un heptapeptide *N*-formylé à son extrémité *N*-terminale qui porte un motif nucléotidique à son extrémité *C*-terminale.

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Structural and functional diversity of microcins, gene-encoded antibacterial peptides from enterobacteria

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Structural and Functional Diversity of Microcins, Gene-Encoded Antibacterial Peptides from Enterobacteria

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Key Words
Microcins · *Escherichia coli* · Siderophore peptide · Nucleotide peptide · Iron siderophore receptors

Abstract
Microcins are a peculiar class of gene-encoded low-molecular-mass antibacterial peptides secreted by enterobacteria. They contribute to the regulation of microbial competitions within the intestinal microbiota. The genetic systems involved in microcin biosynthesis share a conserved organization. Similar to bacteriocins of Gram-positive bacteria, microcins exert potent antibacterial activity directed against phylogenetically-related bacterial strains, with minimal inhibitory concentrations in the nanomolar range. In contrast to bacteriocins, they display a great structural diversity among the few representatives well characterized until now, that makes difficult the description of microcin subclasses. This review focuses on three microcins, MccE492m that carries a *C*-terminal posttranslational modification containing a catechol-type siderophore, MccJ25, a cyclic peptide with a unique 'lasso-type' structure and MccC7 or C51, with a common *N*-formylated heptapeptide-nucleotide structure. We show these microcins exhibit 'Trojan horse' mechanisms of antibacterial activity: either (i) the microcin structure is a mime of an essential element, permitting its recognition by outer membrane receptors used for vital functions in bacteria and further translocation into the periplasmic space, or (ii) it is secreted as a harmless molecule and further processed in susceptible bacteria to form the toxic entity. When inside target bacteria, microcins bind essential enzymes or interact with the inner membrane to form a bacterial killing structure.

Introduction

The bacterial secretion of peptides or proteins (bacteriocins) to fight against other prokaryotic species has appeared as one of the very first examples of biological warfare. These ribosomally synthesized antimicrobial peptides (AMPs) are considered a successful strategy in maintaining equilibrium within a bacterial ecosystem. They kill other bacteria by several mechanisms, including the modification of membrane permeability and depolarization of membrane ion gradients, or the degradation of nucleic acids or cell walls. In literature, the term bacteriocin is usually restricted to peptides produced by Gram-positive bacteria, while in Gram-negative bacteria, mainly enterobacteria, the toxins are called either colicins (i.e. antibiotic proteins targeting *Escherichia coli*) or microcins (characterized by a lower molecular mass) [for reviews, see 1–3]. Bacteriocins from Gram-positive bacteria, particu-

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larly lactic acid bacteria, and microcins from enterobacteria, mostly *E. coli*, share a number of common characteristics, such as a molecular mass below 10 kDa and a mode of secretion into the extracellular medium involving ABC (ATP-binding cassette)-type transporters. In both cases, the producer has a specific immunity mechanism that protects it from being killed by the newly synthesized antibacterial molecule. Both types of peptide essentially inhibit the growth of phylogenetically related species. However, microcins form a much more restricted group with highly diversified structures and mechanisms of action, as exemplified by the six structurally characterized representatives, compared to the very large number of bacteriocins [1] that can be assembled into subgroups according to common structural features or mechanisms of action.

In fact, most of the bacteriocins of Gram-positive bacteria are described as unmodified peptides/proteins [for reviews, see 4, 5], except the highly modified group of lantibiotics, which contain multiple tethering ether bonds [for reviews, see 6–8]. Bacteriocins are usually classified into three main classes: (i) Lantibiotics, or class I bacteriocins, are peptides <5 kDa that contain the unusual amino acids lanthionine (Lan) and β-methyllanthionine (MeLan) (formed when a dehydrated serine or threonine is covalently bridged with a cysteine through the sulfur atom) and a number of dehydrated amino acids. They include two-component lantibiotics that require the presence of two different peptide components to achieve full antibacterial activity [9]. (ii) Class II bacteriocins (sometimes termed pediocin-like AMPs), also <5 kDa, are heat-stable, non-Lan-containing, cationic membrane-active peptides. This class is subdivided into subclass IIa that concerns *Listeria*-active peptides sharing very similar primary structures, especially in the *N*-terminal part (with the *N*-terminal consensus sequence YGNGV), and subclass IIb requiring two components for antibiotic activity. The subclass IIa is itself subdivided into three subgroups according to similarities and differences in the *C*-terminal part [5]. (iii) Class III bacteriocins are large (>30 kDa) heat-labile proteins. Additionally, a class of circular bacteriocins, exemplified by AS48 and circularin A, has recently been proposed [10]. A great number of members of each of these three main classes, and particularly from classes I and IIa, have been described and studied with regard to their common structural aspects and the mechanisms involved in their antibacterial activity. While the class IIa bacteriocins target the inner membrane through its permeabilization [5], leading to disruption of the protonmotive force, the prototypic lantibiotic nisin has a dual mode of action. Nisin uses lipid II (a crucial precursor in peptidoglycan biosynthesis) as a docking molecule, therefore preventing correct cell wall synthesis, as well as initiating within cell membranes the formation of heteromolecular pores that are made up of lipid II and nisin molecules [11, 12]. Rapid and efficient cell death thus results from such a dual mechanism.

By contrast, microcins form a very restricted group of such defense peptides with only 15 representatives identified [for reviews, see 3, 13–15] since their discovery in 1976 [16]. They all inhibit the growth of a variety of Gram-negative bacteria, including *Escherichia, Salmonella, Enterobacter, Klebsiella, Citrobacter* and *Shigella,* with minimal inhibitory concentrations (MICs) in the nanomolar range. They can be low-molecular-mass peptides <3 kDa (microcins B17, C7/C51, D93 and J25) that are generally highly modified, or polypeptides between 7 and 10 kDa that can be modified or not (microcins E492, L, H47, I47, M, 24 and V). Some of these peptides have only been identified on the basis of genetic studies. Despite a high structural heterogeneity, microcins share a conserved organization of their genetic systems. A typical gene cluster, located either on a plasmid or on the bacterial chromosome, includes open reading frames encoding the precursor of the microcin, secretion factors, immunity proteins and, very frequently, modification enzymes [17].

Presumably related to these various structures, microcins have also diverse mechanisms of action, targeting either the inner membrane of target bacteria, or enzymes involved in DNA or RNA structure and synthesis or in protein synthesis. Moreover, to enter the bacteria more efficiently, these peptides use specific receptors designed by all bacteria for essential nutrient uptake. This seems to be a common feature shared by microcins, which is possibly related to the high selectivity of their antibacterial activity. To exemplify the structural and functional diversity of microcins, we have focused on three of them that have been deeply characterized from a biochemical and functional point of view, and that will be discussed in this review: microcins E492 (MccE492), J25 (MccJ25) and C7/C51 (MccC7/C51).

Structural Diversity of Microcins

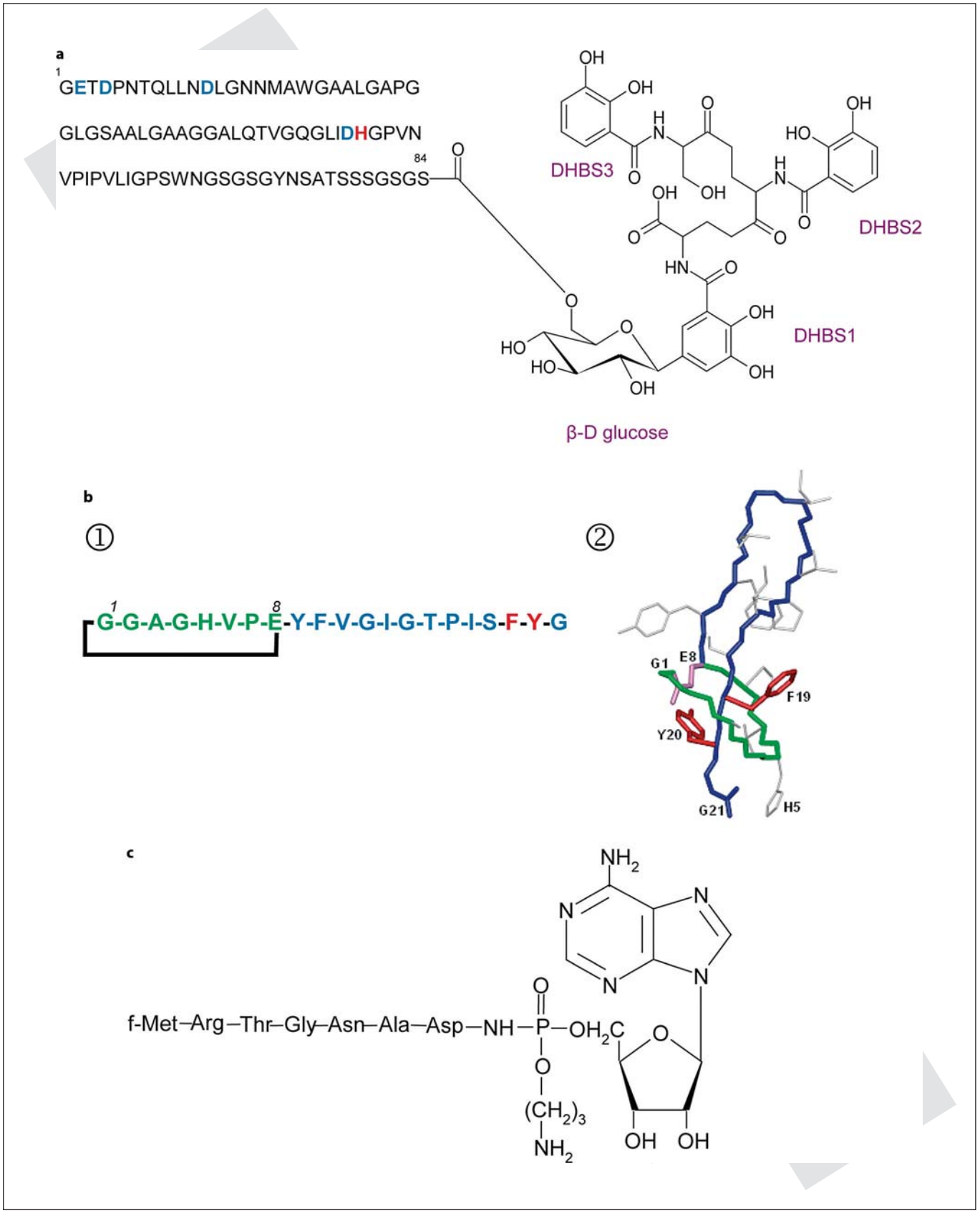
Among the eleven microcins identified until now, only six have been structurally characterized (MccB17, MccC7/C51, MccE492, MccJ25, MccL and MccV [also known as ColV, since initially described as a colicin]), while the others (MccD93, MccH47, MccI47, MccM, Mcc24) have been essentially studied through genetical approaches.

MccB17 is a 43-residue peptide characterized by post-translational modifications consisting of thiazole and oxazole rings [18]. MccC7/C51 is a short nucleotide heptapeptide [19–21] and MccJ25 a cyclic 21-amino-acid peptide [22–25], while MccE492 [26, 27], MccL [28] and MccV [29] are linear polypeptides with molecular masses between 8 and 10 kDa. The structures of microcins have often been the subject of debates in the literature. This is the case of MccE492, MccJ25 and MccC7/C51 that have been selected in this review.

MccE492 is a chromosomally-determined microcin initially characterized from a *Klebsiella pneumoniae* strain [30]. Further cloning in *E. coli* [31] allowed its purification from culture supernatants and primary structure determination [26]. It was identified by mass spectrometry as an unmodified 84-residue peptide [26], in agreement with the amino acid sequence deduced from the gene encoding its precursor MceA [32, 33]. The amino acid residues are mainly uncharged and hydrophobic, with the exception of one histidine, three aspartic acids and one glutamic acid (fig. 1a). Therefore, this peptide is slightly anionic. That differentiates it from both the known bacteriocins from Gram-positive bacteria and most of the AMPs from vertebrates, invertebrates and plants, which all exhibit a strong cationic character. However, we have recently shown that MccE492 could be secreted by both the wild-type *K. pneumoniae* and the recombinant *E. coli* strains under two forms, the unmodified polypeptide previously described and a posttranslationally modified variant that we called MccE492m [27]. This microcin variant, which bears a catechol-type siderophore comprising the linear trimer of dihydroxybenzoylserine (DHBS), exhibits enhanced activity. The siderophore moiety is anchored to the peptide *C*-terminus through a β-D-glucose, which in turn is linked to the Ser84 carboxylate through an *O*-glycosidic bond at C6, and to the first DHBS entity via a *C*-glycosidic bond at C1 (fig. 1a) [27]. This novel posttranslational modification is reminiscent of siderophores, the molecules designed by bacteria to chelate Fe(III), enabling its uptake through the outer membrane via specific receptors [34, 35]. Moreover, mass spectrometry has shown that the modified microcin MccE492m selectively binds ferric iron through the catecholate moiety, confirming it operates as a siderophore and thus establishing a new type of AMP, the siderophore peptides [27]. This modification is in good accordance with the presence of several genes in the genetic system involved in MccE492 biosynthesis, the role of which remained speculative [36] until the finding of this new form of MccE492. In particular, three gene products show sequence homologies to glycosyltransferases (MceC), acyltransferases (MceI) and enterobactin esterases (MceD), which cleaves enterobactin, the cyclic trimer of DHBS, into its monomer, dimer and linear trimer [37], thus providing the corresponding genes *mceC, mceI and mceD* with a very likely role in the acquisition of the posttranslational modification.

MccJ25 has been first described as a head-to-tail macrocyclic linear peptide [38, 39]. The very high stability of the peptide towards high temperatures associated with high concentrations of chaotropic agents has been largely studied in our group [40]. Later on, the peptide was identified as a 'lasso' peptide, with an extraordinary three-dimensional structure, showing that the ring was actually a small cycle resulting from a linkage between the *N*-terminal amino group and the Glu8 side-chain carboxylate (fig. 1b) [22–25]. The 13-residue linear *C*-terminal tail is threaded into the ring, in such a manner similar to the thread through a needle eye, thus forming a loop. Two aromatic bulky side chains from Phe19 and Tyr20, which are each positioned on one side of the ring, tightly lock the tail into the ring (fig. 1b). The tail thus remains firmly entrapped within the ring through this strong steric effect, in combination with non-covalent interactions, in such a manner that it can only be released via the cleavage of the ring. This cleavage is particularly hard to perform, since it can only be accomplished in basic medium [24]. An inference of this peculiar structure is that the ring closure should have been accomplished after acquisition of the spatial structure of the molecule and almost a correct positioning of both (i) the carboxylate and the amino groups involved in the ring closure and (ii) the aromatic side chains responsible for the strong steric hindrance that locks the tail into the ring. Endopeptidases

Fig. 1. The structures of MccE492/MccE492m, MccJ25 and MccC7/C51. **a** The amino acid sequence common to MccE492/MccE492m is indicated in one-letter code and the positively and negatively charged amino acids are figured red and blue, respectively. The posttranslational modification carried by the siderophore peptide MccE492m consists of a trimer of the catechol-type siderophore 2,3-dihydroxybenzoyl serine (DHBS) linked to Ser84 via a β-D-glucose. **b** (1) The cyclic structure of MccJ25 comprises an *N*-terminal ring followed by a *C*-terminal linear tail; the amino acids are colored according to the 'lasso-type' three-dimensional structure (2) that shows the steric hindrance due to two aromatic side chains (Phe19, Tyr20) maintaining the tail entrapped into the ring. **c** Structure of the nucleotide peptide MccC7/C51: the *N*-formylated heptapeptide (in three-letter code) is linked to the adenosine monophosphate moiety through a phosphoramidate bond; the phosphate group bears an *n*-aminopropanol chain.



and strong acidic media do not target the ring, but only cleave bonds in the loop that is subsequently opened. The resulting entities are two-chain peptides [41], the tail (or a shortened tail) remaining firmly anchored to the ring. Such a typical and original lasso structure has never been identified previously among AMPs including bacteriocins and microcins. However, it appears in some enzyme inhibitors synthesized by *Streptomyces* that may be either additionally stabilized or not by a disulfide bonding [25, 42]. Moreover, MccJ25 is the first lasso-type peptide with a glutamic acid involved in the ring closure, rather than an aspartic acid, as found in the *Streptomyces* peptides.

Two microcins sharing a nucleotide peptide character, MccC7 and MccC51, have been described from two different *E. coli* strains [19, 43]. The genetic systems involved in the biosynthesis of these two microcins show only few differences that are located in a gene involved in the immunity to the microcin [21]. A common *N*-formylated heptapeptide had been identified in the two molecules, which differed in both the linkage between the peptide and nucleotide parts and in the nucleotide structure. MccC51 was described as a nebularin 5′-monophosphate *C*-terminus linked to the Asp7 side chain through three methylene bonds. MccC7 contained a modified adenosine monophosphate (AMP) covalently attached to the *C*-terminal aspartic acid residue through a phosphoramidate bond. The phosphoramidate group showed a chiral phosphorus atom, since it was substituted by an *n*-aminopropanol chain. We have optimized the production of MccC51 to reinvestigate its structure and have evidenced that it is actually similar to that of MccC7 [20, 21]. The critical points of the structure, i.e. the presence of the phosphoramidate bond acting as a linker between the heptapeptide and the nucleotide and the location of the *n*-aminopropanol chain, have been unambiguously assigned through typical cross-peaks in two-dimensional ^{1}H-^{31}P NMR spectra [20]. Therefore, MccC7 and MccC51, which arise from two distinct *E. coli* strains that bear closely related genetic systems, share a common structure that will be henceforth denominated MccC7/C51 (fig. 1c). MccC7/C51 is the smallest microcin isolated to date and the only microcin known to carry a nucleotide as a post-translational modification; its three-dimensional structure has never been described.

As previously announced, and now exemplified throughout the description of MccE492, MccJ25 and MccC7/C51 structural characteristics, microcins offer a wide array of peculiar structures. As correlated, a broad diversity of mechanisms of action is exploited by microcins to kill their bacterial targets.

From Structural Diversity to Diverse and Complex Mechanisms of Action

The great differences between eukaryotic and prokaryotic AMPs concern the antibiotic efficacy and the microbial target specificity. The AMPs secreted by bacteria (bacteriocins and microcins) appear to be overall more potent than those from Eukaryota, since they are active in the pico- to nanomolar concentration range, while the latter are active at micromolar concentrations. In addition, they have a narrow spectrum of activity, susceptible strains being essentially closely related to the producing strain, while AMPs from multicellular organisms more often have a broad spectrum of activity directed against Gram-positive and -negative bacteria, yeasts and fungi. It is thus of importance to elucidate the reasons of such an efficiency and selectivity. More refined and complex mechanisms of antibacterial activity, including either a dual mode of action or the involvement of specific receptors could thus be expected for AMPs of bacterial origin.

MccE492 and its siderophore peptide counterpart MccE492m both inhibit selectively the growth of Gram-negative enterobacteria, such as *E. coli*, *Salmonella enterica, Enterobacter cloacae* and *K. pneumoniae*. MccE492m is 2–8 times more active than MccE492 with MICs ranging from 40 to 160 nM against *E. coli* and *S. enterica* strains. The activity of both MccE492 and MccE492m is bactericidal with minimal bactericidal concentration values similar to MIC values [27, 44].

Both MccE492 and MccE492m form ion channels in artificial planar lipid bilayers [44, 45] and permeabilize the inner membrane of Gram-negative bacteria, as shown in *E. coli* ML35 [44, 46]. Interaction with the inner membrane and subsequent permeabilization were considered as responsible for the bactericidal effect [45]. However, we hypothesized that this membrane activity was not sufficient by itself to account for the lethal effect of the microcins [44]. Indeed, despite its more potent antibacterial activity against *E. coli*, MccE492m is less efficient in permeabilizing the membrane bilayer than MccE492, indicating the absence of direct correlation between the rapid decrease in cell viability induced by MccE492m and the rate of membrane permeabilization [46]. Moreover, electron microscopy showed that treated and killed *E. coli* had still intact inner and outer membranes and only showed a swelling of the periplasmic space [46]. Therefore, permeabilization of the inner membrane does not lead to membrane disruption, but rather to a more subtle mechanism, such as the formation of pores, similar to

those observed in artificial bilayers [44, 45]. Consequently, we suspect that an additional step shall be involved in the mechanism of antibacterial activity of MccE492/MccE492m, which could be either the formation of a lethal complex within the bilayer or the interaction of the microcin with a cytoplasmic target (fig. 2a).

Since mutations in *tonB* have resulted in resistance to MccE492, this protein has been suggested to be involved in the mechanism of antibacterial activity of this microcin [47]. TonB, which spans the periplasmic space, is generally found in complex with two other partners ExbB and ExbD, located in the inner membrane, thus forming the TonB-dependent energy transduction system [48–50]. TonB acts as the energy transducer, using the protonmotive force; the role of ExbB and ExbD is less clear. The TonB system is used by bacteria for the uptake of vitamin B_{12} and iron chelators, the siderophores [34, 51], which are recognized at the outer membrane by specific receptors. We have shown that the *tonB* mutation induces a high resistance to MccE492 and MccE492m, and that the antibacterial activity of both microcins is completely restored in the TonB-complemented strain, unambiguously indicating that TonB is required for the antibacterial property of both MccE492 [44] and MccE492m [27]. Moreover, MccE492 and MccE492m activities require the three outer membrane receptors, FepA, Fiu and Cir. These are catechol-type siderophore receptors associated to the TonB system [for reviews, see 34, 52]. At this time, there is still no evidence of the direct interaction of MccE492/MccE492m with any of the three iron siderophore receptors, neither is it not explicitly demonstrated that the microcins are translocated into the periplasmic space through the FepA/Fiu/Cir receptors. Indeed, the translocation step could occur using either a porin, or membrane defects such as membrane islands, which have different lipid compositions, that result in a loose membrane bilayer packing (fig. 2). However, since MccE492/MccE492m are recognized by the receptors involved in the uptake of enterobactin and its breakdown products, the linear trimer, dimer, and monomer of DHBS, which are imported through a TonB- and energy-dependent mechanism [for reviews, see 34, 35], it is highly probable that the microcins undergo the same uptake mechanism across the outer membrane of *E. coli*, involving the TonB/ExbB/ExbD complex and the FepA/Cir/Fiu receptors.

MccJ25 exhibits a potent bactericidal activity with MICs in the 5–500 nM range against a number of enterobacteria including pathogenic *Salmonella, Escherichia* and *Shigella* strains [53, 54]. MccJ25 has been shown to inhibit transcription by binding bacterial RNA polymerase (RNAP), which is targeted at the level of the β′ subunit [55, 56]. MccJ25 binds the RNAP secondary channel, obstructing it and thus preventing the correct positioning of the nucleoside triphosphate substrates, which are therefore prevented from reaching the catalytic center of the enzyme [57, 58]. Indeed, the MccJ25 binding instantaneously stops RNAP progression and thus prevents further elongation of transcription [57]. The first studies on MccJ25 have shown that mutants resistant to MccJ25 were impaired in *fhua*, *tonB* and *sbmA*, indicating that the outer-membrane iron transporter FhuA (the receptor for ferrichrome) and the inner membrane proteins TonB and SbmA were likely involved in the microcin uptake [59, 60]. We recently demonstrated the direct interaction between FhuA and MccJ25 by size-exclusion chromatography and isothermal titration calorimetry, showing that MccJ25 binds to FhuA with a 2:1 stoichiometry and a K_d of 1.2 μM [61]. The differential scanning calorimetry revealed that the interaction of MccJ25 with its receptor involves the external loops of FhuA [61]. To go further in the whole process used by MccJ25 to exert its antibacterial activity, it thus remained to identify both the regions of the molecule involved in the different steps of the mechanism (i.e. recognition, translocation and RNAP targeting) and the other protein partners. The two-chain peptide resulting from MccJ25 thermolysin cleavage (t-MccJ25), which keeps the intact spatial structure of the ring and the positioning of the trapped chain, but does not maintain anymore the β-hairpin loop, was used together with other shorter truncated variants in order to identify the MccJ25 regions involved either in the recognition step by FhuA or in the interaction with RNAP [61, 62]. Since disruption of the Val11-Pro16 β-hairpin did not affect the inhibition of transcription by MccJ25, contribution of this loop to the interaction with RNAP was excluded [62]. Furthermore, t-MccJ25 was unable to bind FhuA, affording direct evidence of the involvement of this region in the recognition step of MccJ25 by the outer membrane receptor FhuA [61]. Before reaching RNAP, MccJ25 should be transported through *E. coli* membranes. Possible partners for this uptake are the outer membrane receptor FhuA itself, and the inner membrane protein SbmA, whose function is still unknown, but which has been shown to be involved in the import of MccB17 [63], MccJ25 [60] and bleomycin into susceptible bacteria. The *E. coli* SbmA protein is homolog of BacA from *Rhizobium meliloti* (64% identity), which is crucial for the complex association between bacteria of the genus *Rhizobium* and leguminous plants [64]. Recently, the His5 residue of MccJ25 has been shown to

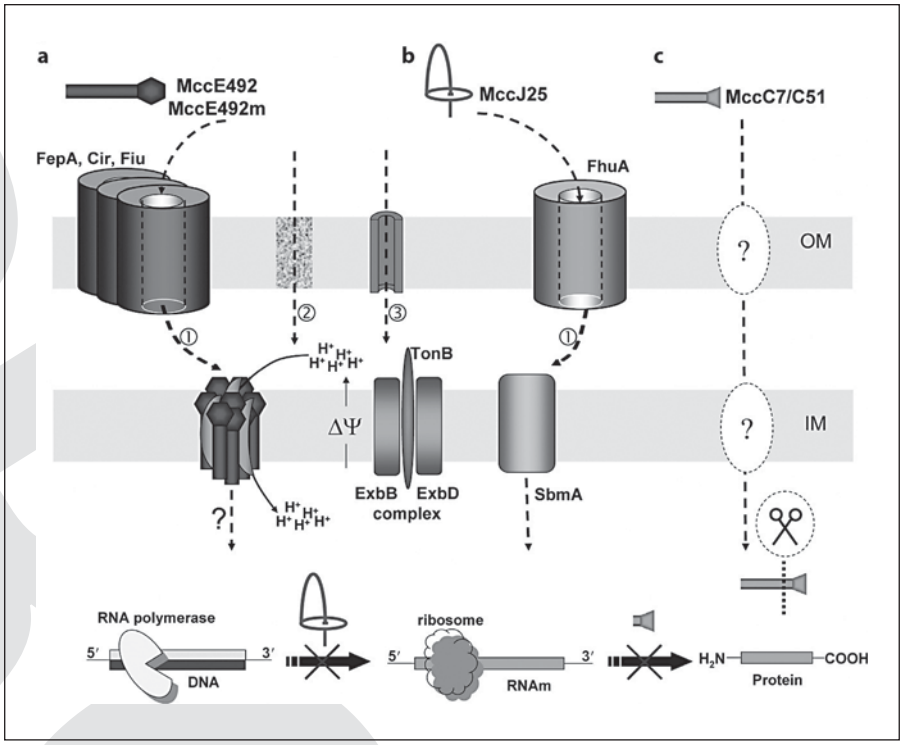


Fig. 2. The 'Trojan horse' mechanisms of action of MccE492/MccE492m, MccJ25 and MccC7/C51. **a** After recognition by the FepA, Cir and Fiu catecholate siderophore receptors, MccE492/MccE492m are translocated across the outer membrane (OM) through an energy- and TonB-dependent mechanism. Translocation may occur via the receptor β-barrel (1), a region with different lipid composition such as a 'membrane island' (2), or a porin (3). Once in the periplasmic space, MccE492/MccE492m insert into the inner membrane (IM) inducing proton leakage and subsequent drop of the IM potential (ΔΨ). At this stage, it is not known whether the microcins recruit other partners, such as inner membrane proteins, to form a toxic supramolecular edifice; it is not known either if they reach an intracytoplasmic target. **b** MccJ25 is recognized by FhuA, the hydroxamate siderophore receptor, before translocation through a TonB-dependent mechanism and further transport through the IM using the inner membrane protein SbmA. Once internalized in the cytoplasm, MccJ25 reaches RNAP, which is inhibited through interaction with the β′ subunit and further obstruction of the secondary channel, which in turn prevents the correct positioning of nucleoside triphosphate substrates. **c** MccC7/C51 is internalized in susceptible bacteria through an unknown mechanism, before being processed by an unknown peptidase that targets the ultimate MccC7/C51 peptide bond (Ala-Asp), leading to a modified unhydrolyzable aspartyl adenylate analogue that contains an *N*-acyl phosphoramidate linkage. The modified aspartyl adenylate thus generated inside the susceptible bacteria inhibits translation by specifically blocking the function of aspartyl-tRNA synthetase via a mechanism that remains to be elucidated.

be critical for the SbmA-dependent transport of MccJ25, and not to be required for RNAP inhibition. Indeed, MccJ25 variants where single amino acids in the ring, and in particular His5, were mutated became unable to kill cells, while still inhibiting RNAP [65]. It was thus proposed that the single histidine residue, which is positioned in the ring, is crucial for the specific interaction of MccJ25 with SbmA, which ensures crossing of the inner membrane, thus achieving the uptake of MccJ25 into the cytoplasm. Therefore, the MccJ25 uptake comprises a first recognition by FhuA, the iron siderophore receptor of the hydroxamate type, and subsequent translocation into the periplasmic space through the TonB/ExbB/ExbD complex, followed by recognition by the inner membrane protein SbmA and further transport through the inner membrane to the cytoplasm, where it can reach RNAP (fig. 2b).

At this stage, it is striking to note that both MccJ25 and MccE492, which do not have any apparent structural similarity to catecholate or hydroxamate siderophores respectively, are however recognized by high-affinity receptors specific for these ligands. This could be made possible by acquisition of specific three-dimensional structures that bring strategic chemical groups at the cor-

rect positioning required for recognition by the receptor. The siderophore moiety carried by MccE492m should then allow a double recognition, resulting in an improved uptake, in agreement with the higher antibacterial activity of MccE492m compared to its unmodified counterpart MccE492.

MccC7/C51 is active against several genera and species of enterobacteria such as *Escherichia*, *Klebsiella*, *Salmonella*, *Shigella, Yersinia* and *Proteus* [66–68]. It inhibits the growth of *E. coli* strains at 100–500 nM. The activity is bactericidal [S. Rebuffat et al., unpubl. results]. MccC7/C51 has been initially proposed to target protein synthesis, inhibiting translation [19]. Since a synthetic peptide with the heptapeptide sequence devoid of the nucleotide moiety mildly inhibited translation in vitro, it was concluded that the peptide was responsible for translation inhibition and that the nucleotide was involved in the recognition and transport into susceptible cells [19]. However, it was recently demonstrated that MccC7/C51 is degraded inside the target bacteria, leading to a modified aspartyl adenylate. This unhydrolyzable aspartyl adenylate strongly inhibits translation by preventing the synthesis of aminoacylated tRNA-Asp by aspartyl-tRNA synthetase, making the nucleotide part of the molecule responsible for cell killing after being processed by a specific protease [69] (fig. 2c). Such a mechanism is reminiscent of that used by other antibiotics containing a nucleotide part such as albomycin, which is taken inside the cells through the ferrichrome uptake system FhuA, before being converted into an active form by peptidase N [70]. Therefore, MccC7/C51 constitutes the first example of a bacterial AMP (either a bacteriocin or a microcin) being processed and converted into the active molecule inside the target bacteria rather than in the producer strain, as it is generally observed. Furthermore, the peptide moiety should consequently play a role in the microcin uptake into susceptible cells. However this remains to be evidenced, as well as the system involved in recognition and uptake.

Concluding Remarks

Despite their completely unrelated structures, the three microcins taken as examples in this paper share a common 'Trojan horse' strategy for killing other bacteria. Either the microcin bears a likeness to essential molecules through its three-dimensional structure, or it disguises itself with a mime of such a useful molecule. This allows MccJ25, MccE492 and MccE492m recognition by naturally specific receptors designed by bacteria for the uptake of essential elements. Alternatively, the microcin is secreted as a harmless molecule (MccC7/C51) that is friendly allowed to enter the susceptible bacteria; but when inside, the inactive microcin is further transformed by the host bacterium itself into a poison fatal to the misled bacterium. Such subtle strategies differ from those adopted by Gram-positive bacteriocins, which prefer to combine two different efficient mechanisms rather than to use dupery and duplicity in order to kill the competitors in their microbial ecosystem. With their outstanding stratagems, microcins and bacteriocins could thus inspire the design of antimicrobial molecules.

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Matériels et Méthodes

I. Souches bactériennes, plasmides et milieux

I.1. Souches bactériennes et plasmides

Les tableaux 1 et 2 récapitulent respectivement les caractéristiques des souches utilisées dans cette étude lors de tests de fonctionnalité et pour la production des protéines recombinantes. Dans le tableau 3 sont listés les différents plasmides ayant servi à nos travaux.

Choix des vecteurs - Le clonage des gènes amplifiés par PCR a été effectué dans pMOSBlue. L'insertion se fait dans le gène *lac*Z, qui code la ß-galactosidase. Cette enzyme dégrade le chromophore X-Gal, dérivé du β-D-galactoside, en un produit bleu. Cette technique de clonage permet donc très aisément de sélectionner visuellement les clones ayant effectivement intégré l'insert. En effet, lorsque le clonage a réussi, les colonies obtenues sur milieu gélosé contenant le X-Gal sont blanches, et non bleues, indiquant une absence d'activité ß-galactosidase.

Le vecteur d'expression choisi est pET28b, dans lequel le gène cloné est sous le contrôle du promoteur fort du phage T7 (reconnu par l'ARN polymérase du phage T7). Il permet de produire une protéine sous forme de fusion avec une étiquette des six histidines en position *N*-terminale. Cette étiquette facilite non seulement la détection de la protéine recombinante grâce à des anticorps commerciaux anti-histidine, mais aussi la purification de la protéine recombinante (voir sections III.2.c)/III.3. et IV.3.). La figure 1 donne le schéma général de la technique de clonage. Les séquences des sites de clonage multiples des plasmides utilisés sont données en figure 2

Choix des souches - Les souches *E. coli* ER2566 et *E. coli* BL21(DE3), ont été choisies pour l'expression des protéines recombinantes, dont le gène est porté par les dérivés du plasmide pET28b. En effet, ces souches portent sur leur génome le gène de l'ARN polymérase du phage T7 sous le contrôle du promoteur *lac*UV5 de l'opéron lactose. La transcription de ce gène peut donc être induite grâce à l'IPTG, un dérivé du galactose qui n'est pas métabolisé par les bactéries. Dans ces conditions, l'ARN polymérase du phage T7 ainsi produite va activer le promoteur du phage T7 et donc induire la synthèse de la protéine recombinante (figure 3). Afin de minimiser la dégradation intracellulaire de la protéine recombinante lors de sa production, nous avons aussi utilisé les souches *E. coli* BL21 (DE3) et *E. coli* ER2566, qui dérivent respectivement de *E. coli* B de *E. coli* K12, car elles ne possèdent pas les gènes codant les protéases Lon et OmpT.

Figure 1 : Clonage dans le vecteur d'expression pET28b

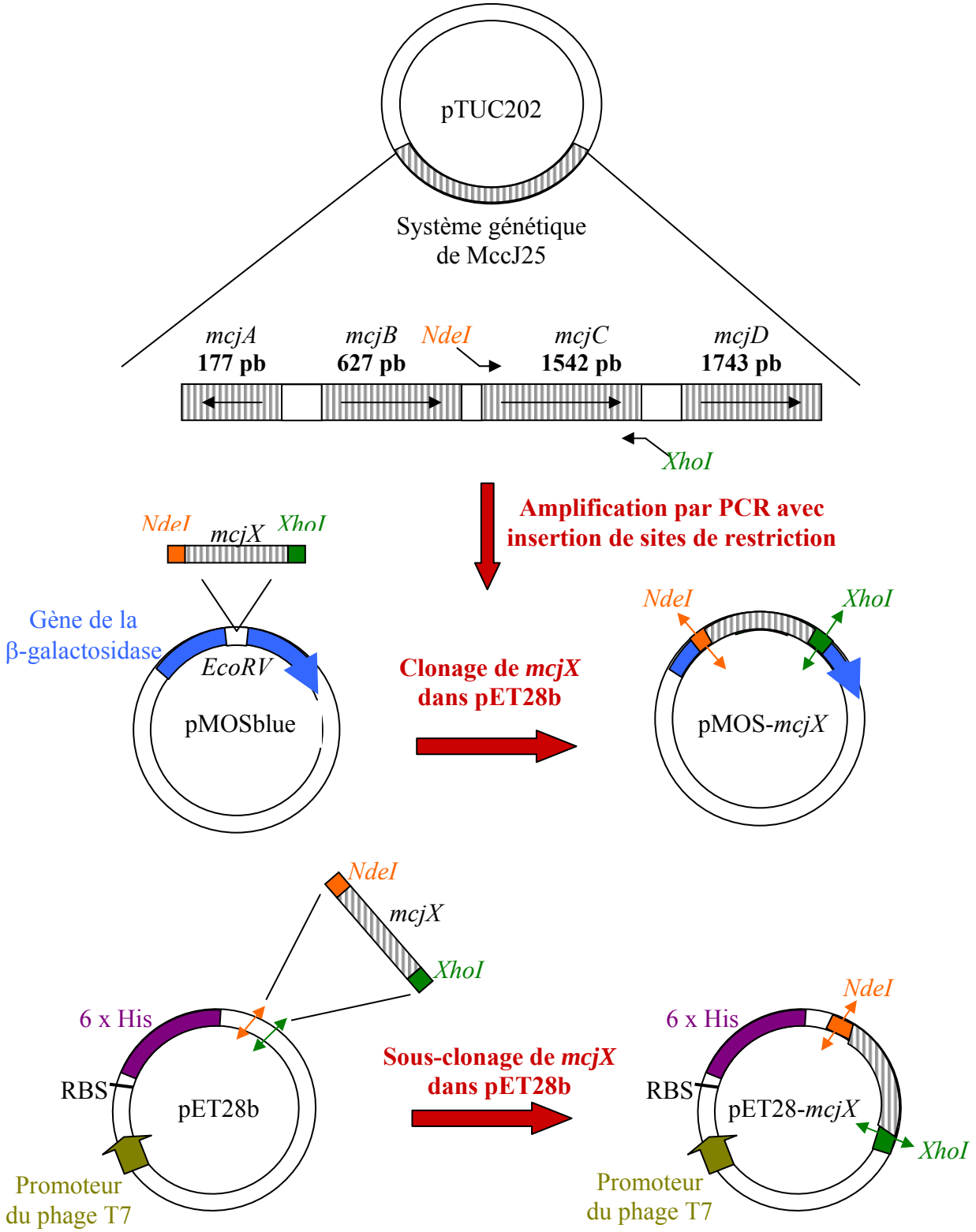
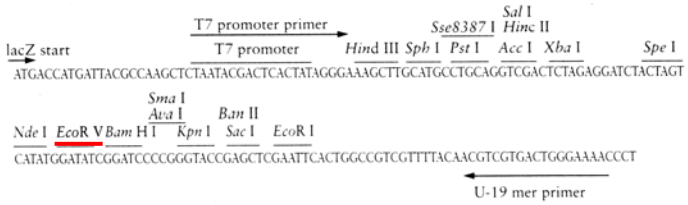


Figure 2 : Site de clonage multiple des plasmides de clonage et d'expression
Les sites d'insertion des gènes sont notés en rouge

pMOSBlue



pET28b

pET22b

pET41b

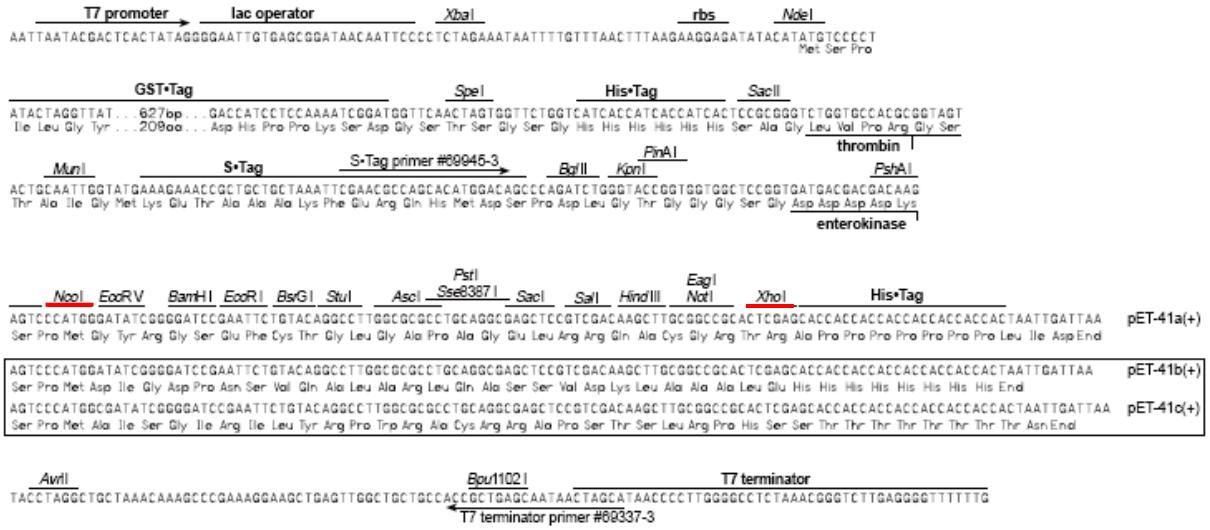
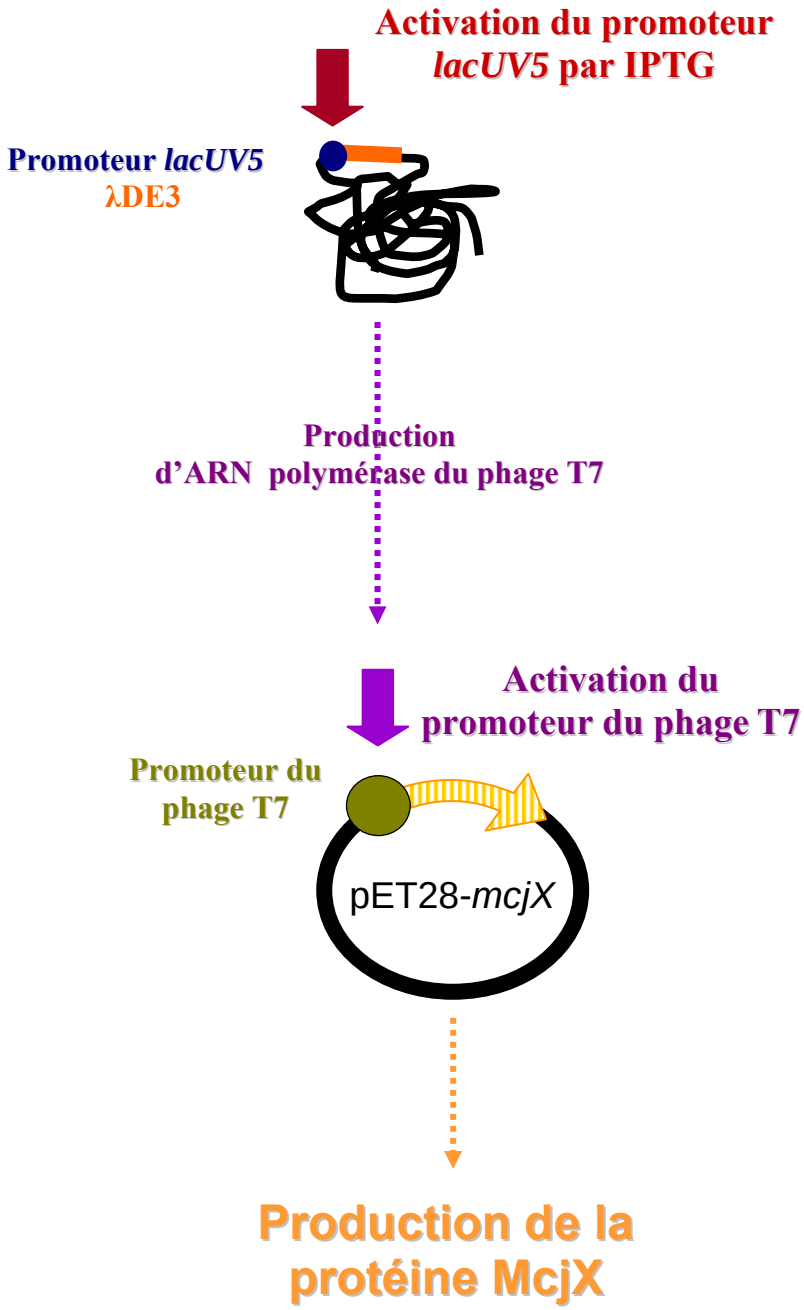


Figure 3 : Production de protéines recombinantes dans la bactérie hôte



I.2. Milieux de culture

- Bouillons de culture

Luria-Bertani Broth (LB) : bactotryptone 10 g/l, extrait de levure 5 g/l, NaCl 5 g/l, pH 7

Milieu Minimum M63 : KH_2PO_4 100 mM, $(NH_4)_2SO_4$ 15 mM pH 7, supplémenté par du glucose 0,2 %, $MgSO_4$ 0,02 %, thiamine 1 mg/l, et 1 g/l d'hydrolysats tryptiques d'acide aminés (Difco) ou d'hydrolysats acide de caséine (Difco).

Milieu Pauvre Poor-Broth (PB) : bactotryptone 10 g/l, NaCl 5 g/l

Les antibiotiques (Sigma) ont été utilisés aux concentrations suivantes :

Ampicilline à 50 µg/ml

Chloramphénicol à 34 µg/ml

Tetracycline à 50 µg/ml

Kanamycine à 50 µg/ml

- Milieux gélosés :

Ils dérivent des bouillons de culture et contiennent 6,5 g/l (gélose molle) ou 15 g/l (gélose dure) d'agar.

Tableau 1 : Souches utilisées pour l'analyse fonctionnelle des protéines

Souche	Génotype	Source
Bactéries à Gram négatif		
Escherichia coli F		Heller et Braun 1979
E. coli W3110	F^- IN(*rrnD-rrnE*)1	Hill et Harnish 1981
E. coli W3110 KP1344	W3110 (*tonB::blaM*)	Larsen *et al.* 1999
E. coli W3110-6	W3110 Δ(*exbB-exbD*)	Howard, unpublished
E. coli C600	*F*$^-$ *sup*E44 *lacY1 thr*-1 *leu*B6 *mcrA thi*-1 *rfb*D1 *fhu*A21 λ^-	Collection L. Letellier (IBBMC, Orsay)
Enterobacter cloacae		Collection P. Bulet (IBMC, Strasbourg)
Erwinia carotovora		Collection P. Bulet
Klebsiella pneumoniae		Collection P. Bulet
Pantoea agglomerans K4		Berner *et al.* 1988
Pseudomonas aeruginosa		ATCC 27853
Salmonella enterica Enteritidis		Portrait *et al.*1999
S. enterica Paratyphi SL369		Graham et Stockler 1977
S. enterica typhimurium LT2		Graham et Stockler 1977
Vibrio harveyi		Institut de recherche et de formation en aquaculture marine (CENAIM, Equateur)
Bactéries à Gram positif		
Aerococcus viridans		Collection P. Bulet
Bacillus megaterium		Collection P. Bulet
Staphylococcus aureus		Collection P. Bulet

Tableau 2 : Souches utilisées pour la production des protéines recombinantes

Souche	Génotype	Source
E. coli MC4100	*araD139* Δ(*argF lac*)U169 *rpsL relA flbB deoC*	Curtis *et al.* 1988
E. coli HO830	*ompF ompC ompA ompB mtl xyl thr aroB leu proA lac Y galK argE rpsL thi*	Bonhivers *et al.* 2001
E. coli Moss (Tet^r)	*end*A1 *hsd*R17 (*rk12 –mk12*)*sup*E44 thi -1 *rec*A1 gyrA96 *rel*A1 lac[F' *pro*A^+B^+lacl^qZΔM15:Tn10(Tc^R)]	GE Healthcare
E. coli DH5α	*F- gyr*A96 *(Nal^r) rec*A1 *rel*A1 *end*A1 *thi*-1 *hsd*R17*(rk12- mk12-) gln*V44 *deo*R *Δ(lacZYA-argF)*U169[Φ80dΔ(*lacZ*)M15]	Invitrogen Corporation
E. coli BL21(DE3)	*F- ompT hsdSB (rB-mB-) gal dcm(DE3)*	Novagen
E. coli ER2566	*F- λ- fhu*A2 *[lon] ompT lacZ:: T7 gene1 gal sulA11* Δ*(mcrC-mrr)114::IS10 R(mcr-73::miniTn*10*-- TetS)2 R(zgb-210::Tn10) (TetS) end*A1 *[dcm]*	Novagen

Tableau 3 : Plasmides utilisés pour la production des peptides et protéines

Plasmide	Caractéristiques	Source
pTUC202 (Chl^r)	Plasmide porteur du système génétique de MccJ25, il dérive du plasmide naturel pTUC100 (EMBL AF061787) et est utilisé dans la souche *E. coli* MC4100 pour produire MccJ25	Solbiati *et al.* 1996
pMOSblue (Amp^r)	Vecteur de clonage, il est utilisé pour transfecter *E. coli* Moss Cells. Ce plasmide est porteur du gène *LacZ*, permettant l'α-complémentation (sélection des clones par criblage blanc/bleu).	GE Healthcare
pET28b, 22b et 41b (Kan^r)	Vecteurs d'expression (promoteur fort Φ10 du phage T7), ils perrmettent d'exprimer, en présence d'IPTG, des protéines sous forme de fusion avec une étiquette His_6, le peptide leader PelB ou la GST en position *N*-terminale.	Novagen
pHX405 (Amp^r)	permet d'exprimer FhuA avec une étiquette His_6	Moeck *et al.* 1996

II. Clonage et transformation

II.1. Purification et dosage d'ADN

II.1.a) Purification d'ADN par lyse alcaline

Les plasmides portés par les différentes souches ont été extraits selon la méthode de la lyse alcaline (Sambrook *et al.* 1989). Les plasmides utilisés pour le séquençage ont été extraits par échange d'ions grâce au procédé commercial « Miniplasmid Prep » (MoBio Laboratories Inc.) ou « Plasmid Midi Kit » (Qiagen).

II.1.b) Purification d'ADN par extraction de gel d'agarose

La purification des fragments d'ADN résultant de digestions enzymatiques a été réalisée par élution de gels d'agarose 0,7 à 2 % (tampon TAE : 40 mM Tris, 0,001 % acide acétique et 0,001 M EDTA, pH 8, coloration au BET), grâce au procédé commercial « Ultra Clean DNA Purification » (MoBio Laboratories Inc.).

II.1.c) Dosage d'ADN

La concentration et la pureté de l'ADN ont été déterminées par spectroscopie UV à 260 et 280 nm sur un spectrophotomètre UVIKON 932. Le rapport de l'absorbance à 260 nm sur celle à 280 nm permet d'estimer la pureté de l'ADN. Lorsque la valeur est inférieure à 1,65 ou supérieure à 2, les solutions sont considérées comme contaminées respectivement par des protéines ou de l'ARN.

II.2. Obtention d'inserts d'ADN

Les amorces oligonucléotidiques utilisées dans cette étude sont résumées dans le tableau 4. Les amorces 1 et 2 ont permis le séquençage du plasmide pTUC202. Les amorces 3 et 4 ont permis le séquençage des divers construits résultant du clonage des gènes *mcjA*, *mcjB* et *mcjC* dans pMOSBlue. Les amorces 5 à 10 ont servi à amplifier les gènes *mcjA*, *mcjB* et *mcjC* à partir de pTUC202. Les amorces 11 à 14 ont servi à inactiver les gènes *mcjB* et *mcjC*. Elles sont complémentaires deux à deux et phosphorylées à leur extrémité 5'.

Les sites de restriction introduits par ces amorces, *Xho*I et *Nde*I, sont respectivement soulignés et soulignés en gras, et les codons stop sont en caractères gras.

Tableau 4 : Caractéristiques des amorces oligonucléotidiques utilisées dans cette étude.

N°	Nom	Séquence 5'-3'	Tm (°C)	Fournisseur
1	pTUC-up	TTCCGCATTCATTAATAC	53,3	Génome Express
2	pTUC-down	TTTTTTCTGCATGTTAATACG	56,95	Génome Express
3	U19	GTTTTCCCAGTCACGACGT	62,6	MWG
4	T7Prom	TAATACGACTCACTATAGGG	50,8	
5	mcjA-down-XhoI	CTCGAGAATATCAGCCATAGAAAG	51	Proligo
6	mcjA-up-NdeI	GCCCATATGATTAAGCATTTT	50	Proligo
7	mcjB-down-XhoI	CTCGAGCTATATCTCTGCAATAAC	49	Proligo
8	mcjB-up-NdeI	CATATGATCCGTTACTGCTTAAC	48	Proligo
9	mcjC-down-XhoI	CTCGAGTTAACCTTTATAATCAATG	49	Proligo
10	mcjC-up-NdeI	CCATATGGAAATATTTAATGTCAAG	49	Proligo
11	mcjB-sens-BstXI	GAGCTCGAG**TAATAGTAG**GGG	49	Proligo
12	mcjB-anti-BstXI	TTCCCC**CTACTATTA**CTCGAG	51	Proligo
13	mcjC-sens-SapI	**TAATAG**TCACTCGAGAGTA	52	Proligo
14	mcjC-anti-Sap	CTCGAG**TGACTATTA**TACT	52	Proligo

II.2.a) Réaction de PCR

Les gènes *mcjA*, *mcjB* et *mcjC* ont été amplifiés par PCR pour clonage et expression recombinante. L'amplification des gènes *mcjA*, *mcjB* et *mcjC* par PCR a été réalisée en utilisant pTUC202 comme matrice d'ADN. 0,3 µg de pTUC202 ont été amplifiés en utilisant les oligonucléotides décrits ci-dessous (160 µM), la polymérase *Taq* ou *Vent* (1 u, New England Biolabs), 200 µM de dNTP (Finnzyme) et 2 mM de $MgSO_4$ dans un volume total de 50 µl de ThermoPol Buffer 1X (New England Biolabs). Pour chacune des réactions, un contrôle négatif a été effectué en absence d'ADN. Le cycle de température [4 min à 94 °C, puis 30 cycles (45 s à 94 °C, 45 s à 45-46 °C, 45 s à 72 °C) et 7 min à 72 °C] a été effectué sur un thermocycleur Mastercycler Personal (Eppendorf) pour les couples d'amorces 5/6, 7/8, 8/9

et 9/10. Le couple d'amorce 8/9 a permis d'amplifier un fragment d'ADN correspondant à *mcjB-mcjC* et noté *mcjBC* par la suite.

Dans certains cas, des réactions de PCR ont été effectuées directement sur colonies bactériennes. Pour cela, les bactéries ont été prélevées sur gélose avec une pipette Pasteur et introduites dans le mélange réactionnel.

II.2.b) Réaction de formation de duplex

Des duplex d'oligonucléotides ont été formés afin d'insérer des codons stop à des positions choisies dans la séquence de gènes à inactiver. Ceux-ci ont été obtenus comme suit. La réaction d'hybridation a été réalisée sur 1 nmole de chacun des oligonucléotides dans 20 µl de tampon Tris-HCl 10 mM pH 7,7, 50 mM NaCl, 1 mM EDTA. Le cycle de température [5 min à 95 °C puis retour à 20 °C en 3 h] a été effectué sur un thermocycleur Mastercycler Personal (Eppendorf).

II.3. Réactions enzymatiques

II.3.a) Digestion

Les enzymes de restriction utilisées sont *Bst*XI, *Nde*I, *Nco*I, *Sap*I, *Xho*I, (New England Biolabs). Elles ont été utilisées dans les conditions recommandées par le fournisseur, en présence de BSA (100 µg/ml) pour *Xho*I. Les digestions ont été effectuées à 37 °C pendant 3 h exceptées les digestions par *Bst*XI, qui ont été réalisées à 55 °C.

II.3.b) Déphosphorylation

L'insertion du duplex destiné à inactiver *mcjC* dans le plasmide pTUC202 linéarisé par l'enzyme *Sap*I ne permet pas d'inactiver ce site de restriction. La ligation du duplex dans pTUC202 linéarisé ne peut donc pas se faire en présence de l'enzyme, ce qui aurait permis d'éliminer les faux positifs correspondant au plasmide religué sur lui même. Dans ce cas, pTUC202 linéarisé a donc été déphosphorylé avant la réaction de ligation.

La réaction de déphosphorylation a été réalisée à l'aide de 0,5 unité de phosphatase alcaline (New England Biolabs) dans 20 µl du tampon fourni préconisé par le fournisseur par µg de plasmide, pendant 1 h à 37 °C. La phosphatase alcaline ne pouvant être inactivée par la chaleur, le plasmide déphosphorylé a ensuite été purifié par extraction au phénol/chloroforme/isoamylalcool (25/24/1) (Sambrook *et al.* 1989).

II.3.c) Ligation

Pour chaque ligation, un contrôle négatif a été réalisé, dans lequel le vecteur linéarisé a été placé seul dans les conditions de ligation utilisées pour les différents plasmides de l'étude, décrites ci-dessous.

- → Vecteur de clonage pMOSblue : La ligation des fragments d'ADN amplifiés par PCR dans le vecteur pMOSblue a été effectuée selon les recommandations du fournisseur (kit de clonage, GE Healthcare). Après une étape de polissage (élimination des dATP sortants en 3') et de phosphorylation enzymatique, les fragments d'ADN amplifiés par PCR ont été puis ligués, par la ligase d'ADN du phage T4, dans le site *Eco*RV du vecteur déphosphorylé (GE Healthcare).

Les plasmides résultants ont par la suite été nommés pMOS-*mcjA*, -*mcjB*, -*mcjC* et -*mcjBC*.

- → Vecteur d'expression pET28b, pET22b et pET41b : Les gènes *mcjA, mcjB, mcjC* et *mcjBC* respectivement issus des vecteurs pMOS-*mcjA*, -*mcjB*, -*mcjC* et -*mcjBC* par double digestion *Nde*I/*Xho*I et purifiés par extraction sur gel d'agarose ont été insérés dans le plasmide pET28b ayant subi le même traitement. De la même manière, *mcjA* issu du vecteur pET28-*mcjA* a été introduit entre les sites *Nco*I et *Xho*I des plasmides pET22b et pET41b. Des rapports molaires insert/vecteur de 10 à 50 ont été utilisés et les réactions ont été faites à 16 °C, pendant 16 h, en présence de 10 à 100 ng de plasmide et d'un excès de ligase d'ADN du phage T4 (New England Biolabs).

Les plasmides résultants ont par la suite été nommés pET28-*mcjA*, -*mcjB*, -*mcjC* et -*mcjBC*, pET41-*mcjA* et pET22-*mcjA*.

- → Plasmide pTUC202: La réaction d'insertion des duplex d'ADN dans pTUC202 a été réalisée à 16 °C pendant 16 h, sur 100 ng de plasmide pTUC202, avec 20 ng de duplex (rapport molaire insert/vecteur de 100) et en présence d'un excès de ligase d'ADN du phage T4.

Les plasmides résultants seront respectivement nommés pTUC202B et pTUC202C, lorsque *mcjB* ou *mcjC* est inactivé.

Après chaque ligation, et avant de transformer des bactéries compétentes avec le produit de la ligation, la ligase d'ADN du phage T4 a été inactivée par une incubation de 20 min à 65 °C.

II.4. Transformation

La transformation des bactéries compétentes *E. coli* Moss (GE Healthcare) par pMOS-*mcjA*, -*mcjB*, -*mcjC* ou -*mcjBC* a été faite par choc thermique selon les recommandations du fournisseur. Les bactéries ont été cultivées sur boite de gélose LB contenant les antibiotiques adéquats ainsi que 175 µg/ml de X-Gal et 50 µg/ml d'IPTG pour permettre une première sélection visuelle des colonies recombinantes.

Les souches d'*E. coli* DH5α, BL21(DE3) et ER2566 ont été rendues compétentes au $CaCl_2$ (Dagert et Ehrlich 1979). Une culture bactérienne de 50 ml dans du LB a été arrêtée à une DO à 600 nm de 0,2 puis centrifugée. Les bactéries ont été traitées pendant 20 min par $CaCl_2$ 0,1 M puis centrifugées avant d'être reprises par 0,5 ml de $CaCl_2$ 0,1 M. A ce stade, les bactéries ont été laissées 16 h à 4 °C. 100 µl de ces bactéries ont alors été transformés avec 10 à 100 ng des plasmides recombinants grâce à une incubation de 10 min sur glace suivie d'un choc thermique de 5 min à 37 °C. Ces bactéries ont ensuite été cultivées à 37 °C dans 1 ml de LB pendant 2 h, puis étalées sur boite de Pétri contenant les antibiotiques de sélection. 100 µl de bactéries n'ayant pas été au contact d'ADN ont été utilisés comme contrôle de stérilité et 100 µl de bactéries transformées avec 10 à 100 ng de plasmide superenroulé ont été utilisés comme contrôle de compétence des bactéries. Enfin, 100 µl de bactéries transformées avec 10 à 100 ng de vecteur seul soumis à ligation ont permis de contrôler l'absence de religation du vecteur sur lui-même.

II.5. Séquençage

Le séquençage des plasmides a été réalisé par les sociétés Génome Express et MWG Biotech. L'interprétation logicielle des chromatogrammes a été vérifiée manuellement.

III. Production et purification de peptides et protéines

III.1. Microcine J25 intacte et clivée à la thermolysine

III.1.a) Microcine J25

La souche *E. coli* MC4100 portant le plasmide pTUC202 a été cultivée 16 h à 37 °C dans i) 1 l de milieu minimum M63, supplémenté par 1 mg/ml de vitamine B1, 0,02 % $MgSO_4$, 0,02 % glucose et 1 g/l d'hydrolysat acides de caséine, pour la production de MccJ25

non radioactive et dans ii) 1 l de milieu M63 supplémenté par 1 mg/ml de vitamine B1, 0,02 % $MgSO_4$, 0,02 % glucose ainsi qu'un mélange d'acides aminés (50 µM chacun) ne contenant pas la glycine et 0,46 MBq de [^{3}H]glycine (592 GBq/mmol, GE Healthcare), pour la production de MccJ25 marquée au tritium.

Dans les deux cas, les bactéries ont été centrifugées à 5 000 *g* pendant 20 min à 4 °C, puis le surnageant a été passé sur cartouche SepPak® C18 (Waters Corp.) préalablement régénérée avec du méthanol et équilibrée avec H_2O-TFA 0,1 % pH 2. La cartouche a ensuite été lavée avec H_2O-TFA 0,1 % puis des élutions à 25 et 30 % d'ACN dans H_2O-TFA 0,1 % ont été effectuées. L'ACN contenu dans la fraction 30 % a été évaporé sous vide dans un concentrateur SpeedVac (Savant), puis la fraction a été lyophilisée. La microcine contenue dans cette fraction a été purifiée par HPLC en phase inverse C18 sur une colonne µBondapak (10 µm, 300 mm × 3.9 mm ; Waters Corp.) à l'aide d'un gradient de 0 à 60 % d'ACN dans H_2O-TFA 0,1 % en 30 min avec un débit de 1 ml/min. La purification a été suivie par mesure de l'absorbance à 226 nm.

La préparation de microcine ainsi obtenue a été dosée par analyse de la composition en acides aminés (voir section IV.1.a)). La radioactivité spécifique de [^{3}H]MccJ25 a été calculée par comptage de la radioactivité associée à une quantité donnée de microcine à l'aide d'un compteur à scintillation Wallac 1410 (GE Healthcare).

III.1.b) Microcine clivée à la thermolysine, t-MccJ25

Le clivage de MccJ25 par la thermolysine a été effectué comme précédemment décrit (Blond *et al.* 2002). 1 µmole de MccJ25 dissoute dans 600 µl d'urée 8 M a été incubée 30 min à 46 °C avant ajout de NH_4CO_3 (1,2 ml, 170 mM final), de $CaCl_2$ (200 µl, 10 mM final) et 40 µg de thermolysine (Boehringer Mannheim). La digestion a été effectuée à 46 °C pendant 1 h, puis la réaction a été arrêtée par ajout de 400 µl d'acide acétique glacial. MccJ25 clivée par la thermolysine, t-MccJ25, a ensuite été purifiée par HPLC en phase inverse C18 sur une colonne Inertsil ODS2 (5 µm, 250 mm × 4.6 mm ; Interchim) par élution isocratique à 31 % d'ACN dans H_2O-TFA 0,1 % avec un débit de 1 ml/min. La purification a été suivie par mesure de l'absorbance à 226 nm.

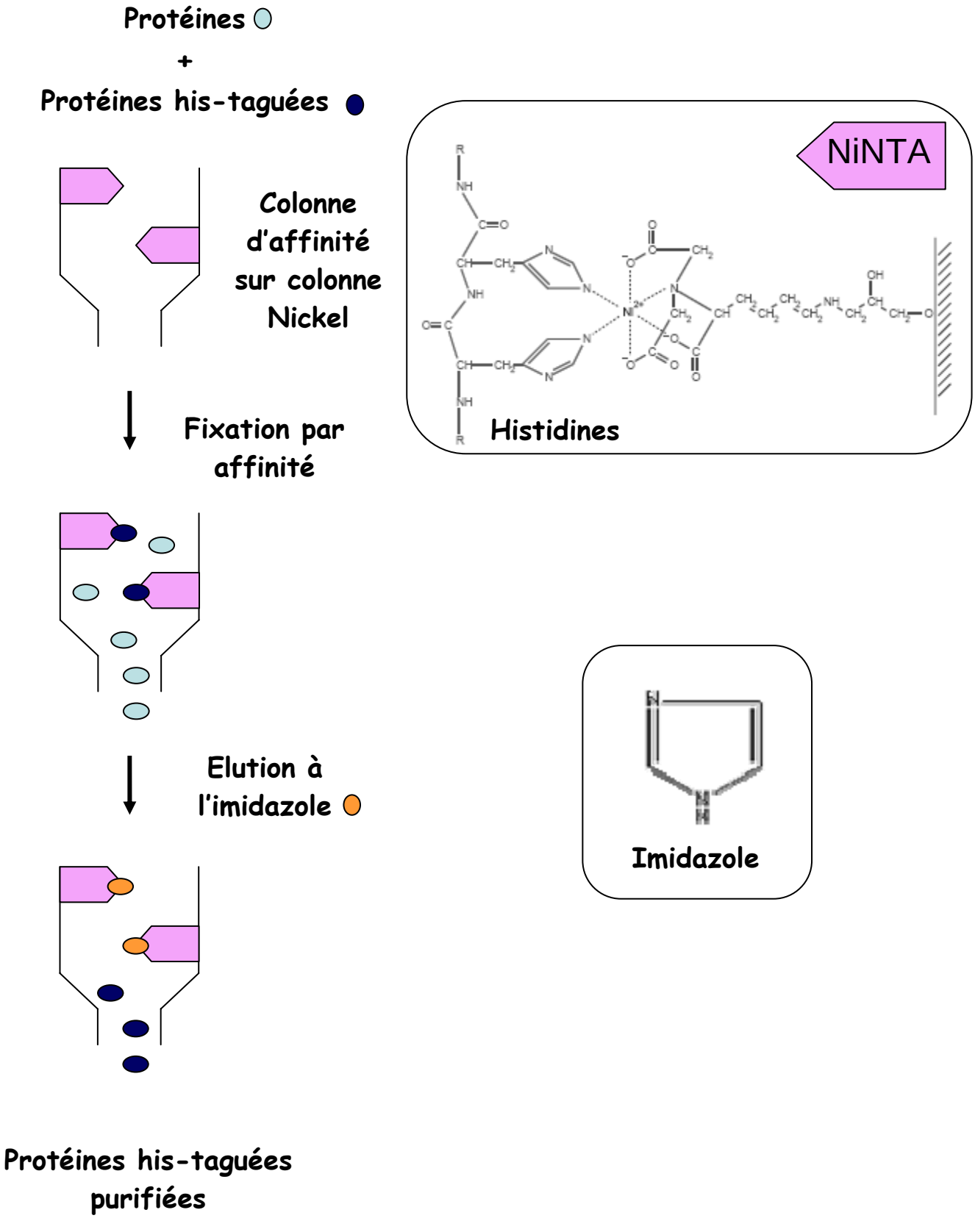
III.2. McjA, précurseur de MccJ25

Dans les sections III.2. et III.3. sont décrites la production et la purification des différentes protéines recombinantes utilisées pour la reconstitution *in vitro* du processus de maturation de MccJ25. L'optimisation des conditions de production nous a amenés à choisir un vecteur d'expression (pET22b, pET28b ou pET41b), une souche hôte (*E. coli* ER2566 ou *E. coli* BL21(DE3)), ainsi qu'une quantité d'IPTG, un temps et une température de culture après induction permettant une production optimale de chaque protéine.

Les protéines ont été produites en conditions natives ou dénaturantes. Ces deux types de conditions diffèrent en fait par l'utilisation, en conditions dénaturantes, d'agents chimiques (SDS) ou d'une étape à haute température risquant de déstructurer la protéine produite.

Les protéines produites grâce au vecteur pET28b ont finalement été purifiées par chromatographie d'affinité sur colonne nickel. Cette chromatographie utilise une phase stationnaire correspondant à de la sépharose couplée à du NiNTA chargé en ion nickel (II). La purification est alors basée sur les interactions entre l'étiquette His_6 des protéines recombinantes et les ions Ni^{2+} immobilisés. En effet, le nickel a une forte affinité pour le noyau imidazole présent dans l'histidine. L'équilibration de la colonne, ainsi que le chargement du lysat bactérien se font dans un tampon contenant peu d'imidazole de manière à minimiser les interactions non spécifiques du lysat avec la phase stationnaire. L'élution se fait par un tampon à forte concentration en imidazole, de manière à décrocher les protéines portant une étiquette His_6 au profit d'une interaction imidazole/Ni^{2+}, la concentration d'imidazole étant supérieure à celle des protéines portant l'étiquette. La figure 4 schématise le principe général de la purification par chromatographie d'affinité sur colonne nickel.

Figure 4 : Purification par chromatographie d'affinité sur colonne nickel



III.2.a) Production

Une préculture de la souche *E. coli* ER2566, porteuse du plasmide pET28-*mcjA* (Kan^R) a été réalisée pendant 16 h, à 37 °C, en milieu LB contenant la kanamycine. Cette préculture a servi à inoculer de 50 ml à 1 l de milieu LB (inoculum 2 %) contenant le même antibiotique. Lorsque la turbidité à 600 nm de la culture a atteint 0,6 à 0,8 (phase exponentielle de croissance), l'IPTG a été ajouté (1 mM final) et la culture a été poursuivie pendant 1 h. Après refroidissement de la culture sur glace, le culot cellulaire a été séparé du surnageant de culture par centrifugation à 5 000 *g* pendant 20 min à 4 °C.

Le culot bactérien a été traité immédiatement après centrifugation ou conservé à -80 °C pour la purification de His_6-McjA respectivement en conditions dénaturantes ou en conditions natives.

III.2.b) Traitement du culot bactérien

III.2.b).i. Conditions natives

Pour la purification en conditions natives, le culot bactérien a été repris sur glace à raison de 10 ml de tampon phosphate de sodium 50 mM pH 8, 500 mM NaCl, contenant 40 mM d'imidazole, supplémenté avec un cocktail commercial d'inhibiteurs de protéases EDTA-free (Roche), de l'ARNase (10 ng/ml final, Sigma) et de l'ADNase (5 µg/ml final, Roche) pour 2,5 g de culot. Les bactéries ont finalement été lysées par un passage à 8 000 psi à travers une presse de French (Thermo Electron Corp.), puis centrifugées à 50 000 *g* pendant 30 min à 4 °C.

III.2.b).ii. Conditions dénaturantes

Le traitement du culot bactérien en vue d'une purification de His_6-McjA en conditions dénaturantes a été réalisée comme suit. Le culot bactérien a été repris sur glace dans un tampon phosphate de sodium 50 mM pH 8, supplémenté avec un cocktail d'inhibiteurs de protéases EDTA-free. La solution ainsi obtenue a été bouillie 10 min avec agitation. Après retour à température ambiante, l'imidazole (40 mM final), le NaCl (500 mM final), le cocktail d'inhibiteurs de protéases EDTA-free, l'ARNase (10 ng/ml final) et l'ADNase (5 µg/ml final) ont été ajoutés.

Les bactéries ont finalement été lysées par un passage à 8 000 psi à travers une presse de French, avant d'être centrifugées 30 min à 50 000 *g*.

III.2.c) Purification de His_6-McjA

La première étape de purification de His_6-McjA, préparée en conditions natives ou dénaturantes, a été réalisée par chromatographie d'affinité sur colonne nickel (NiNTA), sur une colonne His-Trap (1ml, GE Healthcare) à 4 °C. La purification a été suivie par mesure de l'absorbance à 226 nm. Cette colonne a été équilibrée dans le tampon de lyse (phosphate de sodium 50 mM pH 8, 500 mM NaCl, 40 mM imidazole), avec un débit de 1 ml/min jusqu'à obtenir obtention d'une ligne de base stable. L'échantillon a été chargé sur la colonne avec un débit de 0,3 ml/min. La colonne a été lavée avec le tampon de lyse jusqu'au retour de l'absorbance à la ligne de base, puis avec le même tampon contenant 80 mM d'imidazole. His_6-McjA a finalement été éluée avec le tampon contenant 200 mM d'imidazole supplémenté avec un cocktail d'inhibiteurs de protéases contenant de l'EDTA (Roche).

Les deux dernières étapes de purification de His_6-McjA ont impliqué la chromatographie en phase inverse, successivement sur cartouche SepPak® C8, et par HPLC.

Une cartouche SepPak® C8 (Waters Corp.) a été régénérée avec du méthanol, puis équilibrée avec H_2O-TFA 0,1 % (pH 2). L'échantillon a été chargé sur la cartouche. La cartouche a été lavée par 5 ml d'H_2O-TFA 0,1 %, puis par 20 % d'ACN dans H_2O-TFA 0,1 %. L'élution de His_6-McjA a finalement été effectuée à 40 % d'ACN dans H_2O-TFA 0,1 %. La purification se fait sans suivi de l'absorbance.

La dernière étape d'HPLC a été suivie par mesure de l'absorbance à 226 nm, et effectuée sur une colonne C18 µBondapak (10 µm, 300 mm × 3,9 mm ; Waters Corp.) à l'aide d'un gradient de 0 à 25 % d'ACN dans H_2O-TFA 0,1 % en 2 min, puis de 25 à 45 % d'ACN en 25 min et 45 à 60 % d'ACN en 2 min, avec un débit de 1 ml/min.

III.2.d) Clivage à la thrombine

L'étiquette d'histidines en position *N*-terminale de His_6-McjA a été clivée par réaction enzymatique de la thrombine (GE Healthcare); la protéine a été incubée, à raison de 1 µg/µl, dans un tampon Tris 20 mM pH 8,4, 150 mM NaCl et 2,5mM $CaCl_2$, avec la thrombine (0,5 u/µl), dans un volume final de 100 µl et pendant 1 h à 20 °C. McjA a ensuite été purifiée par HPLC en phase inverse sur une colonne µBondapak C18 (10 µm, 300 mm × 3,9 mm ; Waters Corp.) à l'aide d'un gradient de 0 à 25 % d'ACN dans H_2O-TFA 0,1 % en 2 min, puis de 25 à 45 % d'ACN en 25 min et 45 à 60 % d'ACN en 2 min avec un débit de 1 ml/min. La purification a été suivie par mesure de l'absorbance à 226 nm.

III.3. McjB et McjC, enzymes de maturation de MccJ25

III.3.a) Production et purification en conditions natives

Pour la production de His_6-McjB et His_6-McjC, les souches *E. coli* BL21(DE3), respectivement porteuses de pET28-*mcjB* ou pET28-*mcjC*, ont été mises en préculture 16 h à 37 °C, en milieu LB contenant de la kanamycine. Cette préculture a servi à inoculer 1 l de milieu LB stérile contenant le même antibiotique (inoculum 2 %). Lorsque la turbidité à 600 nm de la culture a atteint 0,5, la culture a été équilibrée à 15 °C. Lorsque la turbidité à 600 nm de la culture a atteint 0,6 à 0,8, l'IPTG a été ajouté (1 mM final) puis la culture a été poursuivie pendant 16 h à 48 h. Après refroidissement de la culture sur glace, le culot cellulaire a été séparé du surnageant de culture par centrifugation à 5 000 *g* pendant 20 min à 4 °C.

Le culot bactérien a été repris sur glace à raison de 10 ml de tampon phosphate de sodium 50 mM pH 8, 500 mM NaCl, contenant 20 mM d'imidazole, supplémenté avec un cocktail commercial d'inhibiteurs de protéases EDTA-free, d'ARNase (10 ng/ml final) et d'ADNase (5 µg/ml final) pour 2,5 g de culot. Les bactéries ont été lysées par un passage à la presse de French à 8 000 psi, puis centrifugées à 50 000 *g* pendant 30 min à 4 °C.

De la même manière que pour His_6-McjA, la purification de His_6-McjB et His_6-McjC a été réalisée à 4 °C par chromatographie d'affinité sur colonne nickel His-Trap (1 ml, GE Healthcare), avec suivi de l'absorbance à 280 nm. Brièvement, la colonne a été équilibrée à 1 ml/min dans le tampon de lyse (phosphate de sodium 50 mM pH 8, 500 mM NaCl, 20 mM imidazole). L'échantillon a ensuite été chargé à 4 °C à 0,3 ml/min. La colonne a été lavée avec le tampon de lyse, puis avec le même tampon contenant 80 mM d'imidazole. His_6-McjB ou His_6-McjC a été éluée avec le tampon contenant 200 mM d'imidazole.

La fraction contenant His_6-McjB ou His_6-McjC a finalement été dialysée contre du tampon phosphate de sodium 50 mM pH 8, 100 mM NaCl, dans un boudin de dialyse Spectra/Por 12-16 kDa (Spectrum Medical Industries, Inc.).

III.3.b) Production et purification en conditions dénaturantes

Les souches *E. coli* BL21(DE3) porteuses de pET28-*mcjB* ou pET28-*mcjC* ont été utilisées pour la production de His_6-McjB ou His_6-McjC, suivant un protocole identique à celui décrit pour la production de His_6-McjA (voir section III.2.a)), à l'exception du temps pendant lequel la synthèse des protéines est induite, qui est de 2 h au lieu de 1 h.

Le culot bactérien a ensuite été purifié par électrophorèse préparative. Les protéines bactériennes ont tout d'abord été séparées selon leur poids moléculaire apparent par

électrophorèse sur gel d'acrylamide SDS (voir section IV.2.b)). Un marqueur de poids moléculaire ainsi qu'une des pistes contenant les protéines bactériennes, servant de témoin, ont été colorés au bleu de Coomassie. Cette coloration a permis de déterminer la position de la protéine d'intérêt sur le reste du gel non coloré. La zone de gel contenant la protéine a été découpée, puis placée dans un boudin de dialyse 12-16 kDa immergé dans du tampon d'élution (0,1 M Tris, 0,1 M tricine, 0,1 % SDS, pH 8,5). L'électroélution a été effectuée avec un générateur PowerPac 200 (Biroad), pendant 16 h à 30 mA et à 4 °C. Elle a été suivie d'une dialyse dans un nouveau boudin 12-16 kDa, contre du PBS 1X (Sambrook *et al.* 1989). Les protéines ont finalement été concentrées par ultrafiltration, respectivement sur Centricon 10 kDa (Millipore) et Centriprep 30 kDa (Millipore) pour His_6-McjB et His_6-McjC.

III.4. FhuA, récepteurs aux sidérophores

La purification du récepteur membranaire FhuA a été effectuée par Pascale Boulanger à l'Institut de Biochimie et Biophysique Moléculaire et membranaire, (UMR 8619 CNRS, Orsay), dirigé par Lucienne Letellier.

Le récepteur a été produit sous forme d'une protéine de fusion avec une étiquette contenant six histidines après l'acide aminé 405, et purifié comme précédemment décrit (Plançon *et al.* 2002). La souche *E. coli* HO830 a été transformée avec le plasmide pHX405 puis cultivée 16 h à 37 °C dans du milieu LB en présence de 100 µM de dipyridyl et 125 µg/ml d'ampicilline. Les vésicules de membranes externes ont été préparées par traitement du culot bactérien avec du lysozyme et de l'EDTA, puis solubilisées dans 1 % OG. His_6-FhuA a été élué par le tampon Tris 50 mM, pH 8, 1 % OG, 80 mM imidazole d'une colonne Super Flow chargée en résine NiNTA (Qiagen) à raison de 5 ml pour 14 g de culot bactérien. His_6-FhuA a été chargé sur une colonne Source 30Q (1,5 ml ; GE Healthcare) pré-équilibrée avec le tampon Tris 50 mM pH 8, 1 % OG. His_6-FhuA a finalement été élué par un gradient linéaire de NaCl (0 à 1 M) dans le tampon Tris 50 mM, pH 6,4, 1 % OG, puis dessalée par dialyse dans un boudin de dialyse Spectra/Por 12-16 kDa (Spectrum Medical Industries, Inc.) contre du tampon Tris 50 mM, pH 6,4, 1 % OG.

IV. Caractérisation des peptides et protéines

IV.1. Dosage de solutions de peptides et protéines

IV.1.a) Composition en acides aminés

Cette technique a été utilisée pour confirmer que le produit purifié est bien celui attendu, et pour doser avec précision les solutions pures en peptide.

Après hydrolyse acide, la composition en acides aminés de [^{3}H]MccJ25 a été obtenue suivant le protocole précédemment décrit (Boulanger *et al.* 1996), par Geneviève Auger à l'Institut de Biochimie et Biophysique Moléculaire et membranaire, (UMR 8619 CNRS, Orsay), sur un appareil Biotronik LC 2000 équipé d'une colonne contenant la résine Dionex DC6A (Durrum chemical Corporation) et d'un fluorimètre Spectra-Glo (Gilson).

Après hydrolyse acide, la composition en acides aminés de MccJ25 et de son variant linéaire résultant du clivage à la thermolysine (voir section III.1.b)), t-MccJ25, a été obtenue suivant le protocole précédemment décrit (Destoumieux-Garzón *et al.* 2003) sur un module de séparation Waters 2695 équipé d'une colonne échangeuse d'ions (150 × 4 mm ; Pickering laboratory, LC Tech) et d'un détecteur UV à barrette de diodes (Waters 2996).

IV.1.b) Dosage de Bradford

Cette méthode utilise le fait que le bleu de Coomassie G250 forme avec les protéines un complexe coloré présentant un maximum d'absorbance à 595 nm. Pour une séquence protéique donnée, il existe une bonne corrélation entre la quantité de colorant fixé (donc l'absorbance mesurée) et la concentration de la protéine. Les solutions contenant His_6-McjB et His_6-McjB purifiées ont été quantifiées à l'aide de cette méthode, à l'aide d'un kit de dosage de protéines (Pierce).

IV.1.c) Mesure de l'absorbance à 226 nm et 280 nm

Pour les dosages de routine, les microcines ont été dosées en fonction de leur absorbance UV à 226 nm en sortie d'HPLC. Nous avons comparé pour cela l'absorbance obtenue à celle de solutions standard de MccJ25 et t-MccJ25 précisément dosées par la détermination de leur composition en acides aminés. FhuA a été dosée en routine par spectrophotométrie à 280 nm en fonction de son coefficient d'extinction molaire réel, 103 690 M^{-1} $cm^{-}1$, déduit d'une composition en acides aminés effectuée antérieurement (Boulanger *et al.* 1996).

Le dosage des solutions contenant His_6-McjA et McjA a été effectué par mesure de l'absorbance à 280 nm en utilisant le coefficient d'extinction molaire théorique 2 560 M^{-1} cm^{-1} calculé à partir de la séquence en acides aminés (ProtParam, Expasy, Gasteiger *et al.* 2005).

IV.2. Electrophorèse sur gel d'acrylamide SDS

Les échantillons ont été bouillis pendant 5 min dans un mélange Laemmli/tampon de charge : 80 mM Tris pH 8,8, 200 mM glycine, 2,1 % SDS, 40 mM DTT, 7 % glycérol, 0,1 % bleu de bromophénol pour les électrophorèses Tris-Tricine, ou dans un tampon de charge : Tris 50 mM pH 6,8, 2 % SDS, 100 mM DTT, 10 % glycérol, 0,1 % bleu de bromophénol) pour les électrophorèses Tris-glycine, puis chargés sur gel. Les marqueurs de poids moléculaires utilisés sont : Ultra-Low Color Marker (Sigma), Précision Plus Protein Standards Dual Color ou Précision Protein Standards (Biorad) et Prestained Protein Marker, Broad Range (New England Biolabs).

IV.2.a) Electrophorèse sur gel d'acrylamide SDS Tris-tricine

La tricine est utilisée dans cette technique pour repousser les micelles de SDS au-delà du front de migration. Elle permet une meilleure focalisation des protéines de petite taille (Schagger et von Jagow 1987). Ce type de gel a été utilisé pour l'analyse de His_6-McjA et McjA.

Ainsi, le gel de séparation (16,5 % acrylamide et 0,5 % bis-acrylamide) est surmonté d'un gel de concentration (3,8 % acrylamide et 0,1 % bis-acrylamide). La migration a été effectuée sous 90 V pendant 2 h dans un tampon anode (Tris 0,2 M pH 8,9) et un tampon cathode (0,1 M Tris, 0,1 M tricine, 0,1 % SDS, pH 8,5).

IV.2.b) Electrophorèse sur gel d'acrylamide SDS Tris-glycine

Ce type d'électrophorèse a permis d'analyser His_6-McjB et His_6-McjC. Le gel de séparation (10 à 12 % acrylamide et 0,27 % bis-acrylamide) est surmonté d'un gel de concentration (7,1 % acrylamide et 0,2 % bis-acrylamide). La migration a été effectuée sous 90 V pendant 2 h dans un tampon anode et cathode de composition identique (0,1 M Tris, 0,1 M tricine, 0,1 % SDS, pH 8,5).

Pour les deux types d'électrophorèse, les gels ont été colorés au bleu de Coomassie ou au nitrate d'argent selon les protocoles standard (Sambrook *et al.* 1989). Les gels ont finalement été conservés entre 2 feuilles de cellophane (Biorad). Pour cela, les gels ainsi que

les feuilles de cellophane ont préalablement été immergés 15 min dans une solution aqueuse 4 % glycérol, 10 % éthanol. Le séchage des gels a été effectué durant 2 à 3 jours à température ambiante. Les différents gels ont été photographiés à l'aide d'un système MiniBis Biolmaging (DNR Bio-Imaging Systems, Israel).

IV.3. Western Blot

Les protéines séparées selon leur taille sur un gel d'acrylamide SDS Tris-tricine ou SDS Tris-glycine non fixé et non coloré (protocole décrit ci-dessus) ont été transférées par transfert humide (Mini Trans-Blot Cell, Biorad) sur membrane de nitrocellulose 0,2 µm (Hybond-ECL, GE Healthcare) pendant 1 h à 80 V avec un tampon de transfert Tris 25 mM, 192 mM glycine, 20 % éthanol, 0,05 % SDS, pH 8,3. Les protéines ont ensuite été fixées sur la membrane pendant 30 min à température ambiante dans un tampon Tris-HCl 20 mM pH 7,4, 500 mM NaCl contenant 0,5 % de glutaraldéhyde. Une immunodétection indirecte a ensuite été réalisée. Le blocage des sites non spécifiques de la membrane a été réalisé en incubant la membrane dans une solution de PBS (tampon phosphate de sodium 10 mM pH 7,4, NaCl 0,9 %) et lait écrémé 0,75 %. Ceci permet de minimiser les interactions entre l'anticorps et la membrane. Cette membrane a finalement été incubée pendant 16 h dans une solution de PBS et lait écrémé à 0,25 % contenant 1/1500 d'anticorps primaire dirigé contre l'étiquette d'histidines (monoclonal anti-polyhistidine, IgG2a de souris, Sigma).

Après trois lavages successifs (tampon Tris-HCl 20 mM pH 7,4, 0,1 % BSA, 0,9 % NaCl), l'anticorps secondaire dirigé contre les anticorps de souris et conjugués à la phosphatase alcaline (anticorps anti IgG $F(ab')_2$, Sigma) a été ajouté à une dilution de 1/40000 et incubé 1 h à température ambiante. La révélation a été faite après trois nouveaux lavages par addition d'un mélange de BCIP à 0,15 mg/ml et NBT à 0,30 mg/ml (Sigma).

IV.4. Digestion à la trypsine

Les protéines ont été séparées par électrophorèse sur gel d'acrylamide SDS. Les bandes d'intérêt ont été découpées du gel et lavées deux fois avec du tampon NH_4HCO_3 25 mM pH 8, une fois avec de l'ACN 50 % dans NH_4HCO_3 25 mM pH 8, puis une fois avec NH_4HCO_3 25 mM pH 8, et finalement avec H_2O avant d'être séchées sous vide (SpeedVac, Savant). Les fragments de gel ont été réhydratés dans 50 µl de tampon de clivage (NH_4HCO_3

25 mM pH 8, 5 mM $CaCl_2$, contenant 20 ng/µl de trypsine bovine (T8642, Sigma) et incubés 12 h à 37 °C sous agitation. 50 µl d'H_2O ont été ajoutés, puis les réactions ont été poursuivies 2 h supplémentaires à 37 °C. Les fragments de gel ont été lavés une fois avec H_2O-FA 0,1 % et une fois avec 100 % d'ACN. Les surnageants ont été collectés et réunis, puis séchés sous vide. Les peptides résultant de la digestion par la trypsine ont été resuspendus dans 15 µl d'H_2O-FA 0,1 % puis désalés sur ZipTip C18 (Omix, Varian) avant analyse par spectrométrie de masse MALDI-TOF.

IV.5. Spectrométrie de masse

La spectrométrie de masse consiste à produire des ions en phase gazeuse et permet de déterminer la masse moléculaire des molécules étudiées par des mesures de rapports masse/charge (*m/z*). Elle donne par ailleurs accès à des informations structurales à partir de leurs fragmentations. Deux techniques de désorption/ionisation ont été utilisées. L'ionisation MALDI consiste à irradier à l'aide d'un laser les molécules à étudier, en mélange avec une matrice absorbant fortement à la longueur d'onde du laser, ce qui conduit à la désorption d'un mélange de matrice et d'ions d'intérêt, qui sont formés par transfert de protons entre la matrice et les molécules analysées. L'ionisation ESI se fait à partir d'une molécule à analyser en solution, introduite par un capillaire. L'application d'un champ électrique intense conduit à la formation d'une nébulisation (spray) constitué de gouttelettes chargées. La taille de ces gouttelettes est fortement réduite lors de l'évaporation du solvant, ce qui conduit finalement à des ions désolvatés, généralement multichargés.

Pour ces deux techniques d'ionisation, les ions, accélérés par un champ électrique, ont été séparés selon leur rapport *m/z* dans un analyseur de type temps de vol (TOF). La mesure de *m/z* dans ce type de détecteur consiste à mesurer le temps mis par les ions pour parcourir une région libre de champ, qui est proportionnel à $\sqrt{m/z}$.

- En MALDI-TOF, le TOF est placé dans le prolongement de la source et peut fonctionner en mode linéaire ou en mode réflectron. Ce mode de fonctionnement utilise un miroir électrostatique permettant de corriger l'hétérogénéité des ions en énergie cinétique, et ainsi d'augmenter la résolution de manière significative. L'utilisation du mode réflectron limite cependant la gamme de *m/z* à 5 000.
- En ESI-TOF, le TOF est placé orthogonalement à la source et fonctionne en mode réflectron. L'analyseur utilisé ici est de type Qq-TOF, ce qui permet de réaliser des

expériences de dissociations induites par collision (CID), ou MS/MS. Un quadripôle Q placé en amont du TOF permet d'isoler des ions de rapport *m/z* donné, qui sont alors fragmentés dans la cellule de collision q. Le TOF permet alors de séparer les ions fragments selon leur rapport *m/z*. Ces expériences MS/MS ont été effectuées sur l'ion triplement chargé $[M+3H]^{3+}$ de MccJ25 pour mettre en évidence sa structure en lasso.

Alternativement, un spectromètre de masse de type ESI-piège ionique quadripolaire a été utilisé pour les expériences MS/MS sur MccJ25. Ce type d'analyseur consiste en un assemblage de trois électrodes : une électrode centrale circulaire appelée électrode couronne placée entre deux électrodes identiques appelées électrodes chapeau. Il permet de piéger les ions par l'application d'un potentiel électrique quadripolaire au niveau de l'électrode couronne. Ceux-ci peuvent alors être "manipulés" (éjection du piège, excitation) par l'application des potentiels oscillants de faible amplitude au niveau des électrodes chapeau.

IV.5.a) MALDI TOF

Les expériences MALDI-TOF ont été effectuées sur un appareil Voyager DE-PRO ou 4800 TOF/TOF (Applied Biosystems). 1 µl de solution de matrice acide (alpha-cyano-4-hydroxycinnamique ou acide sinapinique, 10 mg/ml dans une solution 70 % d'ACN dans H_2O-FA 1 %) a été mélangé à 1 µl de la solution de protéine ou 1 µl de digestion trypsique. Le spectromètre a été utilisé en mode positif avec un voltage accélérateur de 25 V, en mode réflectron pour les digestions trypsiques (voir section IV.4.) ou linéaire pour His_6-McjA et McjA. Le spectromètre de masse a été calibré grâce aux ions correspondant aux peptides d'autodigestion de la trypsine pour les digestions trypsiques, ou à un mélange de peptides dont la masse moléculaire varie de 1 à 16 kDa (solution de calibration 3, Applied Biosystems) pour His_6-McjA et McjA.

IV.5.b) ESI-QqTOF

Le couplage HPCL/ESI-MS (HPLC-MS) a été utilisé pour l'analyse des milieux réactionnels de reconstitution *in vitro* de la biosynthèse de MccJ25. La microcine naturellement sécrétée a été utilisée comme référence.

Les expériences ont été effectuées sur une chaîne HPLC (Series 200, Perkin Elmer) connectée à un détecteur UV Agilent 1100 et à un spectromètre de masse Q-STAR Pulsar (Qq-TOF) équipé d'une source ESI (Applied Biosystems). Après dessalage sur cartouche SepPak® C8 (Waters Corp.)(voir section VII.2.), les milieux réactionnels ont été analysés sur une colonne C18 Hypersil Gold (1,9 µm, 50 × 2,1 mm ; Thermo Electron), à l'aide d'un gradient de 10 à 60 % d'ACN dans H_2O-FA 0,1 % en 10 min avec un débit de 0,2 ml/min,

avec suivi de l'absorbance à 226 nm. Le débit a été réduit à 50 l/min en amont du spectromètre de masse à l'aide d'un split. Le spectromètre de masse a été utilisé en mode positif sur une gamme *m/z* de 250 à 1500 en mode standard ou on mode MS/MS en sélectionnant l'ion $[M+3H]^{3+}$ de MccJ25 (*m/z* 703), soumis à une énergie de collision de 22,5 V.

Pour améliorer la sensibilité des expériences MS/MS, un spectromètre de masse de type piège ionique quadripolaire a été utilisé (Esquire 3000, Bruker Daltonics), au Laboratoire de Chimie Structurale Organique et Biologique (UMR 7613, Université Paris VI) dirigé par Pr. Jean-Claude Tabet. Les expériences HPLC-MS/MS sur l'ion $[M+3H]^{3+}$ (*m/z* 703) ont été réalisées à l'aide du même protocole de séparation, après sélection de l'ion précurseur et excitation par application d'une excitation de résonance d'amplitude de 0,85 V_{P-P}.

IV.6. Dichroïsme circulaire

Le DC permet d'étudier la structure secondaire des protéines. Une lumière polarisée rectilignement, correspondant à la somme d'une lumière polarisée circulairement gauche et d'une lumière polarisée circulairement droite, passe à travers la solution à analyser. Après la traversée de la solution optiquement active, l'amplitude des deux composantes de la lumière n'est plus égale. La spectroscopie DC est une spectroscopie d'absorption qui mesure l'ellipticité, liée à la différence d'absorption par l'échantillon de la lumière polarisée circulairement droite et de la lumière polarisée circulairement gauche, en fonction de la longueur d'onde. L'analyse des spectres DC pour des longueurs d'ondes entre 190 et 250 nm permet de déterminer la part des différentes structures secondaires dans la structure globale d'une protéine.

Les expériences de DC ont été effectuées dans l'Unité INSERM U779 du Kremlin-Bicêtre, dirigée Dr. Michael C. Marden.

His_6-McjA a été dissoute à 75 µM dans un tampon 10 mM phosphate de sodium pH 7,4 avec ou sans SDS 8 mM. L'échantillon a été placé dans une cellule en quartz de trajet optique 0,5 mm. Les spectres ont été enregistrés à 25 °C entre 190 et à 250 nm sur un spectropolarimètre Jasco J-810 équipé d'un contrôleur de température à effet Peltier PFD 423S/L.

V. Anticorps polyclonaux anti-[His$_6$-McjB] et anti-[His$_6$-McjC]

La production d'anticorps polyclonaux dirigés contre McjB et McjC a été réalisée par immunisation de souris, au Laboratoire de Biologie Fonctionnelle des Protozoaires (USM 0504, Muséum National d'Histoire Naturelle), dirigé par Pr. Philippe Grellier.

Les sérums des souris (femelles Balb/c de 6 semaines ; IFFA-CREDO, Charles River Laboratories) ont été contrôlés préalablement à l'immunisation afin d'éliminer les animaux dont le sérum présentait une immuno-réactivité croisée avec His6-McjB ou His6-McjC. La détection a été réalisée par Western Blot à l'aide du sérum pré-immun des animaux comme anticorps primaires (voir section IV.3.). Quatre souris ont été sélectionnées pour les immunisations. Les souris ont été immunisées par injection sous-cutanée d'un mélange de 100 µl d'adjuvant complet de Freund (ICN) et de 100 µl de PBS contenant 20 µg de protéine produite en conditions dénaturantes (voir section III.3.b)). Au 20ème jour, un premier rappel a été effectué par injection sous-cutanée d'un mélange identique à celui utilisé lors de la première injection. Un prélèvement de 100 µl de sang du sinus a été réalisé le 35ème jour afin de contrôler par Western Blot l'immunoréactivité du sérum. Un dernier rappel a été effectué le 42ème jour par injection sous-cutanée d'un mélange de 100 µl d'adjuvant incomplet de Freund (ICN) et de 100 µl de PBS contenant 20 µg de protéine. Enfin, les souris ont été sacrifiées le 60ème jour et leur sang a été prélevé par ponction sinusienne. La coagulation du sang a été effectuée pendant 3 h à température ambiante, puis les prélèvements ont été centrifugés (5 000 *g*, 15 min, 4 °C). Le sérum ainsi collecté a été aliquoté puis congelé à -20 °C. Deux sérums contenant des anticorps anti-[His$_6$-McjB] et un sérum contenant des anticorps anti-[His$_6$-McjC] ont été obtenus (respectivement sérums 431/432 et 430).

VI. Mise en évidence d'interactions moléculaires

Les expériences ayant permis de mettre en évidence l'interaction moléculaire entre une microcine, MccJ25, et son récepteur, FhuA, ont été réalisées à l'Institut de Biochimie et Biophysique Moléculaire et membranaire, (UMR 8619 CNRS, Orsay), dirigé par Lucienne Letellier. Les études du complexe MccJ25/FhuA par chromatographie d'exclusion et par calorimétrie, ont été effectuées respectivement dans le Laboratoire de Transport Membranaires de Macromolécules, dirigé par Lucienne Letellier, et le Laboratoire de Modélisation et d'Ingénierie des Protéines, dirigé par Michel Desmadril.

VI.1. Chromatographie d'exclusion

La formation du complexe entre MccJ25 (2 kDa) et FhuA (79 kDa) a été mise en évidence *in vitro* par chromatographie d'exclusion. Etant donné la faible différence de masse moléculaire entre FhuA et un éventuel complexe MccJ25/FhuA, MccJ25 marquée au tritium a été utilisé pour ces expériences. Quatre nmoles de MccJ25 marquée au tritium (3300 cpm/nmol) ou de t-MccJ25 non radiomarquée ont été incubées pendant 10 min à température ambiante avec une quantité croissante de FhuA (4 à 16 nmoles) dans un tampon Tris-HCl 25 mM, pH 7,2 contenant 150 mM NaCl et 1 % OG. Le mélange a ensuite été injecté sur une colonne Superose 12 HR 10/30 (1 × 30 cm, GE Healthcare) de manière à suivre la formation du complexe attendu. La séparation a été effectuée dans le même tampon à un débit de 0,25 ml/min, sur un système FPLC (AKTA GE Healthcare). L'absorbance a été suivie à 226 et 280 nm. Les fractions (0,25 ml) ont été collectées à l'aide d'un collecteur automatique. Trois injections contrôle ont été réalisées avec i) 4 nmoles de FhuA, ii) 4 nmoles de [^{3}H]MccJ25, et iii) 4 nmoles de t-MccJ25. La radioactivité associée (cpm) à chacune des fractions correspondant à la zone d'élution de MccJ25, de FhuA et de MccJ25/FhuA a été mesurée pour chacun des rapports molaires [^{3}H]MccJ25/FhuA à l'aide d'un compteur à scintillation Wallac 1410 (GE Healthcare).

VI.2. Microcalorimétrie

Cette technologie permet de mettre en évidence des interactions moléculaires. Etant donnée la grande sensibilité de la technique, il est nécessaire de minimiser les échanges thermiques dus aux mélanges des tampons contenant ligands et récepteurs. Pour cette raison, le tampon de solubilisation de FhuA a été échangé par dialyse contre un tampon phosphate de sodium 25 mM, pH7,3, 150 mM NaCl, 1 % OG, à l'aide d'un boudin de dialyse Spectra/Por 12-16 kDa (Spectrum Medical Industries, Inc.). Ce tampon a ensuite été utilisé pour solubiliser MccJ25. Les concentrations ont été ajustées en fonction des expériences ; les solutions ont été dégazées à l'hélium avant l'enregistrement des thermogrammes.

VI.2.a) Calorimétrie de titration isotherme (ITC)

Dans ces expériences, la solution contenant FhuA, placée dans la cellule de mesure, est progressivement saturée à température ambiante par microinjections d'une solution très concentrée en MccJ25. A chaque ajout de MccJ25 correspond un échange thermique signant

l'interaction avec FhuA et proportionnel à la quantité de complexe formé. Les quantités de chaleur mesurées permettent d'obtenir l'isotherme de liaison et les paramètres de l'interaction. Une cellule de référence, contenant uniquement le tampon de solubilisation de MccJ25/FhuA de MccJ25 et FhuA, subit le même traitement.

Les expériences d'ITC ont été effectuées sur un appareil VP-ITC (MicroCcal), à 25 °C dans une cellule de 1,43 ml. Une série de 28 injections (10 µl, 1 injection/min) d'une solution à 500 µM de MccJ25 dans une solution à 26,5 µM de FhuA a été réalisée à l'aide d'une micro seringue de 300 µl contrôlée par ordinateur. Les résultats sont visualisés sous forme d'une courbe ΔH (la variation d'enthalpie) en fonction du rapport molaire MccJ25/FhuA.

Une courbe de titration théorique a été superposée aux données expérimentales, grâce au logiciel ORIGIN® (MicroCal). Ce logiciel utilise la relation qui existe entre la chaleur dégagée par chaque injection et ΔH, K_a (la constante d'association), n (le nombre de site de liaison par monomère), la concentration totale en FhuA, la concentration totale en MccJ25, et la concentration en MccJ25 libre.

VI.2.b) Calorimétrie différentielle à balayage (DSC)

La DSC est une technique qui permet de mesurer les variations d'enthalpie qui résultent de changements d'état d'un corps soumis à une variation de température. Dans les expériences décrites ci-dessous, la cellule de mesure, contenant la solution de complexe MccJ25/FhuA, ainsi qu'une cellule de référence, contenant uniquement le tampon de solubilisation de MccJ25/FhuA, sont soumises à une augmentation régulière de température. Des thermocouples placés sous les cellules permettent de déterminer la différence de température entre la cellule de mesure et la cellule de référence. Les résultats sont visualisés sous forme d'une courbe ΔCp (la variation de la capacité calorimétrique) en fonction de la température. La DSC permet donc de suivre la dénaturation thermique du complexe MccJ25/FhuA et de la comparer avec celle de FhuA seul. Les différences observées entre les deux profils de dénaturation thermique sont interprétées pour localiser les régions de FhuA impliquées dans l'interaction avec MccJ25.

Les expériences de DSC ont été effectuées sur un appareil MC2 (MicroCal), avec une vitesse de chauffage de 1 K/min entre 295 et 365 K. Chaque mesure a été précédée par une mesure de la ligne de base correspondant au tampon seul. La capacité calorifique du solvant a été soustraite de celle de l'échantillon protéique avant analyse.

VII. Analyse fonctionnelle des peptides et protéines

VII.1. Activité antimicrobienne

VII.1.a) Tests antimicrobiens en milieu solide

Les tests ont été effectués en milieu solide sur milieu gélosé M63 recouvert d'une gélose molle M63 contenant 10^7 bactéries/ml de la souche *S. enterica* Enteritidis. Le produit à tester a été déposé directement sur la gélose qui a ensuite été placée à 37 °C pendant 16 h. Le lendemain, les diamètres des zones d'inhibition de croissance ont été mesurés.

VII.1.b) Tests antimicrobiens en milieu liquide

La CMI est définie comme étant la plus petite concentration provoquant l'inhibition totale de la croissance bactérienne, alors que la CMB correspond à la plus petite concentration pour laquelle il n'existe aucune bactérie survivante.

Les tests antimicrobiens en milieu liquide ont été réalisés pour déterminer les valeurs des CMI et des CMB caractérisant l'activité antibactérienne de MccJ25 et t-MccJ25 vis-à-vis de diverses souches. Différentes dilutions (0,02 à 10 µM) de MccJ25 et de son variant clivé à la thermolysine, t-MccJ25 (10 µl) ont été incubées sur plaques de 96 puits avec 90 µl d'une suspension diluée de bactéries en milieu de phase exponentielle dans du milieu pauvre PB (DO finale à 620 nm de 0,001). La croissance bactérienne a été estimée par mesure de la DO à 620 nm après 20 h d'incubation à 30 °C, sur un lecteur de plaque Ceres 900 (Bio-Tek Instruments Inc.). 100 µl ont été prélevés des puits où les bactéries avaient apparemment une croissance nulle, puis déposés sur des boites de Pétri contenant du milieu LB gélosé et incubés 16 h à 37 °C. Les colonies bactériennes ont été décomptées.

VII.2. Activité enzymatique de McjB et McjC

His_6-McjB et His_6-McjC ont été incubées avec 0,5 nmole de His_6-McjA dans un tampon phosphate de sodium 50 mM, pH 8, 1 mM DTT, 1 mM ATP et 1mM $MgCl_2$. Pour déterminer quel type de protéase était impliqué dans la réaction, différents inhibiteurs de protéases ont été ajoutés à la réaction. La réaction a aussi été testée en l'absence d'ATP, de DTT ou de $MgCl_2$. Parallèlement, le peptide correspondant au 21 acides aminés *C*-terminaux de McjA (MccJ25 linéaire), obtenu par synthèse en phase solide (Blond *et al.* 2002) a été testé comme substrat. Toutes les réactions ont été incubées à 25 °C pendant 150 min puis désalées par extraction solide sur cartouche SepPak® C8 (Waters Corp.). L'échantillon a été chargé sur la cartouche préalablement régénérée par du méthanol et équilibrée avec H_2O-FA 0,1 %. La

cartouche a été lavée par H_2O-FA 0,1 % puis ACN 10 % dans H_2O-TA 0,1 %, et finalement éluée avec 90 % d'ACN dans H_2O-FA 0,1 %. Cette dernière fraction a été séchée sous vide puis resuspendue dans 50 µl d'ACN 10 % dans H_2O-FA 0,1% avant analyse par HPLC-MS et tests antimicrobiens (voir sections IV.5.b) et VII.1.a)). La recherhce de conditions optimales de fonctionnement des enzymes a été effectuée en modifiant le pH de la réaction (pH 5 et pH 7 comparés au pH 8), ainsi que la température d'incubation (15 °C et 37 °C comparés à 25 °C).

VII.3. Test antiviraux sur phage T5

FhuA est l'unique récepteur du phage T5 à la membrane externe de *E. coli*. Lors du processus d'infection, il se produit une liaison irréversible entre le phage T5 et FhuA qui provoque l'éjection immédiate de l'ADN viral dans les bactéries. Ce système lui permet d'utiliser la machinerie bactérienne à son profit et conduit à la lyse des bactéries. Les expériences décrites ci-dessous ont été conduites pour à étudier *in vivo* l'inhibition par MccJ25 de cette interaction FhuA/phage.

VII.3.a) Détermination de la multiplicité d'infection

La MOI est définie comme étant le rapport du nombre de particules virales (ici le phage T5) sur le nombre de cellules (ici les bactéries *E. coli* W3110).

100 µl d'une préculture d'*E. coli* W3110 ont servi à inoculer 10 ml de LB. Lorsque les bactéries ont atteint leur phase exponentielle de croissance (DO à 600 nm de 0,5), 100 µl de bactéries ont été disposés dans 5 puits (5×10^7 bactéries/puits) d'une plaque 96 puits avec 10 µl d'une suspension de phage T5 en milieu LB pour une concentration finale de 5×10^9, $1{,}25 \times 10^{10}$, $2{,}5 \times 10^{10}$ ou 5×10^{10} phages/ml soit des MOI respectives de 1, 2,5, 5, et 10. La plaque a ensuite été incubée à 37 °C pendant 2 h et une lecture de la DO à 620 nm a été effectuée automatiquement après agitation de la plaque (10 s) toute les 10 min. Les mesures ont été effectuées à l'aide d'un lecteur de plaque Ceres 900 (Bio-Tek Instruments Inc.).

Cette expérience permet de déterminer les conditions expérimentales dans lesquelles se placer (MOI) pour observer la lyse totales des bactéries.

VII.3.b) Inhibition de la lyse bactérienne

L'expérience a été réalisée dans les conditions décrites ci-dessus. 100 µl d'une préculture de *E. coli* W3110 ayant atteint une DO à 600 nm de 0,5 ont ainsi été disposés dans 12 puits (5×10^7 bactéries/puits) d'une plaque 96 puits. 10 µl d'une solution de MccJ25 ou t-

MccJ25 dans du méthanol à 10 %, ou 10 µl d'une solution de méthanol à 10 % ont été ajoutés aux bactéries pour une concentration finale en peptide de 0,25 à 10 µM. Une incubation de 10 min à température ambiante a été réalisée. Dans six des puits servant de témoins pour la croissance bactérienne en absence de phage, 5 µl de milieu LB ont été ajoutés, et dans les six autres, 5 µl d'une solution de phage T5 à $2{,}75 \times 10^{10}$ phages/ml ont été ajoutés (MOI de 2,5). La plaque a alors été incubée à 37 °C pendant 2 h et une lecture de la DO à 620 nm a été effectuée automatiquement après agitation de la plaque (10 s) toutes les 10 min à l'aide d'un lecteur de microplaques Ceres 900.

VII.3.c) Inhibition de l'adhésion du phage T5

Dans ces expériences, *E. coli* F est utilisée car cette souche est connue pour sa grande capacité d'adsorption du phage T5 (exprime très bien FhuA).

100 µl d'une préculture de *E. coli* F ont servi à inoculer 10 ml de milieu LB. Lorsque les bactéries ont atteint leur phase exponentielle de croissance (DO à 600 nm de 0,5), 500 µl de préculture ont été disposés dans 7 tubes Eppendorf stériles avec 25 µl de MccJ25 ou t-MccJ25 pour une concentration finale en peptide de 0,1 à 10 µM. Un contrôle sans microcine a été effectué avec 25 µl de solvant (50 % ACN). Enfin, un contrôle sans bactérie a été effectué dans lequel la culture a été remplacée par 500 µl de milieu LB et la microcine par son solvant.

Les tubes ont été incubés pendant 10 min à 25 °C, puis une solution de phage T5 a été ajoutée pour une concentration finale de $2{,}5 \times 10^{9}$ phages/ml (MOI de 10). Après 15 min à 25 °C, le nombre de phages restant dans le surnageant (non adsorbés) a été compté comme suit : 50 µl d'échantillon ont été dilués dans 500 µl de milieu LB, la solution a été centrifugée pendant 5 min à10 000 *g* puis le surnageant a été dilué 10^{5} fois par du tampon Tris 10 mM, pH 7,4, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$. 50 µl et 100 µl de la dilution ont été étalés sur deux boites de Pétri contenant du LB surmontées de 3 ml de gélose molle contenant 8×10^{5} cfu/ml de *E. coli* F (voir section VII.1.a)). Les boites ont alors été incubées pendant 16 h à 37 °C et les plages de lyse ont été dénombrées pour les différents étalements.

Chapitre I

Mécanisme d’import de MccJ25 dans les bactéries

Une des stratégies décrites dans la littérature pour l'étude du mécanisme d'action des microcines est la recherche de mutants bactériens rendus résistants à l'action de la microcine étudiée. Ainsi, il y a quelques années, la recherche de mutants résistants à MccJ25, a conduit à l'isolement de deux types de mutants.

1. Les mutations les plus fréquemment observées affectaient les gènes codant les protéines membranaires FhuA TonB, ExbB et ExbD (Salomón *et al.* 1993, 1995). Le récepteur membranaire FhuA, appartenant à la famille des récepteurs aux sidérophores de type hydroxamate, est le transporteur du complexe fer/ferrichrome (voir Rappels Bibliographiques). Sa structure tridimensionnelle consiste en un tonneau β obstrué par un domaine globulaire *N*-terminal servant de bouchon (Locher *et al.* 1998). Il est par ailleurs utilisé comme récepteur par différents bactériophages (T1, T5 et Φ80) et par quelques toxines bactériennes (colicine M) ou agents antibiotiques (albomycine) (Letellier et Santamaria 2002). Ces mécanismes requièrent généralement la présence d'un gradient de protons à la membrane interne. L'énergie de la force protomotrice est transférée à la membrane externe par le complexe TonB/ExbB/ExbD, lui-même localisé dans la membrane interne. L'implication de ces protéines dans l'action antibactérienne de MccJ25, corrélée avec une activité antibactérienne très puissante (CMI de l'ordre du nanomolaire), a conduit à proposer que la reconnaissance et/ou l'import de MccJ25 pouvait impliquer le récepteur FhuA.

2. Une mutation rare au niveau du gène codant la sous-unité β' de l'ARN polymérase, *rpoC* a été observée plus récemment (Delgado *et al.* 2001). Elle a permis l'identification de la cible cellulaire de MccJ25. Selon les travaux de Severinov et coll., MccJ25 inhibe l'ARN polymérase en obstruant le canal d'entrée des nucléosides triphosphate (Adelman *et al*. 2004, Mukhopadhyay *et al.* 2004).

Dans l'article présenté ici, nous nous sommes intéressés à l'aspect reconnaissance/import de MccJ25. Sur la base des résultats acquis grâce au premier groupe de mutants, nous avons étudié le rôle de FhuA dans la reconnaissance/l'import de MccJ25. Des études *in vitro* (chromatographie d'exclusion, microcalorimétrie) ont permis de caractériser l'interaction moléculaire directe MccJ25/FhuA. Par ailleurs, nous avons utilisé MccJ25 pour bloquer par compétition les autres fonctions de FhuA, notamment la reconnaissance du phage T5. FhuA est en effet l'unique récepteur du phage T5 sur la membrane externe d'*E. coli*. Lors du processus d'infection, il se produit une liaison irréversible entre le phage T5 et FhuA, qui provoque l'éjection immédiate de l'ADN viral dans les bactéries (Boulanger *et al.* 1996). Ce système lui permet d'utiliser la machinerie bactérienne à son profit et conduit à la lyse des

bactéries. Nous avons ainsi tenté de bloquer l'interaction FhuA/phage T5 à l'aide de concentrations croissantes en microcine dans une série d'expériences *in vitro* et *in vivo*. Finalement, afin d'identifier les régions structurales de MccJ25 importantes pour sa reconnaissance par FhuA, nous avons comparé, dans toutes ces expériences, MccJ25 à son variant obtenu par clivage à la thermolysine, t-MccJ25, dont l'activité antimicrobienne est très fortement réduite, et dans lequel la région en épingle à cheveu β entre les résidus 11 et 16 est déstructurée par le clivage.

I. Publication n°3:

The iron-siderophore transporter FhuA is the receptor for the antimicrobial peptide microcin J25: role of the microcin Val11-Pro16 beta-hairpin region in the recognition mechanism

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The iron–siderophore transporter FhuA is the receptor for the antimicrobial peptide microcin J25: role of the microcin Val[11]–Pro[16] β-hairpin region in the recognition mechanism

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The role of the outer-membrane iron transporter FhuA as a potential receptor for the antimicrobial peptide MccJ25 (microcin J25) 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 demonstrated by antibacterial assays using complementation of an *fhuA*$^-$ strain and by using isogenic strains mutated in genes encoding the protein complex respectively. In addition, MccJ25 was shown to block phage T5 infection of *Escherichia coli*, *in vivo*, by inhibiting phage adhesion, which suggested that MccJ25 prevents the interaction between the phage and its receptor FhuA. 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 K_d of 1.2 μM. Taken together, our 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. Finally, both differential scanning calorimetry and antimicrobial assays showed that MccJ25 binding involves external loops of FhuA. Unlike native MccJ25, a thermolysin-cleaved MccJ25 variant was unable to bind to FhuA and failed to prevent phage T5 infection of *E. coli*. Therefore the Val[11]–Pro[16] β-hairpin region of MccJ25, which is disrupted upon cleavage by thermolysin, is required for microcin recognition.

Key words: antimicrobial peptide, FhuA, iron transporter, microcalorimetry, microcin, phage T5.

INTRODUCTION

Microcins are gene-encoded antimicrobial (poly)peptides secreted by Enterobacteriaceae. Produced under conditions of nutrient depletion, they are active against phylogenetically related microbial strains. Therefore they are considered to play a major role in the regulation of microbial competition within the intestinal flora. MccJ25 (microcin J25) is naturally secreted by the faecal *Escherichia coli* AY25 strain [1]. The 21-residue mature MccJ25 (2.1 kDa) was isolated from culture supernatants [2]. It exhibits a side-chain-to-backbone cyclization involving Glu[8] and the N-terminal glycine, and adopts a lasso-structure in which the C-terminal end of the peptide is threaded into the cyclic backbone [3]. We have shown recently that the two-chain analogue t-MccJ25 (initially called MccJ25-L [2]), obtained by cleavage with thermolysin, retains the lasso-structure of MccJ25 but loses the Val[11]–Pro[16] hairpin-like structure targeted by the enzyme [4] (Figure 1). Interestingly, the highly potent activity of MccJ25 decreases markedly, but is not fully abrogated, upon thermolysin cleavage [5], indicating that the targeted region is required for complete antibacterial activity.

Major progress has been made in characterizing the mechanism of action of MccJ25 in *E. coli*. Indeed, MccJ25 was shown to inhibit transcription by targeting the β' subunit of RNA polymerase [6,7]. The molecular mechanism involves MccJ25 binding and obstruction of the RNA polymerase secondary channel, which in turn prevents the correct positioning of NTP substrates [8,9]. Conversely, few studies have addressed MccJ25 recognition at bacterial membranes. Previous studies have shown that MccJ25-resistant bacteria displayed mutations in the *fhuA*, *tonB* or *sbmA* gene, indicating that the outer-membrane protein FhuA, as well as the inner-membrane proteins TonB and SbmA, are likely to be involved in MccJ25 uptake [10,11].

The *E. coli* outer-membrane protein FhuA (79 kDa) is a high-affinity transporter for iron chelated to the siderophore ferrichrome (for a review, see [12]). FhuA is a multifunctional protein. Indeed, besides its physiological function, FhuA also transports the antibiotics albomycin and rifamycin CGP4832, and serves as a receptor for the bacterial toxin colicin M and for the unrelated phages T1, T5 and Φ80 [13]. Iron–ferrichrome transport across the outer membrane, as well as irreversible binding of phages T1 and Φ80 to the outer membrane, requires an energized cytoplasmic membrane. These functions of FhuA are coupled to the electrochemical gradient of protons via the cytoplasmic membrane-anchored TonB–ExbB–ExbD complex [12,14]. The three-dimensional structure of FhuA reveals a 22-stranded antiparallel β-barrel with an N-terminal globular domain folded inside the barrel. This plug domain spans most of the interior of the barrel and occludes it. The four-stranded β-sheet and the four short helices forming the plug are connected to the β-barrel and to the

Abbreviations used: c.f.u., colony-forming units; DSC, differential scanning calorimetry; ITC, isothermal titration calorimetry; LB, Luria–Bertani; MBC, minimum bactericidal concentration; MccJ25, microcin J25; t-MccJ25, two-chain analogue obtained by cleavage of MccJ25 with thermolysin; MIC, mimimum inhibitory concentration; MOI, multiplicity of infection; p.f.u., plaque-forming units; TFA, trifluoroacetic acid; YO-PRO-1, quinolinium {4-[(3-methyl-2(3H)-benzoxazolylidene)methyl]-1-[3-(trimethylammonio)propyl]}.

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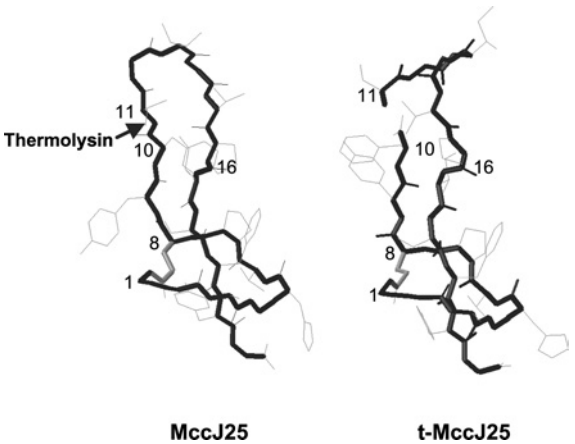

Figure 1 Structural comparison of native MccJ25 (PDB entry 1Q71) and thermolysin-cleaved t-MccJ25 (PDB entry 1S7P)

The Figure illustrates the disruption of the MccJ25 Val^{10}–Pro^{16} β-hairpin upon cleavage by thermolysin. Structures are represented with black backbones and grey side chains using MOLMOL software [38]. Thick covalent bonds were used for the peptide backbones as well as the Glu^{8} side chain engaged in the ring. The Val^{10}–Phe^{11} bond targeted by thermolysin is indicated by an arrow. Selected residues are numbered.

external hydrophilic loops by numerous hydrogen bonds and salt bridges [15]. The binding sites for the different FhuA ligands are located on the external loops of the barrel [16].

The involvement of FhuA in susceptibility to MccJ25 was supported by the observation that a FhuA-deficient *E. coli* strain resistant to MccJ25 was rendered susceptible upon transfection with a plasmid encoding the *E. coli* or *Salmonella enteritidis* Paratyphi FhuA protein, but not when plasmids encoding *S. enteritidis* Typhimurium or *Pantoea agglomerans* (previously *Erwinia herbicola*) FhuA were used [17]. Similarly, when *S. enteritidis* Typhimurium, which is resistant to MccJ25, was transfected with a plasmid encoding the *E. coli* FhuA protein, it became highly susceptible to the microcin [18]. These data strongly suggest that FhuA behaves as a receptor for MccJ25, and that strain susceptibility could vary according to the affinity between MccJ25 and the FhuA protein.

Despite such indirect evidence for a role for FhuA in MccJ25 recognition, previous studies have paid little or no attention to the molecular basis of the recognition mechanism. In the present paper, we have studied the role of FhuA as a receptor for MccJ25, with regard to (i) the conditions for potential MccJ25–FhuA complex formation, (ii) the nature of the regions of MccJ25 and FhuA involved, and (iii) the effect of MccJ25 binding on FhuA-dependent functions. *In vivo*, MccJ25 was used to inhibit FhuA-mediated functions such as phage T5-induced bacterial lysis. *In vitro*, the MccJ25–FhuA molecular interaction was studied and the affinity constant of MccJ25 for FhuA was measured. Finally, the role of the Val^{11}–Pro^{16} β-hairpin region of MccJ25 in the microcin recognition step was evaluated by comparing the inhibitory activity of MccJ25 on FhuA-dependent functions with that of the thermolysin-cleaved t-MccJ25, as well as the affinities of MccJ25 and t-MccJ25 for the FhuA protein.

EXPERIMENTAL

Micro-organisms

Bacterial strains used for MccJ25 and FhuA expression were *E. coli* MC4100 carrying the pTUC202 plasmid [19] (a gift from Professor Felipe Moreno, Hospital Ramón y Cajal, Madrid, Spain) and *E. coli* HO830*fhuA* carrying the pHX405 plasmid [20] respectively. Bioassays were performed with the bacterial strains described previously [21,22], as well as with *S. enterica* Paratyphi SL369 [23], *S. enterica* Typhimurium LT2 [23], *P. agglomerans* K4 [24] (generously provided by Professor Volkmar Braun, University of Tübingen, Germany) and *Pseudomonas aeruginosa* A.T.C.C. 27853 (a gift from Professor Alain Reynaud, Hospital of Nantes, France). Studies on the inhibition of viral adsorption and/or lysis were performed with *E. coli* strains susceptible to phage T5 infection, namely *E. coli* W3110 [25] and *E. coli* F, a fast-adsorbing strain of phage T5 [26]. Stocks of phage T5 [1×10^{12} p.f.u. (plaque-forming units)/ml] were prepared according to [27].

Peptide and protein purification

MccJ25 and FhuA were purified as described previously [2,28] and quantified by amino acid composition analysis as in [21] (MccJ25), or by using the ε_M (103 690 $M^{-1} \cdot cm^{-1}$) deduced from amino acid content (FhuA). Production of ^{3}H-radiolabelled MccJ25 was achieved by growing *E. coli* MC4100 pTUC202 in M63 medium supplemented with a mixture of amino acids (50 μM each) without glycine, containing 1 mg/l thiamine, 0.02 % (w/v) $MgSO_4$, 0.02 % (w/v) glucose, 30 μg/ml chloramphenicol and 0.46 MBq of [^{3}H]glycine (592 GBq/mmol; Amersham Biosciences). After a 16 h incubation at 37 °C, the culture supernatant was subjected to solid-phase extraction on a SepPak C_{18} cartridge (Waters Corp.) pre-equilibrated with 0.1 % (v/v) aqueous TFA (trifluoroacetic acid). After a washing step in 0.1 % TFA, successive elution steps were performed at 25 and 30 % (v/v) acetonitrile in 0.1 % TFA. The 30 % (v/v) acetonitrile fraction containing [^{3}H]MccJ25 was concentrated under vacuum in a SpeedVac concentrator (Savant) and loaded on to a C_{18} μBondapak column (10 μm, 300 mm × 3.9 mm; Waters Corp.) for final purification. Elution was performed with a linear gradient of 0–60 % (v/v) acetonitrile in 0.1 % (v/v) TFA over 30 min at a flow rate of 1 ml/min. Absorbance was monitored at 226 nm and fractions were collected manually. The specific radioactivity of [^{3}H]MccJ25 was determined by radioactivity counting (Pharmacia Wallac 1410 liquid-scintillation counter), and peptide quantification was performed by amino acid composition analysis as described previously [29]. Peptide antibacterial activity was finally controlled according to the protocol described below.

Antibacterial assays

Peptide antibacterial activity was assayed using the liquid growth inhibition assay described previously [21]. Briefly, serial dilutions of MccJ25 or t-MccJ25 (10 μl) were incubated in a 96-well microtitre plate with 90 μl of a mid-exponential phase culture of bacteria at a starting attenuance of 0.001 at 620 nm in Poor-Broth nutrient medium (1 % bactotryptone and 0.5 % NaCl). Bacterial growth was monitored by attenuance measurement at 620 nm in a Ceres 900 (Bio-Tek Instruments) plate recorder after a 20 h incubation at 30 °C. Samples of 100 μl from wells displaying no apparent growth were plated on LB (Luria–Bertani) agar plates and incubated for 16 h at 37 °C in order to detect potential bactericidal effects. The MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) values are expressed as the lowest concentration (μM) that caused 100 % inhibition of growth in liquid medium or on agar plates respectively.

Inhibition of infection with phage T5

A suspension of *E. coli* W3110 at 5×10^8 c.f.u. (colony-forming units)/ml was prepared in fresh LB medium from a culture in the exponential phase of growth. An aliquot of 100 μl of the bacterial

suspension was then incubated for 10 min at room temperature with 10 μl of either MccJ25 or t-MccJ25 dissolved in 10 % (v/v) methanol (final concentration 0.25–10 μM). Phage T5 was added to every well at time zero at an MOI (multiplicity of infection) of 2.5. Plates were then incubated under shaking in a Ceres 900 plate recorder at 37 °C, and bacterial growth and lysis were monitored over 120 min by measurement of the attenuance at 620 nm. A control for bacterial growth was performed in the presence of 0.25–10 μM microcin and in the absence of phage T5.

Inhibition of phage T5 adsorption on to *E. coli*

E. coli F in the exponential phase of growth were diluted to 2.5×10^8 c.f.u./ml in fresh LB medium and incubated further for 10 min at room temperature with MccJ25 or t-MccJ25 (0.1–10 μM), or with the microcin solvent only (50 % acetonitrile) as a control. Phage T5 was added to a final concentration of 2.5×10^9 p.f.u./ml (MOI = 10). After a 15 min incubation, phage adsorbed to the bacteria were eliminated by centrifugation (5 min, 10000 ***g***) and non-adsorbed phage were counted by determination of p.f.u. using a fresh suspension of *E. coli* F as an indicator strain.

Inhibition of phage T5 DNA ejection

FhuA (3 nM) was incubated for 10 min at room temperature with MccJ25 (50 nM–3.1 μM) or t-MccJ25 (3.1 μM) in assay buffer (150 mM NaCl, 1 % octyl glucoside, 25 mM Tris, pH 7.2) containing 4 μM fluorescent DNA intercalant YO-PRO-1 {quinolinium; 4-[(3-methyl-2(3*H*)-benzoxazolylidene)methyl]-1-[3-(trimethylammonio)propyl]; Molecular Probes}. Equal volumes (5 μl) of microcin solvent (50 % methanol) were present in every 800 μl assay. After equilibration of the FhuA/MccJ25 mixture in the spectrofluorimeter (10 min, 37 °C), phage T5 (2.5×10^9 p.f.u./ml) was added and DNA release was monitored over 10 min by fluorescence spectroscopy using a SLM8000 spectrofluorimeter ($\lambda_{excitation}$ 490 nm; $\lambda_{emission}$ 509 nm). The rate of fluorescence increase (V_f), which is directly proportional to the number of phage bound to FhuA in the linear part of the ejection curve, was calculated between 50 and 100 s after the addition of phage T5 [29]. The percentage of adsorbed phage in the presence of microcin (50 nM–3.1 μM) was calculated by comparing the measured V_f with a 100 % reference, determined in the absence of microcin. Data are representative of three independent experiments.

Characterization of the MccJ25–FhuA complex

Analytical size-exclusion chromatography

^{3}H-radiolabelled MccJ25 or t-MccJ25 (4 nmol) resuspended in 10 μl of FhuA buffer (150 mM NaCl, 1 % octyl glucoside, 25 mM Tris, pH 7.2) was incubated for 10 min in the presence of 250 μl of FhuA (4–16 nmol) or FhuA buffer only (control). The sample was then loaded on to a Superose 12 HR 10/30 column (Pharmacia) equilibrated with FhuA buffer. Separation was performed in the same buffer at room temperature at a flow rate of 0.25 ml/min. Absorbance was monitored at 226 nm. Radioactivity in every collected fraction was measured by using a Pharmacia Wallac 1410 liquid-scintillation counter.

For complex stability assays, a [^{3}H]MccJ25–FhuA complex was formed at a ligand/receptor ratio of 1:4 (mol/mol) and separated further from residual [^{3}H]MccJ25 as described above. The fractions containing the [^{3}H]MccJ25–FhuA complex were collected and concentrated by ultrafiltration (4500 ***g***, 45 min, 4 °C) on a MicroSep 10 K Omega spin column (Gelman Lab). The labelled complex was then re-injected on to the Superose 12 HR 10/30 column. Absorbance and radioactivity were monitored as described above.

All separations were performed on a Pharmacia AKTA Basic chromatography system.

Microcalorimetry

Buffers for samples (FhuA, MccJ25 and the MccJ25–FhuA complex) were exchanged by dialysis with 150 mM NaCl, 1 % octyl glucoside and 25 mM sodium phosphate, pH 7.3. Concentrations were adjusted according to the subsequent experiment, and samples were degassed thoroughly prior to recording thermograms. ITC (isothermal titration calorimetry) was performed at 25 °C on a VP-ITC calorimeter (MicroCal). A series of 28 injections of 500 μM MccJ25 (10 μl each) was performed at intervals of 1 min with a computer-controlled 300 μl microsyringe into a 26.5 μM FhuA solution (cell volume = 1.43 ml). A theoretical titration curve was fitted to the experimental data using ORIGIN® software (MicroCal). This software uses the relationship between the heat generated by each injection and ΔH (enthalpy change), K_a (association binding constant), n (number of binding sites per monomer), total protein concentration, and the concentrations of free and total ligand. DSC (differential scanning calorimetry) was carried out on a MicroCal model MC2 at a heating rate of 1 K/min between 20 and 90 °C. Each measurement was preceded by a baseline scan with the buffer. The heat capacity of the solvent was subtracted from that of the protein sample before analysis.

RESULTS

Antibacterial activity of MccJ25 and t-MccJ25

The antibacterial activity of MccJ25 was assayed against a series of Gram-positive and Gram-negative bacteria. Only two Enterobacteriaceae genera known to express the FhuA protein, namely *Escherichia* and *Salmonella*, were found to be susceptible to the microcin, with MIC values in the range $\leqslant$0.02–0.6 μM (Table 1A). The roles of the outer-membrane receptor FhuA as well as the inner-membrane complex TonB–ExbB–ExbD in the mechanism of action of MccJ25 were first investigated by assaying the antibacterial activity of a homogeneous MccJ25 preparation against wild-type and mutant isogenic strains bearing mutations in the *fhuA*, *tonB*, *exbB* and *exbD* genes. Our results showed that *fhuA*$^-$ *E. coli* C600, which is naturally tolerant to MccJ25 (MIC > 10 μM), became highly susceptible to the microcin (MIC $\leqslant$ 0.02 μM) upon transfection with the pHX405 plasmid encoding the *E. coli* FhuA protein (Table 1B). In addition, the loss of TonB or ExbB–ExbD conferred tolerance to *E. coli* W3110 (MIC and MBC > 10 μM), whereas the wild-type strain was susceptible to MccJ25, with MIC and MBC values of 0.6 and 10 μM respectively (Table 1B). Therefore both FhuA and the TonB–ExbB–ExbD complex are required for the antibacterial activity of Mcc25 against *E. coli*.

In order to determine whether the Val^{11}–Pro^{16} β-hairpin region of MccJ25, which is disrupted upon cleavage by thermolysin of the Phe^{10}–Val^{11} bond [4] (Figure 1), is required for the antibacterial activity of the peptide, the MIC and MBC values of MccJ25 were compared with those of its thermolysin-cleaved variant, t-MccJ25. Upon cleavage by thermolysin, MIC values increased at least 15-fold and MBC values shifted over 10 μM, compared with values of 0.04–10 μM respectively for uncleaved MccJ25, for action against the microcin-susceptible bacteria *S. enterica* Paratyphi SL369, *S. enterica* Enteritidis and *E. coli* W3110 (Table 1A). Altogether, these data are indicative of a significant loss of activity of the microcin upon cleavage by thermolysin.

These results prompted us to investigate the role of the thermolysin-targeted region of MccJ25 in the process of recognition by the FhuA receptor. Subsequent *in vivo* assays were consequently

Table 1 Antibacterial activity of MccJ25 and t-Mcc25

(**A**) Spectrum of antibacterial activity; (**B**) antibacterial activity of MccJ25 against wild-type and mutant strains of *E. coli*. MIC values are expressed as the lowest microcin concentration that caused 100 % inhibition in liquid growth-inhibition assays. MBC values are expressed as the lowest microcin concentration for which no activity (c.f.u.) was counted on agar plates. NA, not active in the range 0.02–10 μM.

(A)

	MccJ25 (μM)		t-MccJ25 (μM)	
Bacterium	MIC	MBC	MIC	MBC
Gram-negative bacteria				
Escherichia coli W3110	0.60	10	NA	NA
Enterobacter cloacae	NA	NA	NA	NA
Erwinia carotovora	NA	NA	NA	NA
Klebsiella pneumoniae	NA	NA	NA	NA
Pantoea agglomerans K4	NA	NA	NA	NA
Pseudomonas aeruginosa	NA	NA	NA	NA
Salmonella enterica Enteritidis	0.04	5	1.20	NA
Salmonella enterica Paratyphi SL369	$\leqslant$ 0.02	0.04	0.30	NA
Salmonella enterica Typhimurium LT2	NA	NA	NA	NA
Vibrio harveyi	NA	NA	NA	NA
Gram-positive bacteria				
Aerococcus viridans	NA	NA	NA	NA
Bacillus megaterium	NA	NA	NA	NA
Staphylococcus aureus	NA	NA	NA	NA

(B)

	MccJ25 (μM)	
Strain	MIC	MBC
W3110	0.60	10
W3110 KP1344 (*tonB*$^-$)	NA	NA
W3110-6 (*exbBD*$^-$)	NA	NA
C600 (*fhuA*$^-$)	NA	NA
C600 (*fhuA*$^-$) pHX405	$\leqslant$ 0.02	1.20

designed to characterize MccJ25 recognition without considering its intracellular mechanism of action.

Inhibition of the bacterial lysis by phage T5

Infection of *E. coli* by phage T5 is initiated by the irreversible binding of the phage to FhuA. We therefore investigated whether MccJ25 interferes with phage T5 infection. The phage-induced lysis of *E. coli* F (a wild-type strain for FhuA and TonB–ExbB–ExbD) was measured in the presence of MccJ25. Control experiments showed that MccJ25 or t-MccJ25 alone had little or no effect on *E. coli* F growth at concentrations ranging from 0.5 to 10 μM (results not shown). In the absence of MccJ25, bacterial lysis occurred 50 min after phage T5 addition (Figure 2A). However, addition of MccJ25 prior to phage incubation resulted in a dose-dependent inhibition of lysis. Bacterial growth started to be restored by 0.5 μM MccJ25, and was similar to that observed in the absence of phage T5 when 10 μM MccJ25 was added (Figure 2A). Therefore 10 μM MccJ25 fully inhibited the phage-induced lysis of *E. coli* F. In contrast, bacterial growth could not be restored upon addition of t-MccJ25 over the same range of concentrations (0.5–10 μM) (Figure 2B).

Inhibition of phage T5 adsorption to *E. coli* F

In order to determine whether the MccJ25-mediated inhibition of phage infection was due to inhibition of the interaction between the phage and its receptor FhuA, or to a later event, we measured phage adsorption to *E. coli* F in the presence of MccJ25 or

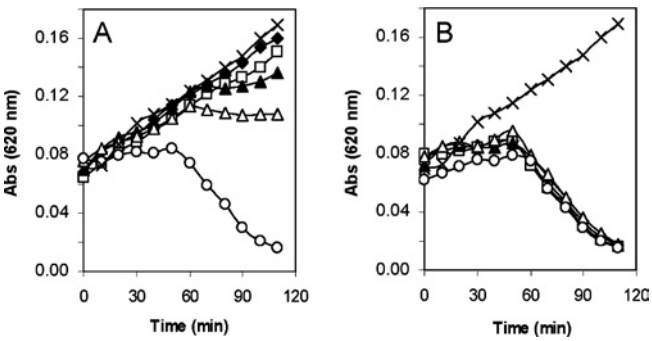

Figure 2 Inhibitory effects of Mcc25 and t-MccJ25 on infection by phage T5

E. coli F was incubated with MccJ25 (**A**) or t-MccJ25 (**B**) at concentrations of 0.5 μM (△), 1 μM (▲), 5 μM (□) and 10 μM (◆), or with solvent only (○). After addition of phage T5, the culture turbidity (Abs) was monitored over 120 min at 620 nm. A control was performed in the absence of phage T5 (×). Data are representative of four independent experiments.

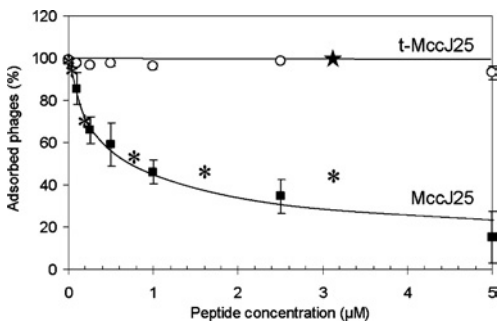

Figure 3 Inhibition of phage T5 adsorption

Phage adsorption was first measured *in vivo* by incubating *E. coli* W3110 for 10 min with 0.1–10 μM MccJ25 (■) or t-MccJ25 (○) before addition of phage T5. The amount of adsorbed phage was determined after a 15 min incubation with phage T5 at MOI = 10. Results are expressed as means ± S.E.M. for three independent experiments. *In vitro*, phage adsorption to FhuA was evaluated by measuring the microcin-induced inhibition of DNA ejection. FhuA (3 nM) was incubated for 10 min with 0.1, 0.2, 0.8, 1.6 or 3.1 μM MccJ25 (∗) or solvent only in the presence of the fluorescent probe YO-PRO-1. The reaction was initiated by phage T5 addition. The inhibitory activity of 3.1 μM t-MccJ25 (black star) was compared with that of MccJ25 at the same concentration. The fluorescence signal indicative of phage DNA release was measured over 10 min ($\lambda_{excitation}$ 490 nm; $\lambda_{emission}$ 509 nm). The rate of fluorescence increase (V_f), which is directly proportional to the number of phage bound to FhuA, was measured in order to calculate the percentage of adsorbed phage. Data are representative of three independent experiments.

t-MccJ25. Upon addition of increasing concentrations of MccJ25 to the bacteria prior to phage T5, the number of adsorbed phage decreased from 80 % at 0.1 μM MccJ25 to 15 % at 5 μM MccJ25 (Figure 3). Conversely, t-MccJ25 did not inhibit phage adhesion over the same range of concentrations (Figure 3).

In vitro inhibition of DNA release induced by binding of phage T5 to FhuA

In order to assess whether the inhibition of phage adhesion to *E. coli* was due solely to binding of MccJ25 to FhuA, the FhuA–phage T5 interaction was monitored *in vitro* after pre-incubation of FhuA with MccJ25. Previous experiments [29] have shown that phage T5 ejects its DNA upon binding to purified FhuA, and DNA release can be measured using the fluorescent DNA intercalant YO-PRO-1. Addition of increasing concentrations of MccJ25 to FhuA prior to incubation with phage T5 resulted in a concomitant decrease in YO-PRO-1 fluorescence (results not shown). From the

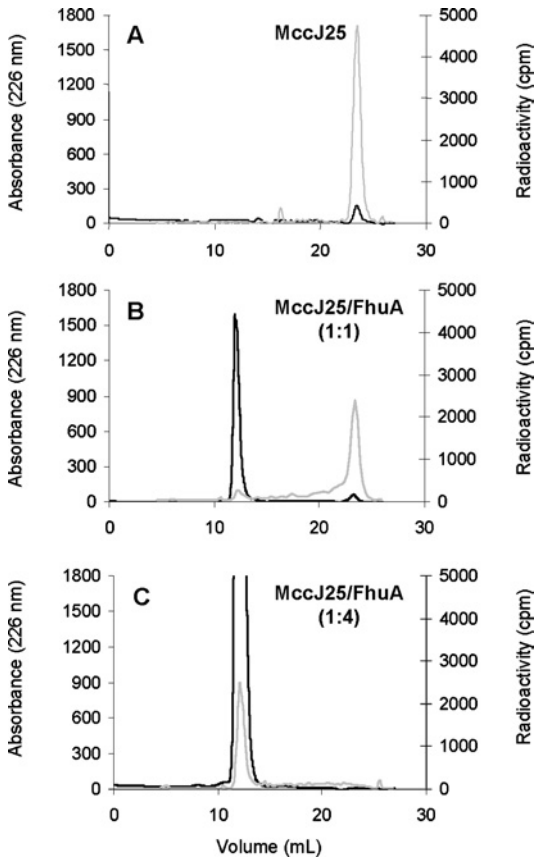

Figure 4 MccJ25–FhuA interaction *in vitro*

^{3}H-labelled MccJ25 (4 nmol) was incubated for 10 min in the presence of 4 nmol (**B**) or 16 nmol (**C**) of FhuA. In a control experiment (**A**), incubation was performed in FhuA buffer only. Samples were analysed by gel-permeation chromatography on a Superose 12 HR 10/30 column. Absorbance was monitored at 226 nm (black line). Radioactivity was measured in every collected fraction by liquid scintillation counting (grey line).

V_f measurements, we calculated that the proportion of adsorbed phage decreased from 100 to 45 % when increasing the MccJ25 concentration from 0.1 to 3.2 μM (Figure 3). These values are in good agreement with the *in vivo* inhibition data (Figure 3). Finally, as shown *in vivo*, addition of t-MccJ25 had no detectable effect on T5 DNA ejection (Figure 3). Taken together, these data suggest that MccJ25 competes with phage T5 for binding to FhuA both *in vivo* and *in vitro*.

In vitro MccJ25–FhuA complex formation

The putative binding of MccJ25 to FhuA was investigated by gel filtration. Purified FhuA (79 kDa) and MccJ25 (2 kDa) eluted as well separated peaks from a Superose 12 column, with retention volumes of 12.0 ml and 23.2 ml respectively (Figures 4A and 4B). Owing to the low molecular mass of MccJ25 compared with that of FhuA, isolated FhuA could not be separated from a putative MccJ25–FhuA complex by gel filtration. Therefore ^{3}H-labelled MccJ25 was used instead of unlabelled MccJ25, and the radioactivity associated with the microcin was measured in the collected fractions. [^{3}H]MccJ25 and FhuA were mixed at molar ratios ranging from 1:1 to 1:4 and incubated prior to gel filtration analysis. The radioactivity initially associated with the [^{3}H]-MccJ25 peak shifted to the FhuA fraction, in which it was fully recovered at a 1:4 [^{3}H]MccJ25/FhuA molar ratio (Figures 4B and 4C). This suggested that [^{3}H]MccJ25 and FhuA formed a complex, and that all of the microcin was associated with FhuA when the receptor was in excess. The strength of the interaction was evaluated by reloading the [^{3}H]MccJ25–FhuA fractions on to the same column after concentration by ultrafiltration. The radioactivity again eluted as a single peak corresponding to the FhuA elution volume, without any significant release of radioactivity at the microcin elution volume (results not shown).

In order to determine whether the lack of activity of t-MccJ25 in the previous assays (antibacterial activity and inhibition of FhuA-dependent functions) could be due to a lack of affinity of the thermolysin-cleaved microcin for the FhuA receptor, we performed the same complex formation assays by incubating t-MccJ25 with FhuA in a 1:4 molar ratio. Unlike MccJ25, t-MccJ25 was unable to bind to FhuA, as indicated by equal absorbance of the t-MccJ25 peak in the presence or in the absence of FhuA (results not shown).

Affinity constant for binding of MccJ25 to FhuA

The MccJ25–FhuA interaction was characterized further using ITC, a powerful technique for determining the affinity, stoichiometry and thermodynamic parameters of receptor–ligand interactions. The titration of FhuA with MccJ25 (described in the Experimental section) was exothermic, with $\Delta H = 14$ kJ/mol (Figure 5A). The best fit to the experimental data was obtained for a binding curve corresponding to a one-class binding site model and a stoichiometry of 1.9, consistent with the binding of two microcins per FhuA (Figure 5A). The threshold at which no further heat was generated upon microcin addition was reached at MccJ25/FhuA molar ratios above 3:1. In agreement with the size-exclusion chromatography data, this indicates that a 3–4-fold molar excess of either the ligand or the receptor shifts the reaction towards complex formation. From the ITC data analysis, this affinity was characterized by a K_d value of 1.2 μM.

To analyse further the effect of binding of MccJ25 to FhuA, thermal denaturation of the complex was compared by DSC with that of FhuA alone. FhuA displays two well resolved thermal transitions, corresponding to the unfolding of the loops and plug, and to the unfolding of the β-barrel [20,28]. Under our experimental conditions, these were observed at 63 and 70 °C respectively (Figure 5B). Interestingly, DSC of the MccJ25–FhuA complex formed under conditions of complete saturation by MccJ25 (80 μM MccJ25 and 20 μM FhuA) showed a higher ΔH for the denaturation of the first domain, which corresponds to the loops and plug (706 kJ/mol, compared with 356 kJ/mol for FhuA alone, as calculated from Figure 5B). The unfolding of the second domain remained almost unchanged (Figure 5B). A fall in ΔCp (excess calorimatric capacity) was observed with both the FhuA and MccJ25–FhuA denaturation curves at 72–73 °C due to heat-induced aggregation. The lack of a signal for unbound MccJ25 is most probably related to its remarkable temperature stability [5]. This was confirmed in a control experiment in which MccJ25 alone gave no enthalpy signal (results not shown).

DISCUSSION

The present study was performed using *in vivo* and *in vitro* quantitative approaches to demonstrate the role of FhuA in the antibacterial activity of MccJ25 and its recognition at the outer membrane of target bacteria.

MccJ25 antimicrobial activity was found to be limited to a few species of Enterobacteriaceae, which were affected by the microcin in the nanomolar range. Unlike most antimicrobial peptides that display low specificity and MIC values in the range 0.1–20 μM associated with a membrane-permeabilizing activity

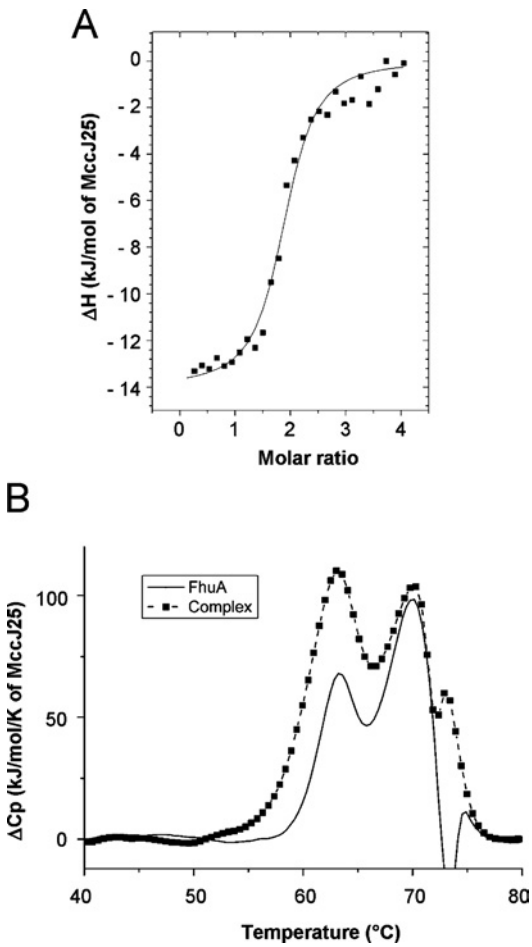

Figure 5 Microcalorimetry analysis

(**A**) Microcalorimetric titration isotherm for the binding of MccJ25 to FhuA. Data were obtained at 25 °C using an automated sequence of 28 injections of 500 μM MccJ25 from a 300 μl syringe into a reaction cell containing 26.5 μM FhuA. The volume of each injection was 10 μl, and injections were made at 1 min intervals. Raw data were treated with ORIGIN® software and the values are plotted against molar ratio. Each point corresponds to the heat generated by the reaction upon each injection. The curve fit to the data (solid line) obtained by the ORIGIN® software yields values for K_d and stoichiometry (see the Experimental section). (**B**) DSC endotherm of the MccJ25–FhuA complex. Baseline-corrected DSC thermograms of FhuA alone (13 μM) (solid line) and the MccJ25–FhuA complex formed by complete saturation of FhuA with MccJ25 (broken line) were recorded in the same buffer as for ITC experiments with a heating rate of 1 K/min.

[30–32], the narrow spectrum of activity and the high potency of MccJ25 are reminiscent of a receptor-mediated mechanism of action. Interestingly, in our antibacterial assays, only four bacterial species belonging to the *Escherichia* and *Salmonella* genera, known to express FhuA, were MccJ25-susceptible. In agreement with previous studies [11], our results with bacterial mutants confirmed that FhuA expression is required for susceptibilty to MccJ25. Indeed, the MccJ25-resistant *E. coli* C600 (*fhuA*$^-$) became highly susceptible (MIC $\leqslant$ 0.02 μM) upon transfection with the FhuA-encoding plasmid pHX405. In addition, we showed that mutations in genes encoding proteins of the TonB–ExbB–ExbD complex also resulted in a complete loss of susceptibility to MccJ25, as revealed by a shift in MIC from 0.6 μM against the wild-type *E. coli* W3110 strain to above 10 μM against the *tonB*$^-$ and *exbBD*$^-$ isogenic strains. This result is in agreement with the isolation of *tonB* mutants of *E. coli* resistant to MccJ25 [10]. The double dependence on FhuA and the TonB complex supports the hypothesis that MccJ25 first binds to the receptor FhuA and then requires the TonB–ExbB–ExbD complex for its uptake into the target bacterium.

Whereas in earlier studies various stable ligand–FhuA complexes could be isolated, providing both thermodynamic and physicochemical parameters of the interaction and structural characterization of the complex [15], no evidence of MccJ25–FhuA complex formation had been reported. The availability of highly pure solutions of MccJ25 and FhuA allowed us to perform both *in vivo* and *in vitro* assays, which permitted us to characterize the interaction between the microcin and its putative receptor independent of any other step of the antibacterial mechanism, such as the inhibition of the host RNA polymerase [6,7] that occurs after the recognition/binding and transport steps. MccJ25 was used to inhibit the well described recognition of phage T5 by FhuA. We demonstrated that, *in vivo*, MccJ25 inhibited phage infection and subsequent bacterial lysis in a dose-dependent manner, and that full inhibition was achieved with 10 μM MccJ25. In addition, pre-incubation of *E. coli* with increasing MccJ25 concentrations (0–10 μM) resulted in decreasing amounts of adsorbed phage. It is therefore very likely that blocking of phage infection results from inhibition of the irreversible binding of phage T5 to FhuA. Interestingly, similar inhibition of phage T5 adsorption was observed *in vitro*. Indeed, MccJ25 was able to inhibit the release of phage T5 DNA induced by the irreversible binding of phage T5 to FhuA that had been previously solubilized and purified in the presence of detergent, and thus deprived of any other bacterial components. Remarkably, *in vitro* and *in vivo* data matched perfectly, as indicated by very similar inhibition values with increasing MccJ25 concentration. Therefore, *in vitro* data can be considered to be reliable for the description of the MccJ25–FhuA interaction *in vivo*. Altogether, these results demonstrate that MccJ25-induced inhibition of phage adhesion results from direct interaction of MccJ25 with the FhuA receptor.

Evidence for actual binding of MccJ25 to FhuA was first obtained by size-exclusion chromatography. In the presence of excess FhuA, ^{3}H-labelled MccJ25 eluted in the FhuA fraction (79 kDa) instead of in the original MccJ25 fraction (2 kDa). The complex was found to be stable, since no detectable MccJ25 release was observed upon re-injection of the complex on to the column. ITC confirmed this complex formation, and gave a MccJ25–FhuA dissociation constant of 1.2 μM. Similar affinities were measured for interactions between colicins (larger-size bacterial toxins) and their outer-membrane receptors. Thus, *in vitro*, ITC gave a K_d value of 2 μM for binding of colicin N to OmpF [33], while *in vivo*, colicins B and D were shown to bind to the enterobactin receptor FepA with K_d values of 0.185 and 0.560 μM respectively [34]. In addition, ITC data indicated that, *in vitro*, MccJ25 binds to one single class of sites, with a stoichiometry consistent with two ligands per receptor ($n = 1.9$). By comparing the thermal denaturation profile of the MccJ25–FhuA complex with that of FhuA, which presents two transitions as characterized previously [20,28], we showed that MccJ25 binding affected only the external loops and plug (first domain) of FhuA, and not the β-barrel (second domain). Indeed, unfolding of the first domain required a much higher enthalpy when MccJ25 was associated with FhuA, indicating that stabilization of the first domain results from binding of MccJ25 to FhuA.

Since MccJ25 is not likely to bind to the plug buried within the β-barrel, our results strongly suggest that binding occurs through the external loops accessible to the various ligands of FhuA. This hypothesis is in agreement with recent findings on the role of these external loops in the transport and receptor functions of FhuA: analysis of the phenotype of *E. coli* mutants expressing different FhuA proteins lacking one of the 11 external loops showed that

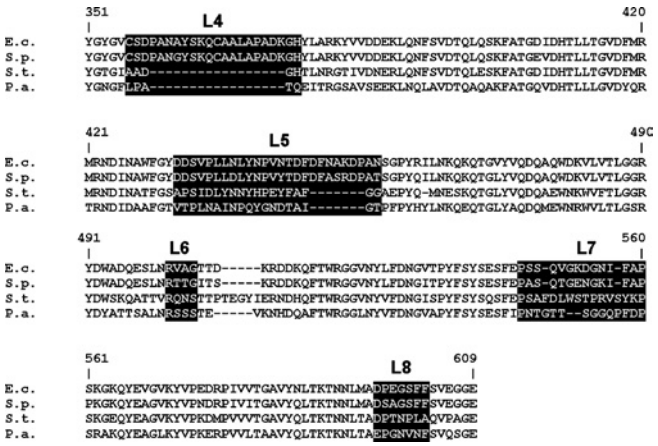

Figure 6 Partial alignment of FhuA amino acid sequences from MccJ25-susceptible (E.c., S.p.) and MccJ25-tolerant (S.t., P.a.) strains

Alignment was performed using the Multalign software [39]. Extracellular loops L4–L8 of the *E. coli* FhuA protein [12] are in black boxes, together with the corresponding sequence in other Enterobacteriaceae. Abbreviations and sources for the sequences are: E.c, *E. coli* K12 W3110 (Swiss-Prot; accession no. P0671); S.p., *S. enterica* Paratyphi SL369 (TrEMBL; accession no O86903); S.t., *S. enterica* Typhimurium LT2 (TrEMBL; accession no O86925); P.a., *P. agglomerans* K4 (TrEMBL; accession no O86924).

loops L5, L7, L8 and L11 are essential for MccJ25 activity, and that loop L8 can be considered as the major and maybe the single binding site for phage T5 [16]. Together with this finding, the ability of MccJ25 to prevent the FhuA–phage T5 interaction in our *in vivo* and *in vitro* assays suggests that MccJ25 and phage T5 bind to the same loop L8 on the FhuA receptor. A major role for the external loops in MccJ25 binding is also supported by a comparative analysis of the sequences of the FhuA proteins originating from both MccJ25-tolerant (*S. enterica* Typhimurium LT2 and *P. agglomerans*) and MccJ25-susceptible (*E. coli* W3110 and *S. enterica* Paratyphi SL369) bacteria (Figure 6). The overall sequence of FhuA is highly conserved among both susceptible and tolerant strains [35], with the exception of the external loops L4–L8. Indeed, the FhuA sequences from tolerant strains show two conserved deletions of 17 and seven amino acids within loops L4 and L5 respectively, as well as significant divergence within loops L7 and L8, as compared with the FhuA sequences from susceptible strains (Figure 6). Altogether, these results suggest that, by modifying the conformation of FhuA loops L5, L7 and/or L8, bacteria could prevent MccJ25 binding and therefore gain resistance to the microcin.

In all of the experiments discussed above, MccJ25 was compared with its thermolysin-cleaved variant t-MccJ25. This latter peptide failed to inhibit FhuA binding properties and did not bind to FhuA under the conditions used for the native peptide. This indicates that binding of MccJ25 to FhuA is specific and does not result artifactually from biophysical characteristics such as hydrophobicity.

The weak antibacterial activity of t-MccJ25 is indicative of a critical role for the Val^{11}–Pro^{16} β-hairpin in the antibacterial activity of MccJ25. Indeed, the three-dimensional structure of the entire region is lost upon cleavage by thermolysin [4]. Recent studies have shown that this region is not required for inhibition of bacterial RNA polymerase [36,37] but did not investigate its potential role in MccJ25 recognition. We show here that the MccJ25–FhuA molecular interaction cannot be observed when the thermolysin-cleaved variant t-MccJ25 is used, indicating that cleavage of MccJ25 induces a major loss of affinity for the FhuA receptor. Moreover, unlike with MccJ25, no inhibitory effect of t-MccJ25 was observed on phage T5 adhesion, DNA ejection and subsequent bacterial lysis. In summary, the present study provides the first evidence for a critical role for the Val^{11}–Pro^{16} region for MccJ25 recognition at the bacterial membrane. It is concluded that the MccJ25 threaded cyclic backbone, the structure of which is retained upon cleavage by thermolysin [4], should play a major role in the RNA polymerase inhibitory activity of the microcin.

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II. Synthèse des résultats

➢ Dans un premier temps, nous avons réalisé un spectre d'activité de MccJ25 et t-MccJ25, ainsi que des mesures de CMI et CMB sur différentes souches, sauvage d'une part, mutées sur les gènes codant FhuA, TonB, ExbB et ExbD d'autre part. Nous avons ainsi pu vérifier que ces protéines étaient nécessaires à l'activité de MccJ25. De plus, **le clivage à la thermolysine induit une chute importante de l'activité antibactérienne**, puisque les CMI mesurées pour t-MccJ25 sont 15 à 100 fois supérieures à celles mesurées pour MccJ25.

➢ Nous avons ensuite voulu déterminer si l'interaction MccJ25/FhuA présumée pouvait interférer avec la fonction de FhuA en tant que récepteur du phage T5. Pour cela, un essai d'inhibition de la lyse bactérienne a été réalisé. La MOI du phage T5 permettant d'induire la lyse de la souche *E. coli* W3110 a été préalablement déterminée : elle est de **2,5 phages T5 par bactérie *E. coli* W3110**. Des bactéries *E. coli* W3110 ont ensuite été incubées avec des concentrations croissantes de MccJ25 ou t-MccJ25, le phage T5 a été ajouté (MOI 10). Le suivi de la turbidité de la suspension bactérienne a permis d'observer que la lyse bactérienne, habituellement induite par le phage T5 au bout de 50 minutes, diminue jusqu'à disparaître quand la concentration en MccJ25, mais pas en t-MccJ25, augmente dans le milieu. Ainsi, **MccJ25 est capable d'inhiber l'infection virale d'*E. coli* par le phage T5 de manière dose-dépendante.** Dans les mêmes conditions, **t-MccJ25 n'a pas cette capacité.**

➢ Une mesure de l'adsorption du phage T5 à la membrane d'*E. coli* en présence et en absence de MccJ25 ou t-MccJ25 a été réalisée par dénombrement des plages de lyse induites par les phages T5 non adsorbés. Nous avons montré que la pré-incubation des bactéries avec des concentrations croissantes en MccJ25 (doses sub-létales) corrèle avec une augmentation du nombre de phages non adsorbés sur les bactéries. t-MccJ25 est sans effet sur ce phénomène. Ceci indique clairement que l'effet inhibiteur de MccJ25 sur l'infection par le phage T5 est dû à une inhibition de l'interaction bactérie/phage. FhuA étant l'unique récepteur du phage T5 sur la membrane externe, nous pouvons conclure que **MccJ25, mais pas t-MccJ25, inhibe *in vivo* l'interaction entre FhuA et le phage T5**.

➢ Enfin, nous avons comparé l'inhibition de l'adsorption *in vivo* à celle observée *in vitro*. Dans ce but, nous avons comparé l'éjection de l'ADN du phage T5, induite par sa liaison irréversible au récepteur FhuA, en présence et en absence de microcine. En accord avec nos résultats *in vivo*, nous avons observé *in vitro* que MccJ25 inhibe le phénomène de

l'éjection d'ADN de manière dose dépendante, alors que t-MccJ25 est sans effet. Une bonne corrélation entre le pourcentage de phages adsorbés sur leur récepteur et la concentration de MccJ25 utilisée pour bloquer l'interaction phage T5/FhuA est observée. **Les données *in vitro* correspondent donc à une réalité biologique *in vivo* et permettent bien de décrire l'interaction MccJ25/FhuA**.

Afin d'étudier plus en détail le mécanisme de reconnaissance de MccJ25 par FhuA, nous avons étudié, *in vitro*, l'interaction moléculaire entre FhuA et MccJ25.

- Etant donnée la faible masse moléculaire de MccJ25 (2,1 kDa) par rapport à celle de FhuA (79 kDa), la liaison de MccJ25 à FhuA ne pouvait pas être visualisée par simple chromatographie d'exclusion, la technique ne permettant pas de différencier FhuA du complexe MccJ25/FhuA. Nous avons donc produit de la microcine marquée au tritium, [^{3}H]MccJ25, pour réaliser ces expériences. Nous avons ainsi pu montrer le déplacement de la radioactivité depuis la zone d'élution de MccJ25 vers la zone d'élution du complexe MccJ25/FhuA, identique à celle de FhuA, lors d'une pré-incubation de MccJ25 avec FhuA. Ce phénomène n'a pas été observé avec t-MccJ25. Ainsi, **MccJ25 se lie à FhuA *in vitro*, pour former un complexe MccJ25/FhuA**. **t-MccJ25, dans laquelle la boucle en épingle à cheveu β est détruite, n'a pas cette capacité de liaison**.

- Les paramètres régissant l'interaction MccJ25/FhuA ont été déterminés par ITC. Ainsi, **MccJ25 se lie à FhuA avec une stœchiométrie MccJ25/FhuA de 2 :1, et une constante d'affinité de 1,2 µM**. Par ailleurs, des expériences de DSC ont permis d'identifier le site de liaison de MccJ25 à FhuA. En effet, la dénaturation thermique de FhuA présente deux transitions : d'abord le bouchon et les boucles extracellulaires, puis à plus haute température, le tonneau β. La stabilisation de la première transition en présence de MccJ25 indique que seule la dénaturation des boucles et du bouchon de FhuA est influencée par la complexation entre MccJ25 et FhuA. La stabilisation observée suggère que **MccJ25 se lie à FhuA via les boucles externes** du récepteur. Cette hypothèse est en accord avec les différences de résistance à MccJ25 observées notamment pour différents serovars de *Salmonella enterica* dont la séquence de FhuA diffère au niveau des boucles.

En conclusion, FhuA est bien le récepteur de MccJ25 sur la membrane externe des bactéries et la région en épingle à cheveu β comprise entre les résidus Val11 et Pro16 est nécessaire pour cette étape de reconnaissance.

Chapitre II

Etude de la voie de maturation de MccJ25

Seuls quelques rares peptides, essentiellement isolés de *Streptomyces*, présentent une structure tridimensionnelle en lasso, semblable à celle de MccJ25 (Frechet *et al.* 1994, Katahira *et al.* 1995, Katahira *et al.* 1996, Iwatsu *et al.* 2006). Cette structure est à l'origine de la stabilité hors du commun de ces peptides (Morishita *et al.* 1994, Blond *et al.* 2002), et de leur activité biologique (Yano *et al.* 1996, Adelman *et al.* 2004).

Ainsi, l'identification et la caractérisation des enzymes responsables de la structuration de tels peptides pourraient représenter une avancée importante en vue, d'une part, de stabiliser des substances bioactives caractérisées mais peu stables, et d'autre part, de mettre au point de nouveaux agents thérapeutiques. De plus, la compréhension des mécanismes mis en jeu par *Escherichia* lors de la biosynthèse de MccJ25 pourrait permettre la recherche d'une voie de synthèse peptidique parallèle chez les *Actinomycetales* et les *Enterobacteriales*.

A ce jour, les mécanismes mis en jeu et les différents effecteurs permettant de générer des peptides structurés en lasso restent inconnus. A ce jour, parmi les peptides en lasso, seul le système génétique de MccJ25, porté par un plasmide, a été séquencé et caractérisé (Solbiati *et al.* 1996, 1999). Même si l'implication de protéines bactériennes chromosomiques dans la biosynthèse de MccJ25 ne peut être écartée, ces données constituent un atout majeur pour l'étude de sa voie de maturation. Ce système comprend quatre gènes, *mcjA*, codant pour un précurseur linéaire de 58 acides aminés, et *mcjD,* codant une protéine d'immunité/export ont été identifiés (Solbiati *et al.* 1996, 1999). Toutefois, au sein du système génétique, deux gènes nécessaires à la production de MccJ25, *mcjB* et *mcjC*, ont été identifiés et codent, selon toute vraisemblance, les enzymes de maturation de MccJ25.

Afin d'isoler et de caractériser les enzymes responsables de la maturation de MccJ25 (clivage du précurseur McjA et cyclisation du peptide résultant), nous nous sommes proposés d'associer approches génétique et biochimique. Dans un premier temps, nous avons inactivé les gènes *mcjB*, puis *mcjC* et réalisé des tests de complémentation homologue afin d'identifier le rôle de ces deux gènes dans le processus de maturation de MccJ25. McjA, le précurseur de MccJ25, et les enzymes de maturation potentielles McjB et McjC ont ensuite été produites de manière recombinante chez *E. coli*, et la voie de biosynthèse de MccJ25 a été reconstituée *in vitro*. L'étude des similarités que présentent McjB et McjC avec les enzymes d'autres espèces bactériennes permet de proposer un rôle pour McjB et McjC dans le processus de maturation de MccJ25.

I. Publication n°4:

Two enzymes catalyze the maturation of a lasso peptide in *Escherichia coli*

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Two Enzymes Catalyze the Maturation of a Lasso Peptide in *Escherichia coli*

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SUMMARY

Microcin J25 (MccJ25) is a gene-encoded lasso peptide secreted by *Escherichia coli* which exerts a potent antibacterial activity by blocking RNA polymerase. Here we demonstrate that McjB and McjC, encoded by genes in the MccJ25 gene cluster, catalyze the maturation of MccJ25. Requirement for both McjB and McjC was shown by gene inactivation and complementation assays. Furthermore, 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 possess proteolytic activity, whereas McjC would be the ATP/Mg^{2+}-dependent enzyme responsible for the formation of the Gly1-Glu8 amide bond. Finally, we show that putative lasso peptides are widespread among Proteobacteria and Actinobacteria.

INTRODUCTION

Many molecules from bacteria endowed with a broad spectrum of biological activities are biosynthesized through a cyclization process [1]. Lasso peptides [2] are 16–21 residue naturally occurring peptides that result from both cyclization and acquisition of a typical and complex structure [2–7]. A side chain to backbone cyclization leads to an amide bond between the amino group of an N-terminal Gly/Cys residue and the carboxyl group of a Glu/Asp residue at position 8 or 9. The C-terminal tail of the peptide is irreversibly trapped in the ring, thus forming a lasso. Lasso peptides are classified according to the occurrence or not of disulfide bonds [8]. Class I is formed by peptides that contain four cysteine residues at conserved positions [2, 4], whereas class II is formed by peptides devoid of cysteine, in which position 1 is always occupied by a glycine [3, 5–8]. Most lasso peptides have been isolated from Actinobacteria and were shown to be enzyme inhibitors [2–6]. Recently, a *Rhodococcus* strain was shown to produce class II lasso peptides with antimycobacterial properties [7]. However, little is known about the enzymes involved in the biosynthesis of lasso peptides.

Microcin J25 (MccJ25) is a typical class II lasso peptide [9–11]. It is a gene-encoded antibacterial peptide secreted by *Escherichia coli* AY25 [12, 13] which uses the iron-siderophore receptor FhuA to enter bacteria [14] and inhibits RNA polymerase [15]. The ring part of the MccJ25 lasso structure (Figure 1) results from an amide bond between Gly1 and Glu8, and the C-terminal tail is sterically blocked in the ring by the two bulky aromatic side chains from Phe19 and Tyr20 on each side of the ring [9], thus creating a β hairpin region over the ring. Cyclization and folding into the particularly compact lasso structure provide MccJ25 with exceptional resistance to high temperatures, proteases, and chaotropic agents [16]. They also define the functional regions of the molecule implicated in recognition by the receptor FhuA at the outer membrane of bacteria [14] and inhibition of transcription by targeting the RNA polymerase β′ subunit [15]. These two properties are conferred by the Val11-Pro16 β hairpin [14] and the ring tail [17] of the MccJ25 lasso structure, respectively.

A major advantage provided by MccJ25 for studying the enzymes able to generate lasso peptides is that the genetic determinants required for its production are known. The *mcjABCD* gene cluster required for MccJ25 biosynthesis has been sequenced entirely [18] (Figure 1). The gene *mcjA* encodes the linear 58 residue precursor of MccJ25, whereas *mcjD* encodes an ATP-binding cassette (ABC) transporter involved both in the export of MccJ25 and in the self-protection of the producing strain against the deleterious effects of its microcin. The last two genes, *mcjB* and *mcjC*, encode proteins of unknown functions. However, they are necessary for the production of active MccJ25 and have been proposed to encode the enzymes converting McjA into MccJ25 [19]. This process comprises 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 microcin (Figure 1).

In this study, we analyzed the effect of *mcjB* and *mcjC* inactivation/complementation on the production of

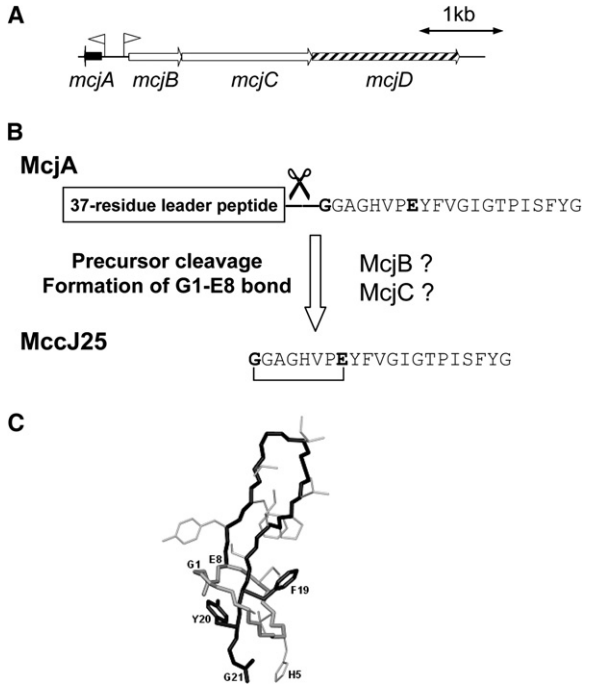


Figure 1. Biosynthesis of MccJ25

(A) Organization of the MccJ25 gene cluster. The genes *mcjA* (black arrow), *mcjB* and *mcjC* (white arrows), and *mcjD* (hatched arrow) encode the MccJ25 precursor McjA, putative maturation enzymes, and the immunity/export protein, respectively. The promoters are indicated by flags.

(B) MccJ25 maturation. McjA is subject to proteolytic cleavage and formation of an amide bond between the amino group of Gly1 and the carboxyl side chain of Glu8.

(C) Three-dimensional structure of MccJ25 (Protein Data Bank code 1Q71).

MccJ25. Furthermore, we cloned and overexpressed McjA, the precursor of MccJ25, as well as McjB and McjC, so as to examine their role in MccJ25 maturation.

RESULTS

McjC Is Expressed as a 513 Residue Protein

The genes *mcjA*, *mcjB*, and *mcjC* were amplified by polymerase chain reaction (PCR) from pTUC202 for cloning into expression vectors. Surprisingly, PCR fragments obtained for *mcjC* systematically displayed a deletion of three bases in the gene encountered in the sequence published for pTUC100, the vector from which the MccJ25 gene cluster was originally sequenced (EMBL accession number AF061787). Following the sequencing of both strands of pTUC202, which derives from pTUC100 [19], it was found that the stop codon located 1327 bases after the initiation codon in the *mcjC* sequence was erroneous (see Figure S1 in the Supplemental Data available with this article online) and was actually displaced 213 bases further (GenBank accession number AM116873). From the sequence data, *mcjC* is separated from *mcjD* by two bases only, and encodes a 513 residue protein (instead of 442) with a molecular mass of 58.7 kDa.

McjB and McjC Are Required for the Production of MccJ25

In order to determine whether *mcjB* and *mcjC* are required for MccJ25 maturation, each gene was inactivated by insertion of a stop codon-containing oligonucleotide duplex at a chosen site in pTUC202 (see Figure S2). The resulting plasmids encoding truncated McjB (127 instead of 208 amino acids) and McjC (52 instead of 513 amino acids) were named pTUC202B and pTUC202C, respectively. Culture supernatants from *E. coli* MC4100 harboring pTUC202, pTUC202B, or pTUC202C plasmids were analyzed for the presence of MccJ25. Whereas the control strain harboring pTUC202 displayed a strong antibacterial activity specifically directed against the MccJ25-susceptible strain, no MccJ25-specific activity could be detected in culture supernatants from strains harboring pTUC202B or pTUC202C (Figure 2). HPLC-MS analysis of the supernatants confirmed that MccJ25, which is characterized by a peak at 10.1 min, was absent from the culture supernatants of strains harboring either pTUC202B or pTUC202C, but present in supernatants from the *E. coli* strain harboring the complete gene cluster (Figure 2). The pellets from strains harboring pTUC202B or pTUC202C were devoid of antibacterial activity (data not shown), which indicated that disruption of either *mcjB* or *mcjC* did not result in export failure but abolished MccJ25 production. The plasmids pET28-*mcjB* and pET28-*mcjC*, encoding His_6-McjB and His_6-McjC, respectively, were further used to complement *E. coli* MC4100 harboring pTUC202B and pTUC202C, respectively. Both antibacterial assays and HPLC-MS experiments clearly demonstrated that MccJ25 production was restored upon complementation (Figure 2). Altogether, these data indicate that both McjB and McjC are required for MccJ25 production. Because the sizes of the inhibition halos produced by complemented strains were smaller than those of wild-type controls, it was speculated that *mcjB* and *mcjC* inactivation had a partial polar effect on their neighbor gene, as one could expect for two genes proposed to be transcriptionally and translationally coupled [18], and as has been observed within the MccC7 gene cluster [20].

Recombinant Expression of His_6-McjA

His_6-McjA was produced as a cytosolic protein in *E. coli* ER2566 harboring pET28-*mcjA*. In the first purification step, using immobilized metal-affinity chromatography (IMAC), His_6-McjA was coeluted with various C-terminally truncated forms due to the high sensitivity of McjA to proteases. An additional RP-HPLC step permitted separation of His_6-McjA from the C-truncated fragments. Purity was checked by MALDI-TOF-MS, which showed two peaks at m/z 8231 and 4117, corresponding to the singly and doubly protonated species, respectively, in agreement with the mass calculated for His_6-McjA (8232 Da; Figure 3A). The yield for His_6-McjA expression and purification was estimated at 150 μg/l of culture. Contrary to MccJ25,

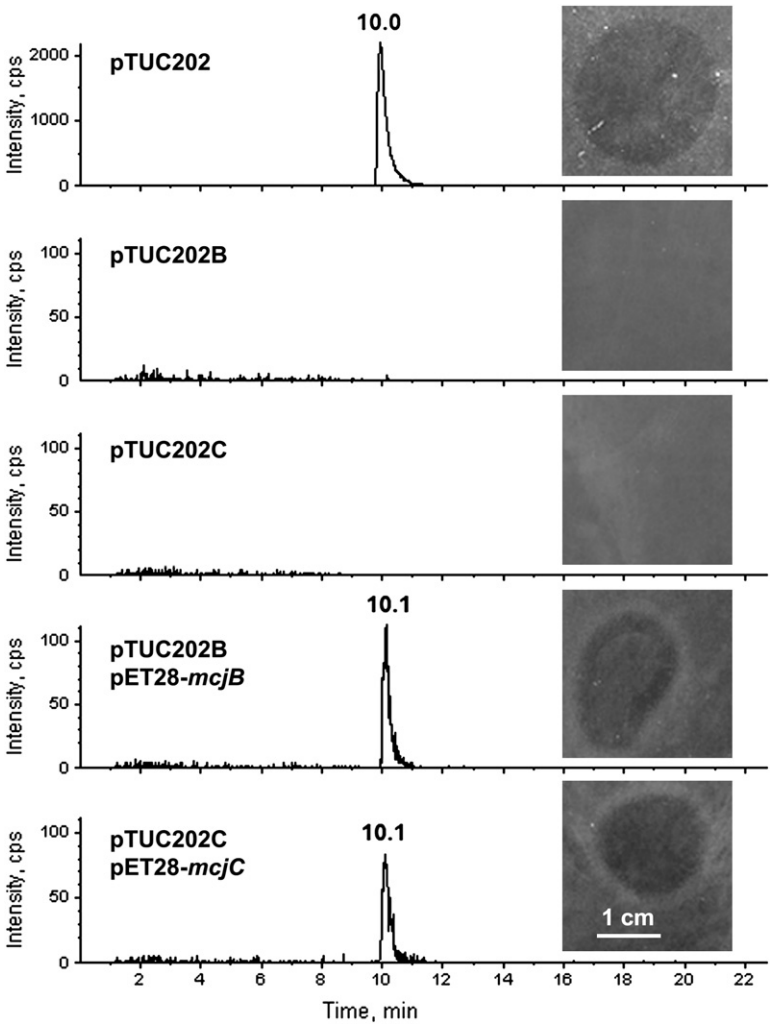


Figure 2. MccJ25 Production Is Abolished upon Inactivation of *mcjB* or *mcjC* and Restored by Complementation

Culture supernatants of *E. coli* MC4100 harboring pTUC202, pTUC202B (inactivated *mcjB*), pTUC202C (inactivated *mcjC*), pTUC202B supplemented with pET28-*mcjB*, and pTUC202C supplemented with pET28-*mcjC* were analyzed by HPLC-MS on a Qq-TOF mass spectrometer. The extracted-ion chromatograms for m/z 703, corresponding to the MccJ25 $[M+3H]^{3+}$ species, are presented together with the antibacterial activities against *S. enterica* serovar Enteritidis. MccJ25 is characterized by the peak at 10.1 min.

His_6-McjA was devoid of antibacterial activity against the MccJ25-susceptible strain (data not shown). The circular dichroism (CD) spectrum of His_6-McjA in aqueous solution displayed a negative band at 198 nm, which indicated the presence of a predominantly random coil conformation (Figure 3B). However, the presence of SDS micelles triggered the folding of the protein, which resulted in two negative bands at 206 and 223 nm, indicative of an α-helical conformation (Figure 3B). Deconvolution of the CD spectrum of His_6-McjA in SDS micelles indicated a helix content in the 10%–15% range.

Recombinant Expression of His_6-McjB and His_6-McjC

His_6-McjB and His_6-McjC were produced in *E. coli* BL21(DE3) harboring pET28-*mcjB* and pET28-*mcjC*, respectively. Generation of inclusion bodies was avoided by growing the strains at 15°C. Soluble His_6-McjB and His_6-McjC purified using IMAC were analyzed by SDS-PAGE. Their electrophoretic mobilities were in accordance with their calculated molecular masses at 26.7 and 60.9 kDa for His_6-McjB and His_6-McjC, respectively (see Figure S3). The identity of the purified proteins was assessed by in-gel digestion with trypsin. The peptide fragments obtained for His_6-McjB and His_6-McjC covered 57% and 30% of the protein sequences, respectively, and were distributed all along the sequences (see Figure S3). The expression/purification yields were estimated at 40 μg and 200 μg/l of culture for His_6-McjB and His_6-McjC, respectively. Given the efficient complementation of inactivated *mcjB* and *mcjC* with DNA sequences encoding His_6-McjB and His_6-McjC (see above), the recombinant proteins were used in further biochemical studies without removal of the N-terminal His tag.

His_6-McjB and His_6-McjC Are Sufficient to Convert His_6-McjA into MccJ25 In Vitro

In vitro reconstitution of MccJ25 biosynthesis was done by incubating His_6-McjA with the potential maturation enzymes His_6-McjB and/or His_6-McjC in the presence of ATP and Mg^{2+}. The ability of both proteins to generate active MccJ25 from its inactive precursor His_6-McjA was monitored by antibacterial assays and HPLC-MS (Figure 4). The incubation of His_6-McjA with either His_6-McjB or His_6-McjC did not generate MccJ25. By contrast, antibacterial activity was detected in the reaction mixture

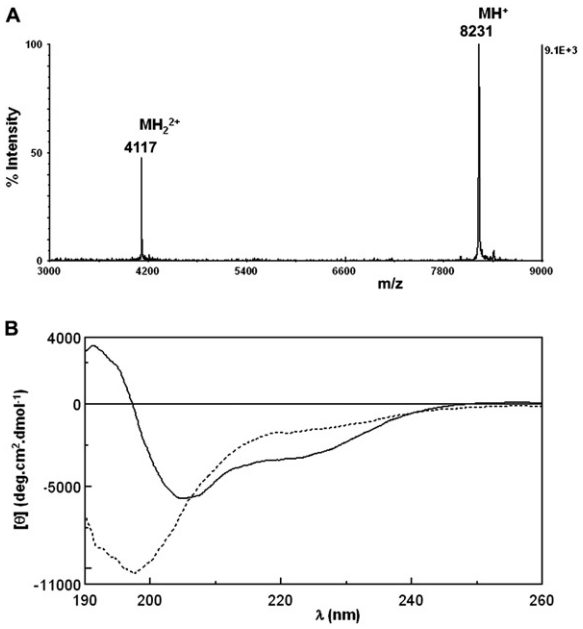


Figure 3. MS and CD Analysis of His_6-McjA

(A) MALDI-TOF spectrum of recombinant His_6-McjA.

(B) CD analysis of His_6-McjA at 75 μM in 10 mM sodium phosphate buffer (pH 7.4) in the absence (dotted line) or presence (solid line) of 8 mM SDS.

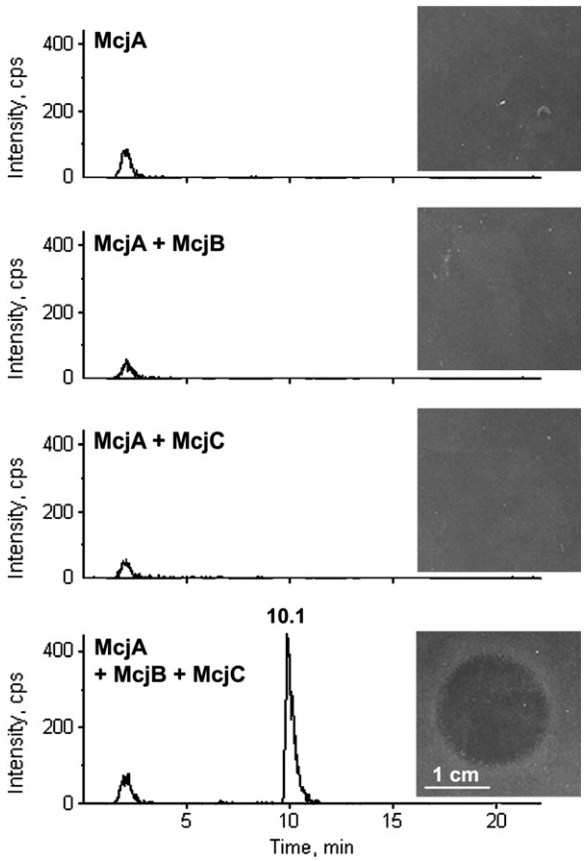


Figure 4. Reconstitution of MccJ25 Biosynthesis In Vitro

HPLC-MS analysis of reaction mixtures containing 0.5 nmol His_6-McjA alone or in the presence of 0.05 nmol His_6-McjB and/or His_6-McjC. The extracted-ion chromatograms for m/z 703, corresponding to the MccJ25 $[M+3H]^{3+}$ species, are presented together with the antibacterial activities against *S. enterica* serovar Enteritidis.

when His_6-McjA was incubated with His_6-McjB and His_6-McjC. This antibacterial activity resulted from the formation of MccJ25, as it was associated with the detection of a triply charged ion at m/z 703 by HPLC-MS, corresponding to the calculated mass for the $[M+3H]^{3+}$ species of MccJ25 (Figure 4). Interestingly, neither antibacterial activity nor MccJ25 could be observed when a synthetic linear MccJ25 corresponding to the 21 residue C-terminal sequence of McjA was used as a substrate instead of His_6-McjA (data not shown).

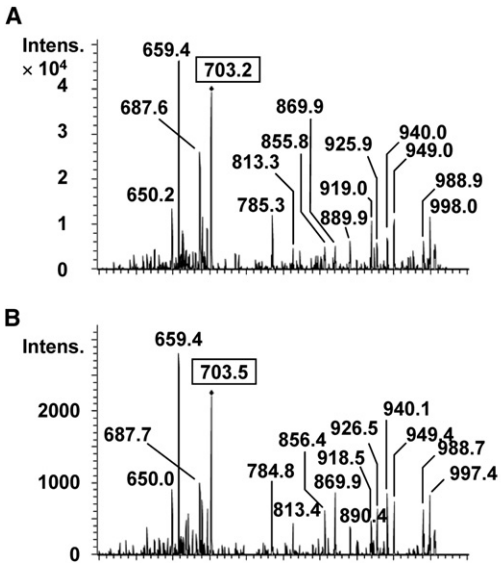
HPLC-MS/MS analysis of the $[M+3H]^{3+}$ ion of MccJ25 (m/z 703) confirmed that the in vitro-synthesized MccJ25 had acquired a lasso structure, that is, that the C-terminal tail was threaded through the Gly1-Glu8 ring. This could be ascertained given the typical fragmentation pattern of lasso peptides [21]. HPLC-MS/MS profiles of MccJ25 secreted by *E. coli* and the in vitro-synthesized peptide displayed identical retention times and fragmentation patterns (Figure 5). Furthermore, several fragment ions characteristic of the lasso structure were detected at m/z 784.8, 813.4, 869.9, 940.1, and 997.4. Such ions were identified as fragments generated by cleavages within the peptide tail which consisted of tail segments trapped in the Gly1-Glu8 ring, already described for the naturally produced MccJ25 [9, 11].

Factors Affecting the Reconstitution of MccJ25 Biosynthesis

Among different pH and temperature conditions tested for MccJ25 biosynthesis, the best were found to be 25°C and pH 8 (data not shown). Withdrawal of DTT, ATP, or $MgCl_2$ from the reaction mixture impaired the conversion of His_6-McjA into MccJ25 by His_6-McjB and His_6-McjC (data not shown). In order to better characterize the mechanism of MccJ25 synthesis, various protease inhibitors were assayed (for a review of protease inhibitor specificity, see http://www.serva.de/products/sheets/proteases.pdf), and the formation of MccJ25 was monitored by antibacterial assays. Pepstatin A, EDTA, phosphoramidon, leupeptin, and E64 did not prevent MccJ25 biosynthesis. By contrast, AEBSF (4-[2-aminoethyl]-benzenesulfonyl-fluoride), as well as TLCK (1-chloro-3-tosylamido-7-amino-2-heptanone) and TPCK (1-chloro-3-tosylamido-4-phenyl-2-butanone), totally inhibited the formation of MccJ25 (data not shown).

Comparative Analysis of Amino Acid Sequences Involved in the Maturation of MccJ25

In order to predict the function of each protein, similarity searches were performed for McjA, McjB, and McjC amino acid sequences using BLAST programs.



Main Fragment ions	Charge state	Calculated *m/z*	Measured *m/z*
$[G^1\text{-}P^{16}][S^{18}\text{-}G^{21}]\text{-}H_2O$	3	659.32	659.4
$[G^1\text{-}V^{11}][S^{18}\text{-}G^{21}]$	2	784.86	784.8
$[G^1\text{-}G^{12}][S^{18}\text{-}G^{21}]$	2	813.37	813.4
$[G^1\text{-}G^{12}][I^{17}\text{-}G^{21}]$ - CO	2	855.92	855.9
$[G^1\text{-}G^{12}][I^{17}\text{-}G^{21}]$	2	869.92	869.9
$[G^1\text{-}G^{12}][P^{16}\text{-}G^{21}]$	2	918.44	918.5
$[G^1\text{-}T^{15}][S^{18}\text{-}G^{21}]\text{-}H_2O$	2	939.94	940.1
$[G^1\text{-}T^{15}][S^{18}\text{-}G^{21}]$	2	948.95	949.4
$[G^1\text{-}P^{16}][S^{18}\text{-}G^{21}]\text{-}H_2O$	2	988.47	988.7
$[G^1\text{-}P^{16}][S^{18}\text{-}G^{21}]$	2	997.48	997.4
$[G^1\text{-}F^{10}][V^{11}\text{-}G^{21}]$	2	1004.48	1004.1

Figure 5. The In Vitro-Synthesized MccJ25 Is a Lasso Peptide

Collision-induced dissociation spectra of the triply charged m/z 703 precursor ion, obtained from HPLC-MS/MS analysis on an ion-trap instrument of (A) MccJ25 purified from culture supernatants of the *E. coli* MC4100 pTUC202 strain and (B) in vitro-synthesized MccJ25 (product of His_6-McjA incubation with His_6-McjB and His_6-McjC). The main fragment ions consisting of two sterically linked peptides (tail fragments trapped in the Gly1-Glu8 ring) are indicated in the table on the right.

McjC (513 residues) exhibits 20%–22% identity (38% similarity) between amino acids 31 and 376 to the C-terminal domain of asparagine synthetase B (AS-B) from various bacteria, including *Burkholderia* species [22, 23]. This domain of AS-B is involved in the synthesis of asparagine from aspartic acid. However, McjC lacks the N-terminal catalytic domain of AS-B which catalyzes the hydrolysis of glutamine to glutamic acid and ammonia. The amino acid sequence of McjC was aligned with those of AS-B from two *Bacillus* species and one *E. coli*, and with a putative AS-B from *Burkholderia thailandensis*. Given the similarity of the Gly1-Glu8 amide bond defining the lasso ring to the amide bond found in β-lactam rings, three β-lactam synthetases (β-LS) were also included in the alignment. These include carbapenam synthetase (CarA) from *Erwinia carotovora* and β-LS from *Streptomyces clavuligerus* and *Streptomyces cattleya*, which catalyze the formation of a β-lactam ring in carbapenems, clavulanic acid, and thienamycin biosynthetic pathways, respectively [24–26]. Two conserved motifs were identified in the multiple alignment (Figure 6A). These motifs are located in the ATP- and substrate-binding sites of the C-terminal synthetase domain, as deduced from the three-dimensional structures of AS-B, CarA, and β-LS [23, 27–29].

McjB (208 residues) contains two putative functional regions. The N-terminal region up to position 133 is similar (25% identity, 42% similarity) to certain adenosine kinases from mammals. Further, the C-terminal region between amino acids 146 and 200 displays 45% identity (62% similarity) with several hypothetical proteins from Betaproteobacteria (*Burkholderia* species), Alphaproteobacteria (*Caulobacter* species, *Sphingopyxis alaskensis*, *Sphingomonas* sp.), Gammaproteobacteria (*Stenotrophomonas maltophilia*), and Actinobacteria (*Streptomyces avermitilis*), one of which (NCBI RefSeqP ZP_01301928) is referred to as a putative transglutaminase-like enzyme. Among other reactions, transglutaminases catalyze the formation of amide crosslinks between glutamine and lysine residues in proteins [30]. Transglutaminases belong to the large family of cysteine proteases characterized by a Cys-His-Asp catalytic triad [31]. This putative catalytic triad is conserved among McjB and McjB-like proteins from various bacteria (Figure 6B).

Surprisingly, as revealed by BLAST against the peptidase database MEROPS [32], McjA (58 amino acids) exhibits similarities with two regions of a large serine C-terminal processing peptidase-3 from *Flavobacteriales bacterium* HTCC2170 (MEROPS accession number S41.004; NCBI RefSeqP ZP_01105541). However, the active site of this peptidase family is not present in McjA.

Gene clusters similar to that of MccJ25 were found in various bacterial genomes. Neighborhood analysis of McjB-like proteins by the STRING program [33] revealed functional association of an McjB-like protein with both an AS-B (Swiss-Prot accession number Q2SVT9) and an ABC transporter (Swiss-Prot accession number Q2SVT8) in *B. thailandensis* E264. These are similar to McjC and McjD, respectively, and encoded, as in the MccJ25 gene cluster, by two genes located downstream of the *mcjB*-like gene in the complete genome (GenBank accession number CP000086). Moreover, a small open reading frame (ORF) was identified upstream of this *mcjB*-like gene. It encodes a putative 47 amino acid

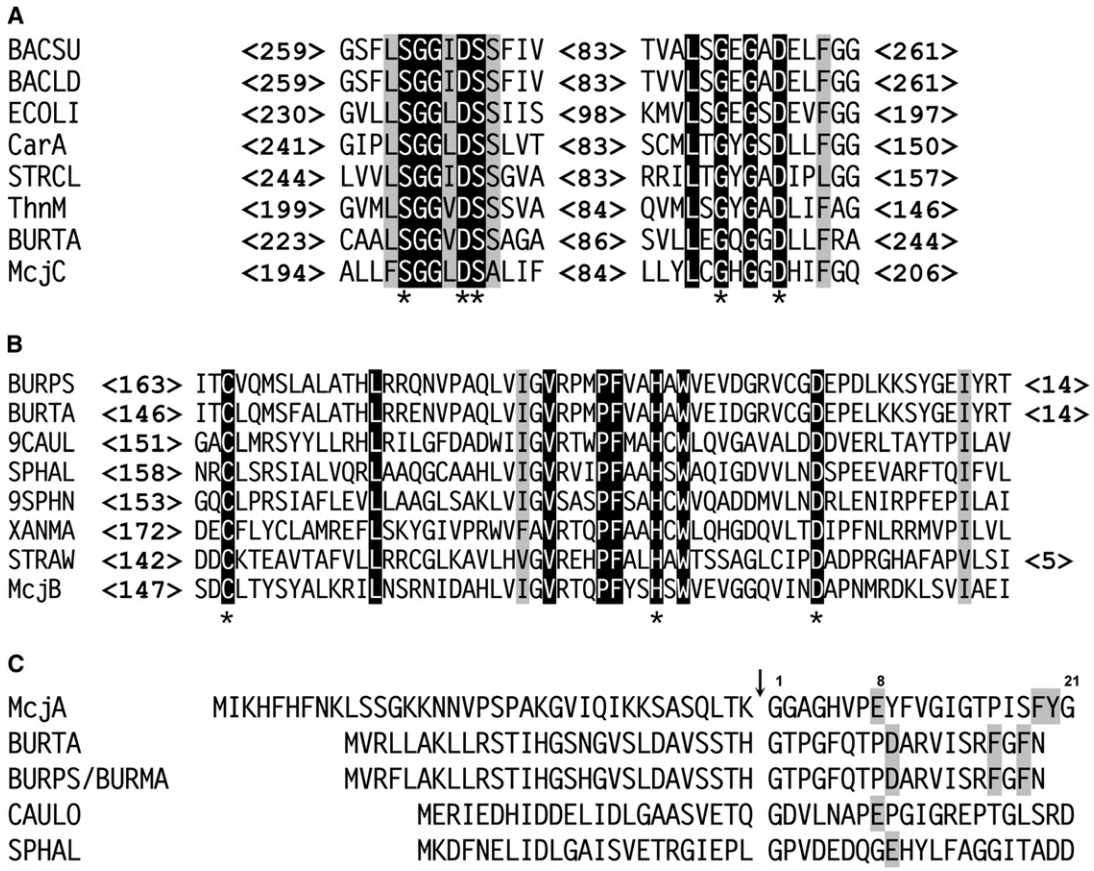


Figure 6. McjC, McjB, and McjA Sequence Similarities

(A and B) Multiple alignments for McjC and McjB. Numbers in angle brackets refer to the length in amino acids of unaligned regions. Letters on black and gray backgrounds highlight identical amino acids and similarity, respectively. All accession numbers of amino acid sequences are from the Swiss-Prot database.

(A) Alignment of similar regions of McjC, asparagine synthetases B (AS-B), and β-lactam synthetases (β-LS). BACSU, AS-B from *Bacillus subtilis* (P54420); BACLD, AS-B from *Bacillus licheniformis* (Q65FV9); ECOLI, AS-B from *E. coli* (P22106); CarA, carbapenem synthetase from *Erwinia carotovora* (Q9XB61); STRCL, β-LS from *Streptomyces clavuligerus* (Q9R8E3); ThnM, β-LS from *Streptomyces cattleya* (Q83XP1); BURTA, putative AS-B from *Burkholderia thailandensis* (Q2SVT9); and McjC, from *E. coli* (Q333M5; this study). The stars indicate amino acids involved in Mg^{2+} coordination and ATP binding.

(B) Alignment of McjB and McjB-like proteins. The stars indicate the catalytic triad of putative cysteine proteases [31]. McjB-like proteins are BURPS, from *Burkholderia pseudomallei* BPSL1795 (Q63U22); BURTA, from *B. thailandensis* E264 (Q2SVU0); 9CAUL, from *Caulobacter* sp. K31 (Q0LZ71); SPHAL, from *Sphingopyxis alaskensis* (Q1GPW3); 9SPHN, from *Sphingomonas* sp. SKA58 (Q1NGU8); XANMA, from *Stenotrophomonas maltophilia* (A1FRG0); STRAW, from *Streptomyces avermitilis* (Q82L14); and McjB, from *E. coli* (Q9X2V8).

(C) Alignment of the MccJ25 precursor, McjA (Q9X2V7), with putative peptides whose C-terminal region displays conserved features of lasso peptides. BURTA, 47 amino acid peptide from *B. thailandensis*; BURPS and BURMA, 47 amino acid peptide from *B. pseudomallei* and *B. mallei*, respectively; CAULO, 44 amino acid peptide from *Caulobacter* sp. K31 (Q0LUL4), SPHAL, 44 amino acid peptide from *Sp. alaskensis*. The arrow indicates the known or putative cleavage site of lasso peptide precursors. Light shading indicates known or putative acid and aromatic residues involved in the amide bond and trapping of the tail in the ring, respectively.

peptide with a 19 residue C-terminal sequence reminiscent of lasso peptide sequences (Figure 6C). In this putative lasso peptide sequence, an N-terminal glycine can be involved in an amide bond with the side chain of aspartic acid at position 9, while, as in MccJ25, two aromatic amino acids (Phe16 and Phe18) located within the C terminus could be involved in the trapping of the tail in the ring. Similar gene clusters encoding the same putative lasso peptide were identified in *Burkholderia pseudomallei* K96243, 1710b, 668, and 1106a and *Burkholderia mallei* ATCC23344 complete genomes (GenBank accession numbers BX571965, CP000124, CP000570, CP000572, and CP000010, respectively). However, an *mcjD*-like gene is lacking in the *B. mallei* genome. It is noteworthy that, contrary to the MccJ25 gene cluster (Figure 1A), the gene clusters encountered in *Burkholderia* species are organized in a single transcription unit. Finally, other putative lasso peptide gene clusters were identified in *Caulobacter* sp. K31 (GenBank accession number AATH01000007) and *Sp. alaskensis* RB2256 (GenBank accession number CP000356) genomes, but, like *B. mallei*, the *mcjD*-like genes are lacking.

DISCUSSION

The unusual lasso three-dimensional structure of MccJ25 has been the subject of major interest [9–11]. In this article, we show that only two enzymes encoded by the MccJ25 gene cluster, namely McjB and McjC, are sufficient for the conversion of McjA, the 58 residue linear precursor of MccJ25, into the lasso-structured MccJ25 endowed with antibacterial activity. To our knowledge, this is the first example of the in vitro reconstitution of a lasso peptide biosynthesis.

A major result from this article is that no other protein but McjB and McjC is necessary to convert McjA into MccJ25. This was clearly shown here by the in vitro generation of a lasso-structured and biologically active MccJ25 from the inactive linear His_6-McjA upon addition of His_6-McjB and His_6-McjC. It therefore appears that the MccJ25-dedicated maturation machinery is encoded by the MccJ25 gene cluster alone. It thus differs from other class I microcins [13], namely microcin B17 (MccB17) and microcin C7/C51 (MccC7/C51), whose precursors are cleaved by proteases that are not encoded by their respective gene cluster but are either chromosome encoded by the producing bacteria (MccB17) or expressed by the target bacteria (MccC7/C51). A relevant inference from our in vitro data is that MccJ25 maturation is completely independent of the export mechanism. Indeed, McjD, which is responsible for the export of MccJ25 into the extracellular medium, is not required to convert His_6-McjA into MccJ25. This finding contrasts with the processing of class II microcins, which requires the complete export machinery to cleave the precursor from its leader peptide concomitant with secretion [13].

The respective function of McjB and McjC was examined using *mcjB*- or *mcjC*-mutated strains. Indeed, in the hypothesis of two enzymes working independently, one of the mutated strains would be expected to accumulate unprocessed McjA while the other would produce linear MccJ25. Unfortunately, we could not detect either McjA or linear MccJ25 in cell pellets and culture supernatants from these strains. However, it cannot be ruled out that inactivation of *mcjB* or *mcjC* affects *mcjA* transcription. The genes *mcjA* and *mcjBCD* are two independent transcription units (Figure 1A), and rapid intracellular degradation of McjA or linear MccJ25 could explain their absence in pellets and supernatants, as observed during the purification of His_6-McjA. Similarly, instability of microcin precursors in strains deficient for the processing machinery has been reported for the precursors of MccB17 and microcin V [34, 35].

Insight into the molecular mechanisms of MccJ25 maturation was obtained by similarity searches and multiple alignments for McjB and McjC. Given its similarity to AS-B and β-LS, McjC is likely to be involved in the formation of the amide bond between Gly1 and Glu8. MccJ25 maturation requires ATP and Mg^{2+}, as AS-B and β-LS do [23, 25, 27–29]. Therefore, we propose that McjC belongs to the family of ATP/Mg^{2+}-dependent amide synthesizing enzymes. Because AS-B and β-LS catalyze intermolecular and intramolecular amide bond formation, respectively, McjC catalytic function is more related to β-LS. As with these enzymes [27–29, 36, 37], the reaction would involve an Mg^{2+}-facilitated adenylation of the γ-carboxylate of Glu8, followed by cyclization via a tetrahedral transition state or an oxyanion intermediate. Based on the conservation of residues located in the β-LS catalytic site (Figure 6A) [23, 27–29], it can be inferred that the side-chain oxygens of Asp203 and Asp302 from McjC coordinate the Mg^{2+} ion, which also interacts with the negatively charged phosphates from ATP. Furthermore, the amide nitrogen of Gly298 and the side-chain hydroxyl groups of Ser199 and Ser204 can interact with adenosine and phosphates from ATP, respectively.

The enzyme responsible for McjA proteolytic cleavage could not be unambiguously identified from this study. On the one hand, we showed that the C-terminal region of McjB contains the Cys150-His182-Asp194 putative catalytic triad of cysteine proteases. On the other hand, MccJ25 in vitro synthesis was blocked by serine protease inhibitors rather than cysteine protease inhibitors. Therefore, the catalytic triad might not involve Cys150 but rather Ser154, located nearby. Further, an autolytic activity of McjA in complex with McjB and McjC cannot be ruled out at this stage, as McjA displays some features of a serine C-terminal processing peptidase-3.

Note that, given the steric trapping of the C-terminal tail in the Gly1-Glu8 ring in MccJ25, the amide bond formation requires the previous positioning of the (1) Gly1 amino group and Glu8 carboxylate and (2) Phe19 and Tyr20 aromatic side chains. As only McjB and McjC are responsible for MccJ25 maturation, the correct positioning of the C-terminal tail is likely to result from the interaction of McjA with one of the modification enzymes, and not to rely on a chaperone protein.

Contrary to McjA, the 21 residue linear MccJ25 was shown not to be a substrate for His_6-McjB and His_6-McjC, suggesting that the 37 residue leader peptide of McjA is required for the maturation. Therefore, the N-terminal leader peptide of the microcin precursor is likely to be involved in the recognition by the modification enzymes rather than the export machinery. This is similar to MccB17 [34] and in contrast with class II microcins [13].

Several peptides displaying the same internal side chain to backbone linkage as MccJ25 were isolated from microbial sources. Although nothing is known about the biosynthesis of these peptides, their occurrence suggests that similar enzymatic machineries could be involved. In this study, putative lasso peptide gene clusters (either complete or partial) were identified in the genome of several Proteobacteria not belonging to the Enterobacteria. Except for MccJ25, all the previously described [7, 8] and the putative lasso peptides are produced by bacteria widely distributed in nature, especially in soil or water. Thus, the MccJ25 gene cluster may have been acquired from these environmental bacteria. The widespread gene clusters encoding putative lasso peptide machineries suggest their conservation during evolution and raise the question of whether lasso peptides observed in various

bacterial strains are involved in bacterial competitions, that is, whether they display antimicrobial properties.

SIGNIFICANCE

Naturally occurring lasso peptides display a complex structure with a side chain to backbone internal linkage forming an N-terminal ring (8 or 9 residues) in which the C-terminal tail is irreversibly threaded. Most of them are enzyme inhibitors produced by *Streptomyces* species. MccJ25 is such a lasso peptide. Gene-encoded and secreted by *Escherichia coli* AY25, it exerts a potent antibacterial activity by blocking bacterial RNA polymerase. Here we demonstrate that two enzymes, namely McjB and McjC, encoded by genes belonging to the MccJ25 gene cluster act in concert to convert the precursor McjA into MccJ25 through a process that requires ATP and Mg^{2+}. To our knowledge, this is the first in vitro reconstitution of a lasso peptide biosynthesis. Our results question the possibility of a common ribosomal biosynthetic pathway leading to lasso peptides in different Proteobacteria and Actinobacteria involving a catalytic mechanism similar to that of β-lactam synthetases.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids

Strains and plasmids are described in Table S1. Production of recombinant proteins was done in either *E. coli* BL21(DE3) or *E. coli* ER2566 (Novagen). MccJ25 production as well as gene inactivation and complementation assays were performed in *E. coli* MC4100 (laboratory collection). For *mcjB* and *mcjC* complementation, bacteria were transformed with either the *mcjB*-encoding pET28-*mcjB* plasmid or the *mcjC*-encoding pET28-*mcjC* plasmid (this study; see construction below). Antibiotics were purchased from Sigma and used at the following concentrations: ampicillin, 50 μg/ml; chloramphenicol, 34 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 50 μg/ml.

Antibacterial Assays

MccJ25-specific antibacterial activity was detected by radial diffusion assay against the microcin-susceptible *Salmonella enterica* serovar Enteritidis and the microcin-resistant *E. coli* MC4100 pTUC202. A gel overlay was prepared by inoculating 10 ml M63 medium (6.5 g/l agar) with 10^7 colony-forming units per ml of bacteria in exponential phase of growth. Petri dishes containing 20 ml M63 medium (15 g/l agar) were overlaid with the bacterial suspension. After solidification, fractions to be analyzed (10 μl of boiled culture supernatant or enzymatic reaction mixtures) were placed onto the overlay. After a 16 hr incubation at 37°C, plates were analyzed for the presence of inhibition halos. Fractions inhibitory to *S. enterica* serovar Enteritidis but not to *E. coli* MC4100 pTUC202 were considered to contain MccJ25.

Gene Disruption of *mcjB* and *mcjC*

The plasmid pTUC202 was digested at one single site within *mcjB* with BstXI. Stop codons were inserted at this site (371 bases after the *mcjB* initiation codon) by insertion of an oligonucleotide duplex complementary to the BstXI-generated cohesive ends (see Figure S2). The duplex was generated with two complementary 5′-phosphorylated oligonucleotides (5′-GAGCTCGAGTAATAGTAGGGG-3′ and 5′-TTCCCC CTACTATTACTCGAG-3′) designed to introduce an XhoI site and abolish the BstXI site after insertion. Prior to transformation, pTUC202 plasmids lacking the insert were eliminated by digestion with BstXI. The positive clones were selected by restriction map comparison. One positive clone carrying an inactivated *mcjB* gene was selected for MccJ25 expression studies. A similar strategy was used for *mcjC* disruption. Briefly, pTUC202 was digested with SapI (156 bases after the *mcjC* initiation codon) and ligated to a duplex generated with 5′-phosphorylated oligonucleotides (5′-TAATAGTCACTCGAGAGTA-3′ and 5′-CTCGAGTGACTATTATACT-3′) (see Figure S2). One positive clone carrying an inactivated *mcjC* gene was selected.

Construction of Plasmids Encoding His_6-McjA, His_6-McjB, and His_6-McjC

Plasmids encoding McjA, McjB, and McjC with an N-terminal fusion of six histidines were constructed. Basically, *mcjA*, *mcjB*, and *mcjC* were amplified from pTUC202 using the forward primers mcjANdeI (5′-GCCCATATGATTAAGCATTTTC-3′), mcjBNdeI (5′-CATATGATCC GTTACTGCTTAAC-3′), and mcjCNdeI (5′-CCATATGGAAATATTTAA TGTCAAG-3′), respectively, as well as the reverse primers mcjAXhoI (5′-CTCGAGAATATCAGCCATAGAAAG-3′), mcjBXhoI (5′-CTCGAG CTATATCTCTGCAATAAC-3′), and mcjCXhoI (5′-CTCGAGTTAACC TTTATAATCAATG-3′), respectively. The forward and reverse primers introduced NdeI and XhoI restriction sites (underlined), respectively. PCR reactions, performed in an Eppendorf Mastercycler, included 30 cycles: 94°C for 45 s, 45°C for 45 s, and 72°C for 45 s. The amplified fragments were cloned into the pMOSBlue plasmid (GE Healthcare) for sequencing and subcloned into the pET28b expression vector (Novagen) by NdeI/XhoI double digestion.

Expression and Purification of Recombinant His_6-McjA, His_6-McjB, and His_6-McjC

The pET28b derivatives encoding His_6-McjA, His_6-McjB, and His_6-McjC were used to freshly transform *E. coli* ER2566 (for pET28-*mcjA*) or *E. coli* BL21 (for pET28-*mcjB* and pET28-*mcjC*). Cells were grown at 37°C in LB medium supplemented with kanamycin. At the optical density of 0.6 at 600 nm, 1 mM IPTG (QBiogen) was added and cells were further incubated for 1 hr at 37°C (His_6-McjA) or 16 hr at 15°C (His_6-McjB and His_6-McjC). Cells were harvested by centrifugation at 4°C (5000 × g, 20 min) and resuspended in chilled lysis buffer (50 mM sodium phosphate [pH 8], 500 mM NaCl) supplemented with an EDTA-free protease inhibitor cocktail (Roche), 5 μg/ml DNase (Roche), and 10 μg/ml RNase (Sigma). His_6-McjA-producing cells were heated at 100°C for 10 min to prevent enzymatic degradation. Cells were broken in a French press (Thermo Electron) and cell debris were removed by centrifugation (50,000 × g, 30 min). Supernatants were subjected to affinity chromatography on a HisTrap HP column (GE Healthcare) pre-equilibrated with 20 mM (His_6-McjB and His_6-McjC) or 40 mM imidazole (His_6-McjA) in lysis buffer. After loading of the bacterial lysate supernatants, the resin was washed with 80 mM imidazole in lysis buffer and the His-tagged proteins were eluted by increasing the imidazole concentration to 200 mM. Purification at 4°C was monitored by measuring absorbance at 280 nm.

Fractions containing His_6-McjA were desalted by solid-phase extraction on a SepPak C_8 cartridge (Waters Corporation) pre-equilibrated with 0.1% aqueous TFA (trifluoroacetic acid). The cartridge was washed with 0.1% aqueous TFA prior to elution with 20% and 40% acetonitrile (ACN) in 0.1% aqueous TFA. The 40% SepPak fraction was vacuum dried (SpeedVac; Savant) and His_6-McjA was purified by RP-HPLC on a μBondapak C_{18} column (10 μm, 300 × 3.9 mm; Waters Corporation). Separation was performed at a flow rate of 1 ml/min under the following biphasic gradient: 0%–25% ACN in 0.1% aqueous TFA in 2 min and 25%–45% ACN in 0.1% aqueous TFA in 25 min. Purification was monitored by UV detection at 226 nm and fractions were hand collected. Purity was controlled by SDS-PAGE and MALDI-TOF-MS.

Fractions containing His_6-McjB or His_6-McjC were desalted by extensive dialysis against 50 mM sodium phosphate (pH 8), 100 mM NaCl. Purity was assessed by SDS-PAGE.

Protein Quantification

His_6-McjA was quantified on a Uvikon 932 spectrophotometer (Kontron Instruments), assuming a theoretical molar extinction coefficient of 2560 M^{-1} cm^{-1} at 280 nm. His_6-McjB and His_6-McjC were quantified by the Bradford assay with a Coomassie protein assay kit (Pierce).

SDS-PAGE Analysis

Tris-tricine SDS-PAGE (16.5% acrylamide) was performed for His_6-McjA [38], whereas Tris-glycine SDS-PAGE (10%–12% acrylamide) was performed for His_6-McjB and His_6-McjC [39]. Proteins were visualized by silver or Coomassie blue staining. The molecular-weight markers were a polypeptide standard (Bio-Rad) for His_6-McjA and a broad-range protein marker (New England Biolabs) for His_6-McjB and His_6-McjC.

In-Gel Trypsin Digestion

Protein bands were excised from SDS-PAGE and washed twice with 25 mM NH_4HCO_3 (pH 8), once with 50% ACN in 25 mM NH_4HCO_3 (pH 8), once again with 25 mM NH_4HCO_3 (pH 8), and finally with H_2O before being vacuum dried. Gel slices were rehydrated with 50 μl digestion buffer (25 mM NH_4HCO_3 [pH 8.0], 5 mM $CaCl_2$ containing 20 ng/μl trypsin from bovine pancreas [Sigma T8642]) and incubated for 16 hr at 37°C with vigorous shaking. Supernatants were collected. Gels were washed once with 0.1% aqueous FA (formic acid) and once with ACN, and extracts were combined to the formerly collected supernatants. The pooled supernatants were vacuum dried, resuspended in 15 μl 0.1% aqueous FA, and desalted on C_{18} tips (Omix; Varian) before MALDI-TOF-MS analysis.

In Vitro Enzymatic Assays for MccJ25 Synthesis

Freshly prepared His_6-McjA or linear MccJ25, the 21 residue C-terminal sequence of McjA [16] (0.5 nmol), was incubated for 150 min at 25°C in the absence or presence of 0.05 nmol His_6-McjB and/or His_6-McjC in 50 mM sodium phosphate (pH 8), 1 mM DTT, 1 mM ATP, 1 mM $MgCl_2$ (1 ml). To determine the nature of the enzymatic activities, different protease inhibitors were added to the reaction mixture, namely AEBSF (0.25 mM), TPCK (3.5 μM), TLCK (3.5 μM), E64 (12.5 μM), leupeptin (2.5 μM), phosphoramidon (12.5 μM), EDTA (0.5 mM), or pepstatin (25 μM). All were from Sigma, except AEBSF (Alexis Biochemicals). Assays were also done in the absence of ATP or $MgCl_2$, or at 15°C or 25°C, or pH 5 or 7. Solid-phase extraction on SepPak C_8 cartridges (Waters Corporation) was performed for desalting. The reaction mixtures, acidified with 0.1% aqueous FA, were loaded and successively eluted with 5 ml 0.1% aqueous FA, and 5 ml 10% ACN and 3 ml 90% ACN in 0.1% aqueous FA. The last fraction was vacuum dried and resuspended in 50 μl 10% ACN in 0.1% aqueous FA prior to HPLC-MS analysis.

MALDI-TOF Mass Spectrometry

Experiments were done on a Voyager DE-PRO or a 4800 TOF/TOF instrument (Applied Biosystems). One microliter of matrix solution (α-cyano-4-hydroxycinnamic acid or sinapinic acid dissolved at 10 mg/ml in 30% ACN in 1% aqueous FA) was mixed with 1 μl of protein solution or 1 μl of trypsin digest. The mass spectrometer was operated in positive-ion mode with a 25 kV accelerating voltage, either in reflectron mode for His_6-McjB and His_6-McjC trypsin digests or in linear mode for His_6-McjA. The mass spectrometer was calibrated either internally with the ions corresponding to trypsin autodigestion for trypsin digests or externally with a peptide mixture (calibration mixture 3; Applied Biosystems) for His_6-McjA.

HPLC-MS and HPLC-MS/MS Experiments

HPLC-MS experiments were done on a Perkin Elmer chromatographic system (Series 200) connected to an Agilent 1100 UV detector and a Q-STAR Pulsar Qq-TOF mass spectrometer equipped with an ionspray source (Applied Biosystems). The separation was achieved on a Hypersil Gold C_{18} column (1.9 μm, 50 × 2.1 mm; Thermo Electron). The elution gradient was 10%–60% ACN in 0.1% FA over 10 min at a flow rate of 0.25 ml/min. The UV detection was set up at 226 nm and a post-UV split directed 50 μl/min of the effluent to the MS instrument, operated in positive mode over the range m/z 250–1500. HPLC-MS/MS experiments were done on the species at m/z 703 with a 22.5 V collision energy. Alternatively, HPLC-MS and HPLC-MS/MS experiments were conducted on an Agilent 1100 series HPLC system connected to an Esquire 3000 ion trap mass spectrometer equipped with an electrospray ionization (ESI) source, with the same separation conditions. HPLC-MS/MS experiments were done on the species at m/z 703 with a resonant excitation amplitude of 0.85 V_{P-P}.

Circular Dichroism

CD spectra were acquired on a Jasco J-810 spectropolarimeter equipped with a PFD 423S/L Peltier-type temperature controller. His_6-McjA samples, dissolved at 75 μM in 10 mM sodium phosphate buffer (pH 7.4) in the absence or presence of 8 mM SDS, were placed in a 0.05 cm path quartz cell and analyzed at 25°C over the 190–250 nm range. The spectra were acquired at 50 nm/min, with 1 s response time, 2 nm band width, and three scans averaged per sample. CD contribution from the buffer was subtracted, and CD signals were normalized to protein concentration and expressed as the mean residue weight ellipticity, [θ]. The CD spectra were deconvoluted with the different programs provided by DICHROWEB [40], DICHROPROT [41], and CDPro [42] to assess the helix content in the protein structure.

Sequence Analysis

Similarity searches for McjB and McjC (this study) were performed using the BLAST programs [43, 44] at the National Center for Biotechnology Information. Multiple sequence alignment was performed using the CLUSTAL W program [45] with the Gonnet 250 matrix to find highly conserved amino acid sequences. ORFs of more than 100 bases were predicted by ORF Finder at NCBI followed by a similarity search with BLASTP. Searches of proteins associated with McjB-like proteins were performed using the STRING (Search Tool for the Retrieval of Interacting Proteins) program [33] at the European Molecular Biology Laboratory (http://string.embl.de/). Searches for genes neighboring the *mcjB*-like genes were also performed manually. Putative proteolytic activity of McjA was investigated by a BLASTP search against the peptidase database MEROPS [32] at the Sanger Institute (http://merops.sanger.ac.uk/).

Supplemental Data

Supplemental Data include three figures and one table and can be found with this article online at http://www.chembiol.com/cgi/content/full/14/7/793/DC1/.

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II. Synthèse des résultats

➢ En vue d'effectuer l'expression recombinante de McjA, McjB et McjC, les trois gènes d'intérêt, *mcjA*, *mcjB* et *mcjC* ont été amplifiés par PCR et clonés. Une erreur récurrente nous est alors apparue au niveau du codon stop de *mcjC*. Le plasmide utilisé comme matrice de PCR, pTUC202, dérive du plasmide pTUC100, porteur du système génétique de MccJ25, originellement séquencé (EMBL AF061787). Le séquençage double brin de pTUC202 a permis de montrer que le codon stop de *mcjC* est en réalité situé 213 bases plus loin que dans la séquence publiée. Ainsi, ***mcjC* est un gène de 1542 et non 1329 bases, codant une protéine de 513 au lieu de 442 acides aminés. La séquence nucléotidique de *mcjC* a été déposée dans GenBank sous le numéro d'accès AM116873**.

➢ En vue d'identifier la fonction de McjB et McjC dans le processus de maturation de MccJ25, nous avons inactivé dans pTUC202 les deux gènes codant ces protéines, par introduction d'un codon stop dans leur séquence. L'analyse par HPLC-MS et test d'activité antibactérienne spécifique de MccJ25 a montré que MccJ25 était présente dans les surnageants de culture et culots bactériens de la souche *E. coli* MC4100 porteuse du plasmide pTUC202, mais absente des surnageants de culture et culots bactériens d'*E. coli* MC4100 portant pTUC202B et pTUC202C, les plasmides respectivement mutés sur *mcjB* et *mcjC*. Les vecteurs d'expression pET28-*mcjB* et pET28-*mcjC*, codant respectivement His_6-McjB et His_6-McjC ont permis de restaurer la production de MccJ25 dans *E. coli* MC4100 pTUC202B et pTUC202C. **Ceci indique que les deux protéines McjB et McjC sont nécessaires à la biosynthèse et non à l'export de MccJ25.**

➢ **His_6-McjA,** utilisé comme substrat dans les tests de reconstitution de la biosynthèse de MccJ25 *in vitro*, **a été produite** à partir d'*E. coli* ER256 pET28-*mcjA*, **puis purifiée** par chromatographie d'affinité et HLPC sur colonne de phase inverse. La pureté de His_6-McjA a été vérifiée par spectrométrie de masse MALDI-TOF. Après quantification du peptide grâce à son coefficient d'extinction molaire théorique à 280 nm, **le rendement de production et de purification estimé pour His_6-McjA est de 150 µg/l de culture**. His_6-McjA ne possède **pas d'activité antibactérienne**. L'analyse de la structure secondaire de His_6-McjA par dichroïsme circulaire montre un changement de **structuration en présence de micelles de SDS**. Le spectre présente alors deux bandes négatives à 208 et 222 nm, typiques d'une **hélice α**. La déconvolution de ce spectre de dichroïsme circulaire indique **10-15 % d'hélice**.

➢ **His_6-McjB** et **His_6-McjC**, les enzymes de modification potentiellement impliquées dans le processus de maturation de MccJ25, **ont été produites** respectivement à partir d'*E. coli* BL21 pET28-*mcjB* et pET28-*mcjC*, **puis purifiées** par chromatographie d'affinité. Leur mobilité électrophorétique correspond aux masses moléculaires de 26,7 kDa et 60,9 kDa calculée respectivement pour His_6-McjB et His_6-McjC. De plus, **les cartes peptidiques obtenues pour His_6-McjB et His_6-McjC par digestion sur gel par la trypsine couvrent respectivement 57% et 30% de leur séquence**. Après quantification par dosage de Bradford, **les rendements de production/purification de His_6-McjB et His_6-McjC sont estimés, respectivement, à 40 et 200 µg / l de culture**.

➢ **La biosynthèse de MccJ25 a été reconstituée *in vitro***. La présence de MccJ25 a d'abord été détectée par l'apparition d'une activité antibactérienne spécifique de MccJ25. Celle-ci a été uniquement observée lorsque His_6-McjA a été incubée conjointement avec His_6-McjB et His_6-McjC pendant 2h30. L'analyse des réactions par HPLC-MS a montré la présence d'**un ion triplement chargé $[M+3H]^{3+}$ à *m/z* 703, caractéristique de MccJ25. Ceci a permis de confirmer que l'entité active générée était bien MccJ25**. De plus, le **profil de fragmentation HPLC-MS/MS de cet ion** est identique à celui obtenu pour l'ion $[M+3H]^{3+}$ à *m/z* 703 obtenu pour MccJ25 sécrétée par *E. coli*. Ces expériences ont permis de montrer que **His_6-McjB et His_6-McjC suffisent à convertir le précurseur McjA en MccJ25 structurée en lasso et active.**

➢ Dans le but de caractériser plus en détails le processus de maturation, nous avons testé différents paramètres et analysé la génération d'une activité antibactérienne spécifique de MccJ25. Ainsi, nous avons montré que la réaction n'était inhibée ni par la pepstatine A, l'EDTA ou le phosphoramidon, ni par la leupeptine et l'E64, mais par l'AEBSF, le TPCK ou le TLKC. De plus, le retrait de **l'ATP** ou du **$MgCl_2$** dans la réaction empêche le processus de maturation. Enfin, les conditions optimales de fonctionnement des enzymes ont été recherchée et sont **25 °C** et **pH 8**.

➢ De manière à déterminer le rôle de la région *N*-terminale de McjA dans le processus de maturation, le peptide synthétique linéaire correspondant aux 21 acides aminés *C*-terminaux de McjA a été utilisé comme substrat de la réaction enzymatique. **His_6-McjB et His_6-McjC ne convertissent pas ce peptide en MccJ25**. Cette forme linéaire de la microcine n'est donc pas libérée, puis reprise en charge, et n'a donc pas de réalité *in vivo*. De plus, ceci suggère que **la région *N*-terminale du précurseur est impliquée dans le processus de maturation**, et est susceptible de constituer une **zone de reconnaissance** par les enzymes.

➢ L'utilisation de programmes Blast a permis d'étudier les **similarités entre McjB/McjC et des protéines identifiées à ce jour**. Ainsi, McjC possède deux motifs similaires à ceux présents dans les **asparagines synthétases de classe B** et retrouvés dans les **β-lactame synthétases**. Ces deux familles d'enzymes permettent respectivement de catalyser la formation d'une liaison amide inter- et intramoléculaire. De plus, les deux motifs conservés sont localisés au niveau des **sites de fixation des ligands et de l'ATP** dans les **asparagines synthétases de classe B** et dans les **β-lactame synthétases**. La région *C*-terminale de McjB est similaire à différentes protéines hypothétiques de protéobactéries, dont l'une est assimilée à une **transglutaminase**. L'analyse d'alignements multiples de McjB avec ces dernières protéines a notamment permis d'identifier huit résidus conservés parmi lesquels une **triade Cys-His-Asp**. L'étude plus en détail du voisinage des gènes codant des protéines similaires à McjB dans les génomes de diverses protéobactéries a également permis de mettre en évidence l'existence de **clusters de gènes qui pourraient être responsables de la biosynthèse de peptides en lasso**.

III. Résultats complémentaires

➢ De manière à tenter de **minimiser la dégradation de His_6-McjA** lors de sa production chez *E. coli*, différentes méthodes ont été comparées.

La première a consisté en l'utilisation de différents inhibiteurs de protéases. Cependant, l'utilisation ni de cocktails d'inhibiteurs de protéases disponibles dans le commerce, ni d'autres préparations d'inhibiteurs n'a permis d'éliminer ce phénomène. La seconde stratégie à été la production de cette protéine *via* les **vecteurs d'expression pET22b et pET41b**. pET22b devait permettre de produire la protéine McjA sous forme de fusion avec le peptide signal PelB en position *N*-terminale (21 acides aminés), et ainsi d'exporter McjA dans le périplasme bactérien ou dans le milieu de culture. L'étude des culots et surnageants de culture d'*E. coli* ER2566 pET22-*mcjA* cultivées en présence d'IPTG n'a pas permis de mettre en évidence une amélioration significative. pET41b devait permettre de produire McjA sous forme de fusion avec la gluthatione-S-transferase (GST, 220 acides aminés) en position *N*-terminale. L'expression de GST-McjA dans *E. coli* ER2566 pET41-*mcjA* a pu être vérifiée, cependant l'extrémité *C*-terminale de McjA est toujours sujette à une dégradation importante.

➢ Des expériences complémentaires ont aussi été effectuées dans le but d'étudier la **possibilité d'une complexation entre McjB et McjC**. Il est en effet possible que les deux protéines forment un complexe assurant simultanément la coupure du précurseur McjA, et la formation de la liaison Gly1 et Glu8 conduisant à la future MccJ25.

Afin de mettre en évidence un tel complexe, **des anticorps polyclonaux dirigés contre His_6-McjB et His_6-McjC ont été produits** qui devraient permettre de co-immunoprécipiter les deux protéines si elles sont en complexe. Afin d'obtenir de grandes quantités de His_6-McjB et His_6-McjC, les corps d'inclusions de ces deux protéines, produites dans *E. coli* BL21 à 37°C ont été séparés des autres protéines par SDS-PAGE, puis électroéluées. Ces conditions ont permis d'obtenir de **grandes quantités de protéines pures mais inactives enzymatiquement**. Des **anticorps polyclonaux** Anti-His_6-McjB et Anti-His_6-McjC ont été produits par immunisation de souris. Alors que **l'immunisation n'a pas abouti pour His_6-McjB, les anticorps polyclonaux anti-His_6-McjC obtenus sont beaucoup plus sensibles que les anticorps anti-His_6 commerciaux** (Figure 5). Ils devraient donc pouvoir être utilisés dans de futures expériences d'immunoprécipitation.

(A)
1. McjC
2. mutant pTUC202B
3. mutant pTUC202C
4. pTUC202
5. Culture IPTG$^+$ 15 °C 16h
6. Culture IPTG$^+$ 37 °C 2h
7. Mwm

(B)
1. Mwm
2. Culture IPTG$^+$ 37 °C 2h
3. Culture IPTG$^-$ 15 °C 16h
4. Culture IPTG$^+$ 15 °C 16h

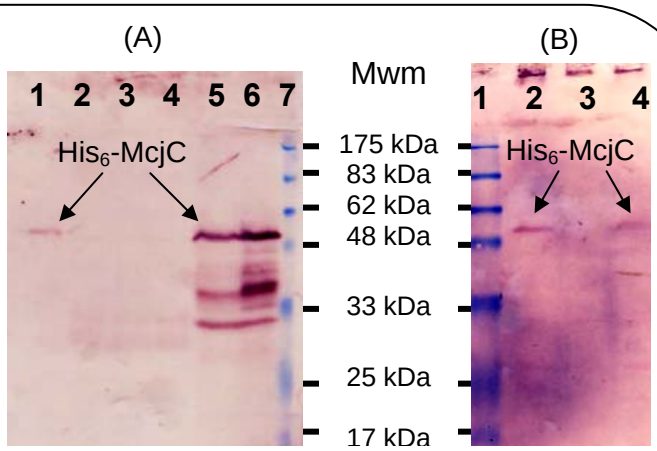

Figure 5 : Comparaison par western blot de l'immunoréactivité vis-à-vis de McjC des anticorps anti-McjC (A) et anti-histidine (B)

Gel d'électrophorèse SDS Tris-glycine 12 % transféré sur membrane de nitrocellulose ; dépôt de 1,5 x 10^8 bactéries par puits/ 0,5 µg McjC ; Mwm (Marqueur de poids moléculaire)

De manière à mettre en évidence, selon une autre approche, un complexe potentiel formé par McjB et McjC, le vecteur pET28-*mcjBC* a été construit. Un fragment d'ADN contenant les gènes *mcjB* et *mcjC* a ainsi été amplifié par PCR à partir de pTUC202, puis cloné dans le vecteur d'expression pET28b. Le vecteur d'expression ainsi construit, **pET28-**

mcjBC, permet d'induire la production de His_6-McjB d'une part, grâce au RBS commercial optimisé contenu dans pET28b, et de produire McjC, d'autre part, grâce au RBS naturel contenu dans le système génétique de MccJ25 (Figure 6). Seule **la production des deux protéines His_6-McjB et McjC dans la souche *E. coli* BL21 pET28-*mcjBC* a été vérifiée** à ce jour (Figure 7).

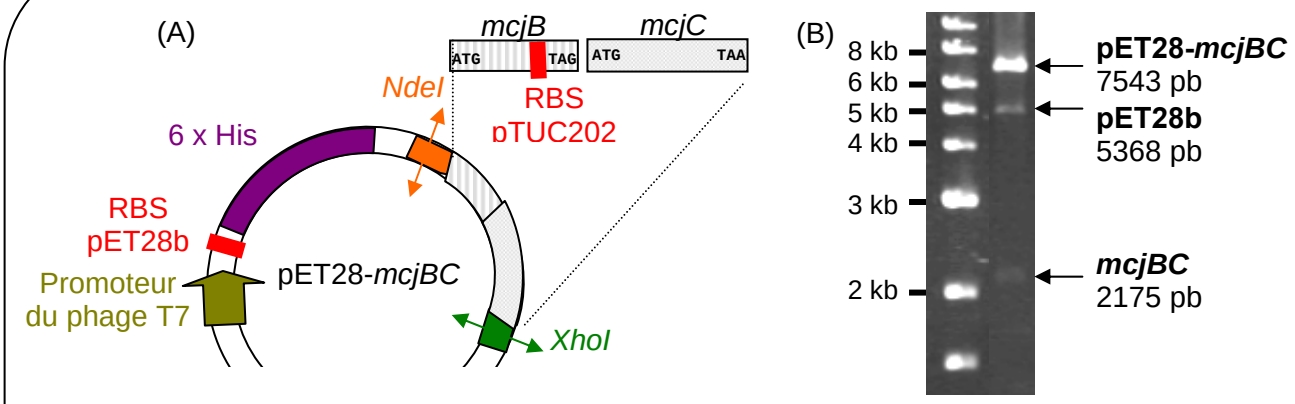

Figure 6 : Construction du plasmide pET28-*mcjBC* permettant la coexpression de McjB et McjC

(A) Principe de la construction

(B) Electrophorèse sur gel d'agarose TAE 1 % de pET28-*mcjBC* après digestion *Nde*I/*Xho*I

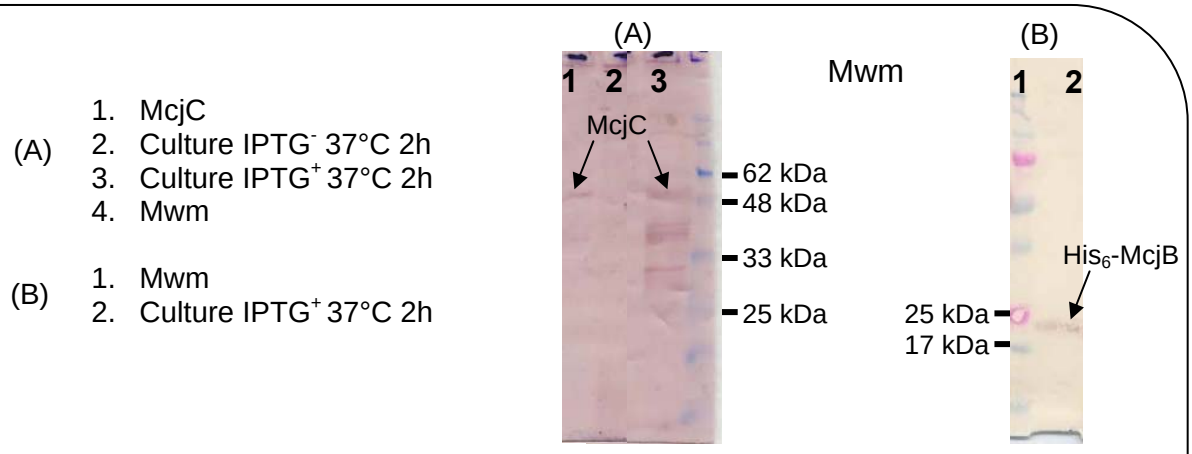

Figure 7 : Analyse de l'expression de McjB et McjC, dans la souche *E. coli* BL21(DE3) porteuse de pET28-*mcjBC*, par western blot avec des anticorps anti-McjC (A) et anti-histidine (B)

Gel d'électrophorèse SDS-PAGE Tris-glycine 12 % transféré sur membrane de nitrocellulose

Avec dépôt de 1,5 × 10^8 bactéries par puits/ 0,5 µg McjC. ; Mwm (Marqueur de poids moléculaire)

Conclusions et Perspectives

Conclusions et Perspectives

Ce travail de thèse représente une contribution à la connaissance de MccJ25, depuis sa biosynthèse jusqu'à son mécanisme d'action. Grâce aux travaux des groupes de Salomón, Ebright et Severinov, ces dernières années ont vu l'identification de la cible cellulaire de MccJ25 et du mécanisme générant cette activité. Ainsi, il est maintenant établi que MccJ25 inhibe l'ARN polymérase en obstruant le canal d'entrée des nucléotides triphosphate (Adelman *et al.* 2004, Mukhopadhyay *et al.* 2004). Nos travaux ont, quant à eux, permis de définir les bases moléculaires du mécanisme de la reconnaissance de MccJ25 à la membrane externe des bactéries et d'appréhender sa voie de maturation.

Dans le **Chapitre I**, nous avons présenté, au travers d'un article paru dans *Biochemical Journal*, les résultats obtenus sur l'étude du mécanisme d'import de MccJ25 dans les bactéries. Ce type d'étude a pu être mis en œuvre grâce à l'obtention, par notre laboratoire et par l'Institut de Biochimie et Biophysique Moléculaire et Cellulaire (IBBMC, Orsay), de préparations pures de MccJ25 et FhuA.

Nous avons montré que, tout comme le phage T5 ou la colicine M, MccJ25 parasitait le transporteur biologique du complexe fer/ferrichrome, FhuA. *In vivo*, l'étude de la sensibilité de différentes souches sauvages et mutées sur les gènes de FhuA, TonB, ExbB, ExbD a montré que ces protéines étaient impliquées dans l'activité de MccJ25. De plus, le franchissement des membranes nécessite, pour MccJ25 comme pour de nombreux ligands des récepteurs aux sidérophores, le complexe TonB/ExbB/ExbD sur la membrane interne. Le rôle de ce complexe consiste à transférer l'énergie de la force protomotrice de la membrane interne à la membrane externe (pour revue, consulter Postle et Kadner 2003).

Nous avons montré l'interaction MccJ25/FhuA *in vivo*, en bloquant par MccJ25 l'une des fonctions de FhuA, sa capacité à être l'unique récepteur du phage T5. *In vitro*, les paramètres thermodynamiques régissant l'interaction MccJ25/FhuA ont été déterminés par ITC. Ainsi, MccJ25 se lie à FhuA avec une constante d'affinité de 1,2 µM, pour former un complexe MccJ25/FhuA de stœchiométrie 2 :1. De plus, nous avons pu observer par DCS que cette complexation stabilisait la région des boucles externes et du bouchon de FhuA, impliquant une interaction probable de MccJ25 avec les boucles externes du récepteur. Ce résultat est en accord avec le fait que les souches de *Salmonella* sensibles ou résistantes à

MccJ25 expriment. un récepteur FhuA dont la séquence diffère au niveau des boucles Enfin, la même étude a été effectuée avec un variant de MccJ25 clivé à la thermolysine, t-MccJ25, dans lequel la région en épingle à cheveux ß entre les résidus 11 et 16 est clivée et déstructurée. Cette étude a montré que t-MccJ25 est 15 à 100 fois moins active que MccJ25 et que ceci est dû à une baisse considérable de l'affinité pour son récepteur FhuA.

Toutefois, différents points restent encore à préciser. Ainsi, après l'étape de reconnaissance par FhuA à la membrane externe des bactéries, le passage physique des membranes par MccJ25 pourrait s'effectuer par le tonneau ß que constitue FhuA. Cependant, il n'est pas possible d'exclure qu'il fasse intervenir une porine ou encore une zone de composition atypique de la membrane externe. En effet, plusieurs mécanismes ont été envisagés concernant le passage de ligands par les récepteurs de la membrane externe eux mêmes, la principale hypothèse étant un changement de conformation drastique du bouchon plutôt que son expulsion totale dans le périplasme, permettant ainsi au ligand de franchir le tonneau ß (Eisenhauer *et al.* 2005, Chakraborty *et al.* 2006). De plus, il a été montré que certains ligands, comme la colicine E3, utilisaient une porine pour franchir la membrane externe après une étape de reconnaissance (Kurisu *et al*. 2003). Enfin, l'étude de mutants résistants à MccJ25 a montré qu'une autre protéine de la membrane interne, SbmA (Laviña *et al.* 1986), était nécessaire à l'activité de MccJ25 (Salomón *et al.* 1995). Son rôle précis dans la voie d'import de MccJ25 reste à déterminer.

L'étude de la relation entre la structure de MccJ25 et son activité a été débutée, et reste à poursuivre. Ainsi, la région Val11-Pro16 structurée en épingle à cheveux ß est nécessaire à l'interaction MccJ25/FhuA (Destoumieux-Garzón *et al.* 2005), alors que cette région n'influence pas l'activité de MccJ25 sur l'ARN polymérase (Semenova *et al.* 2005). De plus, la région cyclique de MccJ25, et en particulier l'histidine en position 5, semblent être impliquées dans l'interaction de la microcine avec SbmA (de Cristóbal *et al.* 2006).

Par ailleurs, les boucles externes du récepteur FhuA apparaissent être impliquées dans l'interaction MccJ25/FhuA, mais rien n'est connu quant à l'interaction de SbmA avec MccJ25, et en particulier les régions de SbmA impliquées dans cette interaction.

La figure 8 résume la connaissance actuelle du mécanisme d'action de MccJ25, depuis son export hors des bactéries productrices jusqu'à sa cible cytoplasmique. Afin de pouvoir compléter cette figure synthétique, les perspectives de recherche à envisager sont nombreuses.

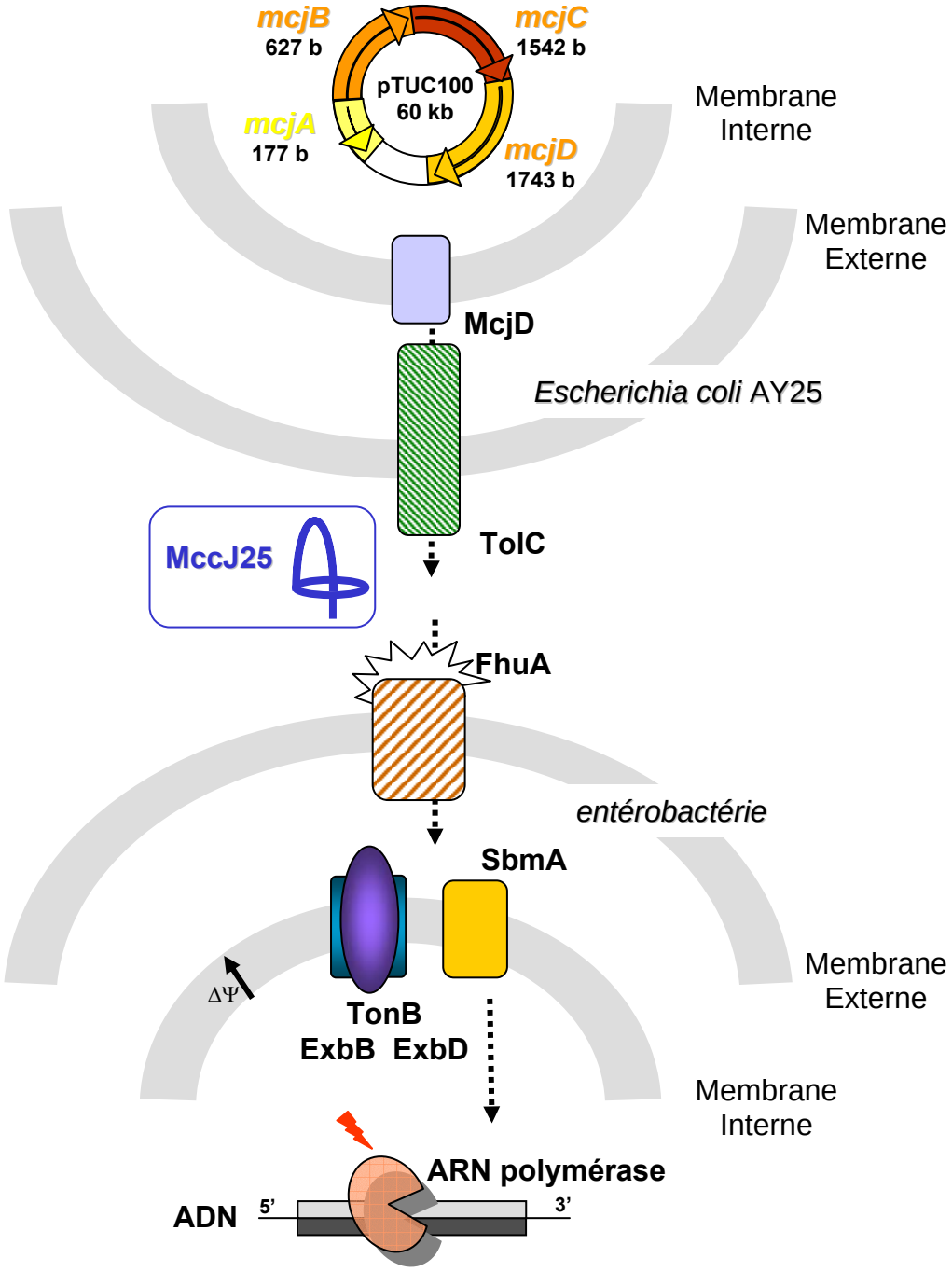

Figure 8. Le système génétique responsable de la biosynthèse de MccJ25, constitué de quatre gènes, est porté par le plasmide naturel pTUC100 (EMBL AF061787). MccJ25 est vraisemblablement exportée des bactéries productrices *via* McjD et TolC. MccJ25 pénètre dans les bactéries après une étape de reconnaissance par le récepteur FhuA, dépendante du complexe TonB/ExbB/ExbD. SbmA interviendrait dans le franchissement de la membrane interne par MccJ25. Dans le cytoplasme, MccJ25 inhibe l'ARN polymérase.

Concernant l'interaction MccJ25/FhuA, les régions du peptide et de la protéine impliquées dans l'interaction devraient pouvoir être identifiées grâce à la **cristallisation du complexe MccJ25/FhuA**, comme ceci a été effectué pour le complexe FhuA/ferrichrome (Locher *et al.* 1998). De plus, l'obtention des CMI ainsi que des paramètres régissant l'interaction MccJ25/FhuA, déterminés pour une solution pure de MccJ25 sur FhuA présentant diverses substitutions d'acides aminés obtenues par **mutagénèse dirigée** sur le gène *fhuA*, précisera l'**implication des différentes régions impliquées** et complètera l'étude qualitative réalisée (Endriss et Braun 2004). Cette technique a déjà été mise en œuvre avec succès dans l'étude de l'interaction de FepA, un récepteur aux sidérophores de type catécholate, avec ses différents ligands (Annamalai *et al.* 2004).

Un autre point qui reste à éclaircir est l'étape suivant la biosynthèse de MccJ25. En effet, MccJ25 serait exportée via l'action conjuguée de McjD, une protéine homologue à un ABC transporteur et codée par le système génétique de MccJ25 et de la protéine chromosomique TolC. Cependant, si le système d'export des microcines de classe II, et notamment de la colicine V, a été étudié *in vivo* et *in vitro* (Van Belkum *et al.* 1997, Guo *et al.* 2006), l'implication des protéines McjD et TolC dans l'export de MccJ25 n'a été déduite que d'études génétiques (Solbiati *et al.* 1996, Delgado *et al.* 1999). Ainsi, la **purification de McjD** sera à envisager, de manière à **étudier, *in vitro*, le mécanisme d'export de MccJ25**. Ceci pourrait être effectué en collaboration avec l'équipe de Jean-Marie Pagès (INSERM, Marseille) travaillant sur TolC.

Enfin, des études du même type que celle menée sur la voie d'import de MccJ25 doivent être envisagées pour d'autres microcines. En effet, des études génétiques préliminaires ont montré que l'activité de MccE492, sous sa forme non modifiée et modifiée par un sidérophore en position *C*-terminale, ainsi que de MccM, dépendaient des récepteurs aux sidérophores de type catécholate FepA, Cir et Fiu (Thomas *et al.* 2004, Vassiliadis *et al.* non publié). Ceci suggère que les récepteurs aux sidérophores pourraient être impliqués dans la reconnaissance et l'import de microcines aux structures particulièrement diverses. Il serait donc intéressant d'étudier précisément l'**interaction de MccE492 et MccM avec ces récepteurs**. *In vivo*, l'utilisation de microcines radiomarquées permettra d'accéder aux **paramètres d'adsorption (K_d) et de transport (K_m, V_{max} et k_{cat})**, comme ceci a été effectué précédemment, en présence de ^{59}Fe, pour l'interaction FepA/FeEnt (Annamalai *et al.* 2004). Ces paramètres devront ensuite être comparés à ceux obtenus *in vitro*, par mesures calorimétriques ou par **résonance plasmonique de surface**, par exemple. L'influence de la

complexation de MccE492 au fer, ainsi que celle de la présence du sidérophore pourront être finalement étudiées.

Dans le **Chapitre II**, nous avons présenté, au travers d'un article soumis à *Chemistry and Biology*, les résultats obtenus concernant l'étude de la voie de maturation de MccJ25. Nous avons inactivé *mcjB* et *mcjC*, soit chacun des deux gènes du système génétique de MccJ25 codant des protéines de fonction inconnues, et avons ainsi montré que les protéines McjB et McjC, était impliquées dans le processus de maturation de MccJ25. Une complémentation de ces mutants a été effectuée avec les vecteurs pET28-*mcjB* et pET28-*mcjC*, permettant respectivement la synthèse de His_6-McjB et His_6-McjC. McjB et McjC, ainsi que leur substrat, McjA, le précurseur linéaire de MccJ25, ont été produites de manière recombinante chez *E. coli*, puis purifiées et caractérisées. Tout d'abord, nous avons montré que la séquence anciennement publiée pour *mcjC* (EMBL AF061787) était erronée, et que *mcjC* est en fait un gène de 1542 b et non 1329 b (GenBank AM116873), codant une protéine de 513 acides aminés au lieu de 442. De plus, nous avons mis en évidence une structuration de McjA en hélice α dans un environnement micellaire. Mais surtout, nous avons montré que McjB et McjC sont non seulement nécessaires, mais suffisantes à la conversion de McjA, inactif sur *E. coli*, en MccJ25 active et structurée en lasso.

Les alignements de séquences de McjB et McjC ont permis d'émettre des hypothèses quant au mécanisme de conversion de McjA en MccJ25. En effet, la région *N*-terminale de McjC est similaire au domaine *C*-terminal des asparagines synthétases de la classe B, qui catalysent la formation de liaisons amide intermoléculaires, ainsi qu'aux ß-lactame synthétases, qui catalysent la formation de liaisons amide intramoléculaires, ces deux réactions étant dépendantes de l'ATP et de Mg^{2+}. Ceci est parfaitement en accord avec le fait que notre réaction enzymatique nécessite elle aussi l'ATP et le Mg^{2+}. De plus, la région *C*-terminale de McjB est, quant à elle, similaire à différentes protéines hypothétiques dont l'une est décrite comme une transglutaminase, enzyme qui catalyse la formation d'une liaison amide entre un acide glutamique et une lysine, et qui fait partie de la famille des protéases à cystéine dont la triade catalytique est Cys-His-Asp. La triade Cys150-His182-Asp194 est présente sur la séquence de McjB, toutefois, les résultats obtenus avec les inhibiteurs de protéases à cystéine/sérine utilisés au cours de cette étude semblent suggérer que la protéase impliquée dans le mécanisme enzymatique est une protéase à sérine. La sérine 154 pourrait remplacer la cystéine située en amont, et constituer la triade catalytique potentielle Ser154-His182-Asp194.

Nous proposons donc que McjB serait la protéase responsable du clivage de McjA et que McjC serait l'enzyme responsable de la formation de la liaison Gly1-Glu8.

Ce travail ouvre de nombreuses perspectives. En effet, il a permis de montrer que MccJ25 pouvait être synthétisée *in vitro*, grâce à l'action de deux enzymes uniquement, McjB et McjC. De plus, ces enzymes peuvent être produites de manière recombinante chez *E. coli*, puis purifiées tout en conservant leur activité enzymatique. L'intérêt biotechnologique que présente ce projet a été reconnu au niveau européen, puisqu'il a fait l'objet d'un financement PAI européen (**PROCOPE**) en collaboration avec le laboratoire « Fachbereich Chemie-Biochemie » de l'Université de Marburg (Allemagne) dirigé par le Pr. M. Marahiel.

A ce stade, le principal problème qui devra être résolu pour étudier plus avant ce mécanisme enzymatique concerne les rendements obtenus pour la production/purification de chacun des composants McjA, McjB et McjC. Les différents essais visant à diminuer la dégradation de McjA dans les bactéries productrices (inhibiteurs de protéases, vecteurs d'expression et hôtes multiples) ont été vains. L'obtention de quantités suffisantes des trois protéines constituant un pré-requis à l'étude du mécanisme de la réaction, il faudra donc envisager de mettre au point une méthode de **synthèse chimique** ou de **production « cell-free » de McjA**. En effet, cette dernière technique, qui peut permettre la synthèse rapide de quelques milligrammes de protéine (Klammt *et al.* 2004), offre également la possibilité d'enrichir McjA en ^{15}N et en ^{13}C. Ceci permettra de compléter l'étude structurale préliminaire réalisée par dichroïsme circulaire, par une **étude approfondie par RMN** de la **structure tridimensionnelle de McjA** et ainsi de déterminer la **région de la protéine se structurant en hélice α** dans un environnement micellaire.

La **co-production de McjB et McjC** dans le même hôte bactérien devra être étudiée plus en détail, car elle pourrait pallier l'agrégation et la dégradation des protéines lors de leur production. Il est en effet probable que les deux enzymes forment un complexe, et que celui-ci stabilise les deux protéines. Cette production pourra être réalisée à l'aide du vecteur **pET28-*mcjBC*** déjà construit dans le cadre de cette étude. Toutefois, la construction d'un vecteur permettant de produire les deux protéines sous forme chacune de fusion avec une étiquette donnée pour faciliter la purification, à partir de RBS optimisés, devra être envisagée, à l'aide par exemple des vecteurs **pETDuet ou pACYCDuet** (Novagen).

Différentes techniques permettront alors de mettre en évidence la formation d'un **complexe entre McjB et McjC** : i) la **purification concomitante de McjC et His_6-McjB**,

par chromatographie d'affinité sur colonne nickel, à partir du cytoplasme de bactéries portant pET28-*mcjBC*, ii) la **co-immunnoprécipitation** de His_6-McjB et His_6-McjC à l'aide des anticorps anti-McjC produits chez la souris au cours de ce travail, et iii) l'utilisation de **gels d'acrylamide natifs** couplés au western-blot. Par ailleurs, l'**influence de McjA et de l'ATP** sur le phénomène de complexation de McjB avec McjC devra être étudiée. L**'état d'oligomérisation** de McjB et McjC, ainsi que **la stœchiométrie du complexe catalytique** devront être caractérisés par **chromatographie d'exclusion**. Enfin, les **caractéristiques cinétiques** *(K_m, k_{cat}* et *V_{max})* **du complexe catalytique** devront être déterminées.

Une caractérisation plus poussée des interactions moléculaires mises en jeu au cours du processus de maturation pourra être entreprise. Ainsi, afin de mettre en évidence les **résidus de McjB et McjC impliqués dans la réaction enzymatique**, la conversion *in vitro* de McjA en MccJ25 sera réalisée en présence d'enzymes présentant des substitutions d'acides ainés, obtenues par mutagenèse dirigée sue les gènes codant McjB et McjC. Les résidus potentiellement responsables du mécanisme réactionnel, *i.e.* les résidus Cys150/Ser154, His182 et Asp194 constituant le site catalytique suggéré pour McjB, ainsi que les résidus localisés dans le site présumé de fixation de l'ATP et de Mg^{2+} à McjC (Ser199, Asp203, Ser204, Gly298 et Asp302) seront principalement étudiés. De même, l'étude de la région de McjA constituant la **zone de reconnaissance** des enzymes sera examinée. Nos résultats suggèrent que cette région serait située sur le peptide leader (*N*-terminal). Ainsi, des peptides mimant un fragment plus ou moins long du peptide leader pourront être utilisés pour inhiber, par compétition, la conversion de McjA en MccJ25. De même, l'aptitude de formes tronquées de McjA, dont le peptide leader aura été raccourci, à être converties en MccJ25 sera évaluée.

Au délà des microcines, l'élucidation pour la première fois de la voie de maturation d'un peptide lasso pose la question de la **conservation de cette voie** chez les autres espèces synthétisant de telles molécules. En effet, comme mentionné au chapitre 2, non seulement des structures semblables à MccJ25 ont pu être isolées dans différents ordres bactériens, mais des clusters de gènes éventuellement responsables de la biosynthèse de peptides en lasso ont été identifiés dans le génome de diverses Protéobactéries. L'étude de ces systèmes pourra être envisagée *in vitro*, en construisant des vecteurs permettant l'expression des enzymes et du substrat hypothétiques à partir de gènes synthétiques. Les mécanismes de biosynthèse pourront alors être comparés au système McjA/McjB/McjC, et la permutation des enzymes appartenant à ces différents systèmes permettra de cerner **l'adaptabilité de ce système enzymatique**.

Conclusions and Prospects

This PhD work represents a contribution to the knowledge of MccJ25, from its biosynthesis to its mechanism of action. Thanks to the research work accomplished since the past few years by the groups of Salomón, Ebright and Severinov, the cellular target of MccJ25 and the mechanism responsible for this activity were identified. Thus, it is now established that MccJ25 inhibits RNA polymerase by blocking the triphosphate nucleotides input channel (Adelman *et al.* 2004, Mukhopadhyay *et al.* 2004). Our work made it possible to define the molecular bases of MccJ25 mechanism of recognition at the outer membrane of target bacteria and to lighten its maturation process.

Chapter I presented, through an article published in *Biochemical Journal*, the results obtained on the study of MccJ25 uptake mechanism in target bacteria. This study could be achieved thanks to the preparation, by our laboratory and the Institute of Biochemistry and Molecular and Cellular Biophysics (IBBMC, Orsay), of pure MccJ25 and FhuA solutions, respectively. We showed that, just like phage T5 or colicin M, MccJ25 parasitizes the biological transporter of the iron/ferrichrome complex, FhuA. *In vivo*, the sensitivity of various wild type strains and isogenic strains mutated on genes encoding FhuA, TonB, ExbB, ExbD was studied, and showed that these proteins are involved in MccJ25 activity. Moreover, the uptake of MccJ25 requires the TonB/ExbB/ExbD complex within the inner membrane, as the uptake of most ligands of the siderophores transporters do. This complex is widely used amongst bacteria to transfer protomotive force energy from the inner to the outer membrane (for a review, see Postle and Kadner 2003).

We showed that MccJ25 interacts with FhuA *in vivo*, using MccJ25 to block the function of FhuA as the sole receptor of phage T5 at the outer membrane. *In vitro*, the thermodynamic parameters governing the MccJ25/FhuA interaction were determined by ITC. Thus, MccJ25 binds to FhuA with a 2: 1 stoichiometry and an affinity constant of 1.2 µM. Moreover, using DSC, we could observe that this binding stabilizes the external loops and plug of FhuA, suggesting a probable interaction of MccJ25 with the external loops of the transporter. This result is in agreement with the finding that *Salmonella* strains producing FhuA whose sequences display differences on the loops are either sensitive or resistant to MccJ25. Finally, the same study was carried out with a thermolysin-cleaved variant of MccJ25, t-MccJ25, in which the ß-hairpin Val11-Pro16 region is cleaved and disrupted. This

study showed that t-MccJ25 is 15 to 100 times less active than MccJ25 and than this is du to a considerable decrease of its affinity for FhuA.

However, various points still remain to be elucidated. Thus, after the recognition step by FhuA at the bacterial outer membrane, the physical crossing of the membranes by MccJ25 could be achieved either through the FhuA ß-barrel itself, through a porine or even through a region with different lipid composition such as a "membrane island". On the one hand, several mechanisms were considered concerning the crossing of the outer membrane through transporters, the principal assumption being a drastic conformational change of the plug rather than its total expulsion into the periplasm, thus making it possible for the ligand to cross the ß-barrel (Eisenhauer *et al.* 2005, Chakraborty *et al.*l 2006). In addition, it was shown that several ligands, such as colicin E3, used a porine to cross the outer membrane after the recognition step by the outer membrane receptor (Kurisu *et al.* 2003). Moreover, the study of mutants resistant to MccJ25 showed that another protein of the inner membrane, SbmA, was required for MccJ25 activity (Salomón *et al.*1995), but its precise role in the uptake of MccJ25 remains to be determined.

The structure/activity relationship study was started for MccJ25, and remains to be continued. Thus, the Val11-Pro16 ß-hairpin region is involved in the FhuA/MccJ25 interaction (Destoumieux-Garzón *et al.* 2005), whereas this region does not influence the activity of MccJ25 on RNA polymerase (Semenova *et al.* 2005). Moreover, the ring-tail region of MccJ25 and the histidine in position 5 particularly, seem to be implied in the interaction of MccJ25 with SbmA (de Cristóbal *et al* 2006).

Furthermore, the external loops of FhuA seem to be involved in MccJ25/FhuA interaction, but nothing is known to date regarding the interaction of SbmA with MccJ25, and in particular the region of SbmA implicated in this interaction.

Figure 8 summarizes the current knowledge on the mechanism of action of MccJ25, since its export from the producing bacteria to its target. In order to supplement this synthetic figure, the research prospects to be considered are numerous.

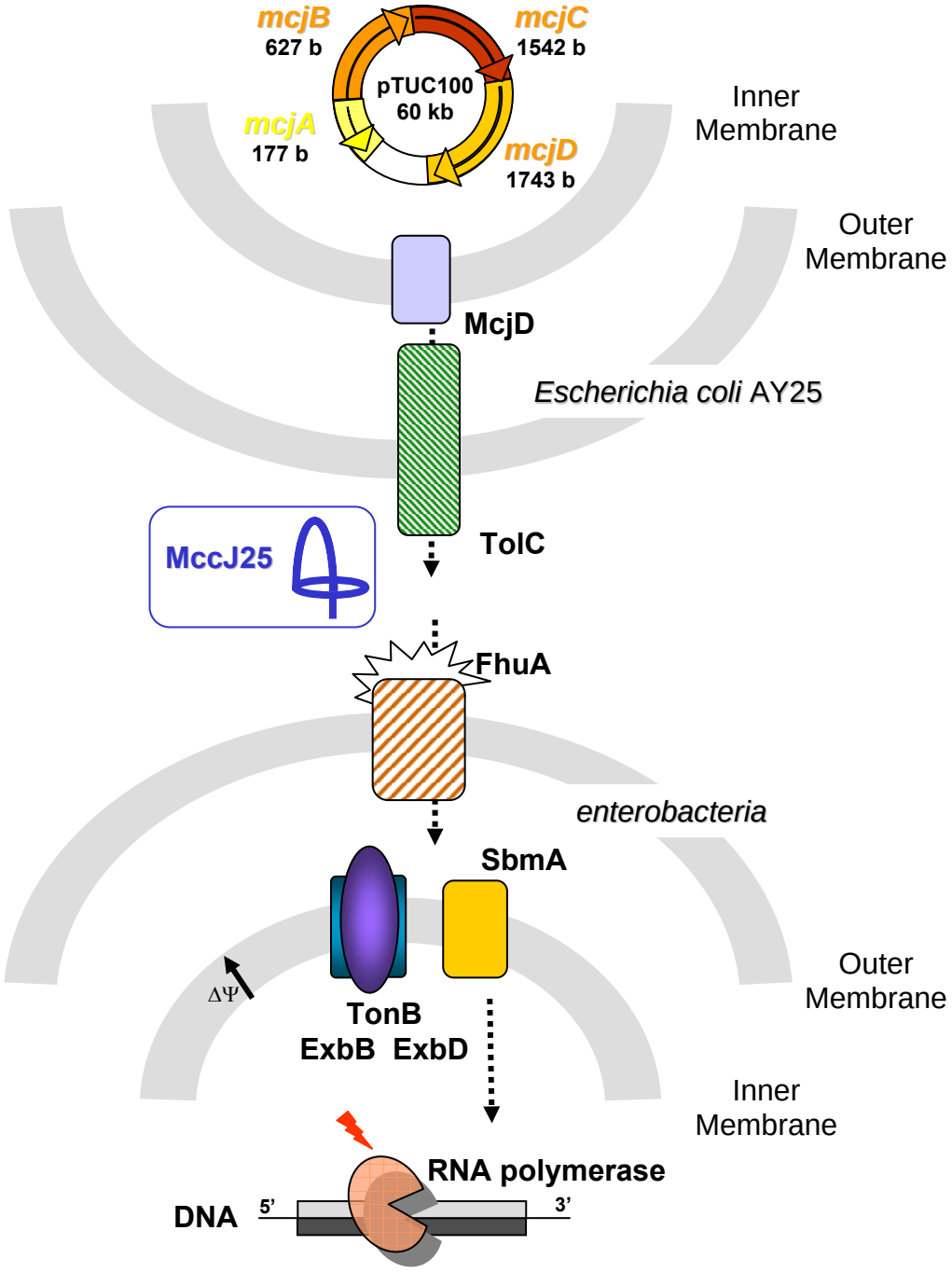

Figure 8. The plasmid-borne genetic system responsible for MccJ25 production consists of four genes (EMBL AF061787). The export of MccJ25 out of the producing strain is thought to be mediated by McjD and TolC. The uptake mechanism of MccJ25 in bacteria involves a TonB/ExbB/ExbD-dependent recognition step by FhuA at the outer membrane. SbmA would play a role in McJ25 inner membrane crossing. MccJ25 then inhibits bacterial RNA polymerase in the cytoplasm.

Concerning the MccJ25/FhuA interaction, the regions involved in both the peptide and the protein should be identified thanks to the **crystallization of the FhuA/MccJ25 complex**, as it was achieved for the iron/ferrichrome complex (Locher *et al.* 1998). Moreover, determination of MIC obtained for a pure solution of MccJ25 on various mutants obtained for FhuA by **site-directed mutagenesis**, as well as of the kinetic and thermodynamic parameters governing the interaction of MccJ25 with such mutants, will state **the influence of the various regions** involved and thus supplement the qualitative study carried out yet (Endriss and Braun 2004). This technique was successfully used for the study of the interaction of FepA, a catecholate siderophore receptor, with different ligands (Annamalai *et al.* 2004).

The step following the biosynthesis of MccJ25, *i. e.* its export into the extracellular medium, is still to elucidate. Indeed, MccJ25 would be exported via the combined action of McjD, a protein homologous to an ABC transporter and encoded by the MccJ25 genetic system and of the chromosomally encoded TolC. However, while the export system of class II microcins, and colicin V in particular, were deeply studied *in vivo* and *in vitro* (Van Belkum *et al.* 1997, Guo *et al.* 2006), the involvement of McjD and TolC in MccJ25 export was only deduced from genetic studies (Solbiati *et al.* 1996, Delgado *et al.* 1999). Thus, **purifying McjD** is to be considered, so as **to study, *in vitro*, the export mechanism of MccJ25**. This could be carried out in collaboration with the group of Jean-Marie Pagès (INSERM, Marseilles) working on TolC.

Finally, the type of study that was undertaken on the uptake mechanism of MccJ25 must be considered for others microcins. Indeed, preliminary genetic studies showed that the antibacterial activity of MccE492, bearing or not the siderophore-modification in *C*-terminal position, as well as of MccM, involved the catecholate siderophore receptors FepA, Cir and Fiu (Thomas *et al.* 2004, Vassiliadis *et al.* submitted). This suggests that siderophore receptors could be responsible for the recognition and uptake of various microcins displaying diverse structures. It would thus be interesting to precisely **study the interaction of MccE492 and MccM with these receptors**. *In vivo*, the use of radio-labelled microcins will help determine the **adsorption (K_d) and transport (k_m, V_{max} and k_{cat}) parameters**, as it was achieved previously, using ^{59}Fe, for the FepA/FeEnt interaction (Annamalai *et al.* 2004). These parameters will then have to be compared with those obtained *in vitro*, by calorimetry measurements or **surface plasmon resonance**, for example. Then, the influence of MccE492 complexation to iron, as well as of the presence of the siderophore-modification might be studied.

Chapter II presented, through an article submitted to *Chemistry and Biology*, the results obtained on MccJ25 maturation process. We inactivated *mcjB* and *mcjC*, two genes of the MccJ25 genetic system whose products have undetermined function(s), and thus showed that McjB and McjC were involved in MccJ25 maturation process. Complementation of these mutated strains was carried out with the pET28-*mcjB* and pET28-*mcjC* plasmids, allowing the synthesis of His_6-McjB and His_6-McjC, respectively. McjB and McjC, as well as their substrate, McjA, the MccJ25 58-residue long linear precursor, were produced as recombinant proteins in *E. coli*, and purified for further characterization. First of all, we showed that the sequence formerly published for *mcjC* (EMBL AF061787) was erroneous, and that *mcjC* is in fact a 1542 b and not 1329 b gene (GenBank AM116873), encoding a 513- instead of 442-residue protein. Moreover, we highlighted an α-helical structuration of McjA in SDS micelles. But above all, we showed that McjB and McjC are not only necessary, but are sufficient for the conversion of McjA, inactive on *E. coli* strains, in active and lasso-structured MccJ25.

The study of similarities displayed by McjB and McjC sequences made it possible to assign them with putative functions. Indeed, the *N*-terminal region of McjC is similar to the *C*-terminal domain of asparagin synthetases class B, which catalyse the formation of intermolecular amide bonds, as well as to ß-lactam synthetases, which catalyse the formation of intramolecular amide bonds, these two reactions being ATP- and Mg^{2+}-dependent. This is in agreement with our enzymatic reaction requiring ATP and Mg^{2+}. Moreover, the *C*-terminal region of McjB is similar to various putative proteins, one of which is described as a transglutaminase catalysing the formation of an amide bond between glutamic acid and lysine, and which belongs to the family of cysteine proteases whose catalytic triad is Cys-His-Asp. The Cys150-His182-Asp194 triad is also found in McjB sequence, however, results obtained in this study with cysteine and serine protease inhibitors seem to suggest that the protease implied in the enzymatic mechanism is a serine protease. A serine in position 154 could replace Cys150 in this triad, leading to the Ser154-His182-Asp194 putative catalytic triad

We thus propose that McjB would be the protease responsible for the cleavage of McjA and that McjC would be the enzyme responsible for the Gly1-Glu8 lactam linkage.

This work gives rise to many prospects. Indeed, we showed that MccJ25 could be synthesized *in vitro*, thanks to the combined action of two enzymes only, McjB and McjC. Moreover, these enzymes can be produced as recombinant proteins in *E. coli*, and then purified while preserving their enzymatic activity. The biotechnological significance of this project was recognized by the European scientific community, since it was the subject of a

PAI European funding (**PROCOPE**) in collaboration with the "Fachbereich Chemie-Biochemie" laboratory of the University of Marburg, Germany, supervised by Pr. Mr. Marahiel.

At this stage, the main problems to be solved to study this enzymatic mechanism in great details are the production/purification yields of McjA, McjB and McjC. Various experiments aiming at decreasing McjA degradation in the producing bacteria (use of diverse protease inhibitors cocktails, different expression plasmids and multiple bacterial hosts) were useless. As the production of sufficient amounts of all three proteins constitutes a prerequisite for the study of the reaction mechanism, it will be necessary to develop an efficient method to produce high amounts of McjA, either by **chemical synthesis** or **cell-free protein expression**. Indeed, this latter technique, which allows the *in vitro* synthesis of few milligrams of recombinant protein (Klammt *et al.* 2004), would also make it possible to label McjA with ^{15}N and ^{13}C in order to **complement the preliminary CD structural study with NMR experiments**, and thus **confirm the α helical structuration of McjA in SDS micelles and determine the position of the helix** in the protein.

The **co-production of McjB and McjC** in the same bacterial host will be studied in more details, as it might prevent the aggregation and degradation of both proteins during their production. It is indeed likely that the two enzymes form a complex, stabilizing each protein. This production could be achieved using the **pET28-*mcjBC*** plasmid constructed during this PhD work. However, the construction of a plasmid enabling the production of the two proteins, each one bearing a specific tag, from optimized RBS, would facilitate the purification and could thus be considered, using the **pETDuet or pACYCDuet** plasmids (Novagen), for examples.

Various techniques will then enable to evidence the formation of a McjB/McjC complex: i) **concomitant purification of McjC and His_6-McjB**, by nickel affinity chromatography, starting from cytoplasm of bacteria harbouring the pET28-*mcjBC* plasmid, ii) His_6-McjB and His_6-McjC **coimmunnoprecipitation** using the anti-McjC antibodies produced in mouse during this PhD work, and iii) combined use of **native gel electrophoresis** and western-blots. In addition, the influence of McjA and ATP on the McjB/McjC complex formation shall be studied. Subsequently, McjB and McjC **oligomerization state**, as well as the **stoichiometry of the catalytic complex** will be characterized by **size-exclusion chromatography**. Finally, the **kinetic parameters (K_m, k_{cat} and V_{max}) of the catalytic complex** will be determined.

A more detailed characterization of the molecular interactions underlying the maturation process will be undertaken. Thus, in order to highlight **McjB and McjC residues involved in the enzymatic reaction**, *in vitro* conversion of McjA into MccJ25 will be carried out with mutated enzymes obtained by site-directed mutagenesis. We shall focus on the residues potentially supporting the mechanism, *i.e* the Cys150/Ser154, His182, Asp194 catalytic triad suggested for McjB, as well as residues located the hypothetical ATP and Mg^{2+} binding site in McjC (Ser199, Asp203, Ser204, Gly298 and Asp302). Furthermore, the region of McjA constituting the **prime determinant for recognition** by the enzymes will be examined. Our data suggest that this region would be located in the peptide leader (*N*-terminal). Thus, small peptides mimicking a size-varying fragment of the McjA leader peptide could be used to inhibit, by competition, the conversion of McjA into MccJ25. Similarly, the aptitude for being converted into MccJ25 of McjA truncated forms, whose peptide leader will have been shortened, will be evaluated.

Apart from microcins, the elucidation, for the first time, of a lasso-peptide maturation process raises the question of **the conservation of such a process** in the other species shown to synthesized lasso peptides. Indeed, as mentioned in chapter 2, peptides displaying lasso structures similar to MccJ25 were isolated from *Streptomyces* and *Rhodococcus* sp., and putative lasso peptides were found encoded on diverse genomes from Proteobacteria. The *in vitro* study of these systems should be considered, by constructing plasmids allowing the expression of the hypothetical enzymes and substrate starting from synthetic genes. Lasso peptide maturation mechanisms could thus be compared and permutation of the enzymes from these different systems will determine **the adaptability of this enzymatic system**.

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