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### **4.2.3. Class II Microcins**

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#### **Abstract:**

Class II microcins are 4.9 to 8.9 kDa polypeptides produced by and active against enterobacteria. They are classified into two subfamilies according to their structure and their gene cluster arrangement. While class IIa microcins undergo no post-translational modification, class IIb microcins show a conserved C-terminal sequence that carries a salmochelin-like siderophore motif as a posttranslational modification. Aside from this C-terminal end, which is the signature of class IIb microcins, some sequence similarities can be observed within and between class II subclasses, suggesting the existence of common ancestors. Their mechanisms of action are still under investigations, but several class II microcins use inner membrane proteins as cellular targets, and some of them are membraneactive. Like group B colicins, many if not all class II microcins are TonB- and energy-dependent and use catecholate siderophore receptors for recognition/translocation across the outer membrane. In that context, class IIb microcins are considered to have developed molecular mimicry to increase their affinity for their outer membrane receptors through their salmochelin-like post-translational modification.

#### 4.2.3.1 Introduction

Class II microcins are **higher molecular mass microcins** (4.9 to 8.9 kDa) and their peptide backbone does not undergo extensive post-translational modifications (Duquesne *et al.*, 2007). It has been recently shown that class II microcins were markers of virulence patterns in uropathogenic *Escherichia coli* (Azpiroz, Poey and Laviña, 2009). In this review, we have successively detailed the structures, the genetics, the biosynthesis, and the mechanisms of action of class II microcins. The organization of class II microcin gene clusters involves at least four clustered genes organized into one single or several transcription units. The minimal structure is composed of (i) the gene encoding the microcin precursor, (ii) an adjacent self-immunity gene, which encodes the self-immunity protein that protects the producing strain from its own antibacterial substance, and (iii) at least two genes encoding an inner membrane ABC (ATP-binding cassette) transporter and its accessory protein responsible for the secretion of the microcin. The nonspecialists should be aware that the name given to each gene is not standardized throughout the different microcin gene clusters. While class IIa microcins are not post-translationally modified, class IIb microcins show a conserved C-terminal sequence that carries a salmochelin-like siderophore motif as a post-translational modification. They are therefore considered as antibacterial siderophore-peptides (Thomas *et al.*, 2004). Siderophores are small iron chelators synthesized by bacteria and fungi for the uptake of the very low amount of iron available in physiological media (Andrews, Robinson and Rodriguez-Quinones, 2003). The enzymatic pathways leading to the class IIb microcin post-translational modification have been recently studied (Nolan *et al.*, 2007; Vassiliadis *et al.*, 2007; Nolan and Walsh, 2008; Mercado *et al.*, 2008) and various steps of class IIb microcin biosynthesis could be achieved *in vitro*. The mechanisms of action of class II microcins are still under investigations, most of the knowledge being acquired on their recognition/uptake. Like group B colicins, many if not all class II microcins are TonB- and energy-dependent and use catecholate siderophore receptors for recognition/translocation across the outer membrane (Chehade and Braun, 1988; Trujillo, Rodríguez and Laviña, 2001; Patzer *et al.*, 2003; Thomas *et al.*, 2004; Strahsburger *et al.*, 2005). As a consequence, the salmochelin-like post-translational modification of class IIb microcins increases their affinity for their outer membrane receptors (Thomas *et al.*, 2004). Such a molecular mimicry is a unique case among antimicrobial peptides.

#### 4.2.3.2 Class IIa microcins

Microcins (Mcc) belonging to this subclass are characterized by the absence of post-translational modification. MccV and MccL contain disulfide bond(s) whereas Mcc24 would be a linear unmodified peptide lacking such bond.

##### 4.2.3.2.1 Genetics and structure

Class IIa microcin gene clusters (Fig. 1) are composed of only **four plasmid-borne genes**. MccV, formerly colicin V (ColV) (Fredericq, 1949) was the first antibiotic substance reported to be produced by *E. coli* (Gratia, 1925). MccV is secreted by various *E. coli* strains harbouring large (> 80 kb) and low copy number pColV plasmids (Waters and Crosa, 1991). MccL is produced by *E. coli* LR05 isolated from poultry intestine (Gaillard-Gendron *et al.*, 2000), while Mcc24 (formerly colicin 24) is secreted by the uropathogenic *E. coli* 2424 and its genetic determinants are located on the 43.5-kb conjugative plasmid p24-2 (O'Brien and Mahanty, 1994). MccV and MccL gene clusters are composed of four genes organized in two converging transcription units (Gilson, Mahanty and Kolter, 1987; Chehade and Braun, 1988; Gilson, Mahanty and Kolter, 1990; Pons *et al.*, 2004). The genes *cvaC* and *mclC*, encoding a 103-amino acid MccV and a 105-amino acid MccL precursor, respectively, as well as the self-immunity genes *cvi* and *mclI* form the first operon. The second operon contains two genes (*cvaA/mclA* and *cvaB/mclB*) involved in the MccV and MccL export, respectively (Gilson, Mahanty and Kolter 1990; Pons *et al.*, 2004). In MccL gene cluster, downstream of *mclI*, one open reading frame (ORF) exhibits 98% identity with *cvi*, which makes MccL-producing strains resistant to MccV (Sablé *et al.*, 2003). Mcc24 gene cluster (GenBank accession number U47048) revealed four genes that would belong to one single operon (O'Brien and Mahanty, 1996, unpublished work). The genes *mtfS* and *mtfI* encode the putative 88-amino acid precursor and self-immunity protein of Mcc24, respectively, whereas *mtfA* and *mtfB* encode proteins similar to CvaA and CvaB, respectively.

The mature **MccV** is a 88-amino acid peptide, originating from a 103-amino acid precursor (CvaC) that possesses a single disulfide bond located in the C-terminal sequence (Table 1) (Håvarstein, Holo and Nes 1994). **MccL**, which is generated from a 105-amino acid precursor (MclC), is composed of 90 unmodified amino acids (Pons *et al.*, 2004). MccL is an anionic and highly hydrophobic peptide characterized by two disulfide bonds (Pons *et al.*, 2004). MccL and MccV share

an identical 13-amino acid C-terminal sequence that contains one disulfide bond (Table 1). **Mcc24** has neither been isolated nor characterized. Nevertheless, its precursor amino acid sequence, which shares homology with MceA, a class IIb microcin precursor (Table 1), but lacks the 10-amino acid C-terminal sequence typical of class IIb microcins (see 4.2.3.3.2), and its gene cluster, which contains four genes only, suggest strongly that Mcc24 belongs to class IIa microcin. The putative mature Mcc24 corresponds to a 73-amino acid peptide devoid of disulfide bond.

#### 4.2.3.2.2 Maturation and export

Many bacterial proteins are translocated across the inner membrane by the Sec pathway (de Keyzer, van der Does and Driessen, 2003). Nevertheless, some bacteriocins, like class II microcins, use ATP-binding cassette (ABC) transporters (Davidson and Maloney, 2007; Hollenstein, Dawson and Locher 2007; Moussatova *et al.*, 2008). For all class II microcins, maturation (cleavage of the leader peptide) and export appear to occur simultaneously. Except for MccV, whose mechanism of export has been partially characterized (Zhong, Kolter and Tai, 1996), the class II microcin export machineries have been mainly identified based on genetics and sequence identity analysis. The class II microcin export machinery displays a canonical structure consisting of three components.

The first component is an **ABC transporter**. Encoded by the microcin gene cluster, this protein is an homodimer carrying a nucleotide fixation domain. CvaB would be responsible for the export of MccV and, based on their highly similar sequence (up to 70% identity), MclB and MtfB, for the export of MccL and Mcc24, respectively. All three proteins are similar to ABC transporters responsible for the export of other antibacterials such as class II bacteriocins from Gram positive bacteria (Håvarstein, Diep and Nes 1995). Typically, ABC transporters exhibit three domains: (i) an N-terminal domain, supposed to be located in the cytoplasm (Franke *et al.*, 1999; Wu *et al.*, 2004), with a protease activity likely involved in the leader peptide cleavage during export, (ii) a central transmembrane domain, poorly conserved, and (iii) a C-terminal domain carrying a highly conserved nucleotide-binding cassette required for ATP binding (Benabdelhak *et al.*, 2003). A model was proposed in which the binding of the MccV precursor to CvaB promotes a transition between an inactive dimer bound to nucleotide diphosphate to a high-energy dimer bound to nucleotide triphosphate (Guo, Harrison and Tai, 2006).

The second component of the export machinery is the **ABC transporter accessory protein**. Based on bioinformatics studies, this protein, which is encoded by the microcin gene cluster, would be periplasmic with an N-terminal transmembrane helix that serves as an anchor at the inner membrane (Skvirsky, Reginald and Shen, 1995; Franke *et al.*, 1996). Like ABC transporters, accessory proteins are also highly conserved. CvaA, MclA and MtfA are accessory proteins for MccV, MccL, and Mcc24, respectively. Their function in microcin secretion remains unclear. However, they have been proposed to serve as a connector between the ABC transporter and the third component of the class II microcin export machinery (Gilson, Mahanty and Kolter, 1990; Pons *et al.*, 2004).

The third component of the machinery is **TolC**, located at the outer membrane. Unlike genes encoding the ABC transporter and the accessory protein, *tolC* is located on the bacterial chromosome outside of the microcin gene cluster. This protein forms a trimeric channel with an  $\alpha$  helix in the periplasmic space, and a  $\beta$  barrel channel spanning the outer membrane (Koronakis *et al.*, 2000; Koronakis, Eswaran and Hughes, 2004). The expression of TolC is required for the production of extracellular antimicrobials by MccV- and MccL-producing strains (Gilson, Mahanty and Kolter, 1990; Pons *et al.*, 2004). Therefore, TolC is considered to be required for the export of class II microcins.

#### 4.2.3.2.3 Recognition/uptake and mechanism of action

Class IIa microcins have been poorly studied in terms of mechanism of action compared to class IIb. Most of the data are speculative and based on sequence similarities with microcins with known mechanism of action.

**MccV** (ColV) is active against Gram-negative bacteria belonging to the genera *Escherichia*, *Klebsiella*, *Salmonella* and *Shigella* (Håvarstein, Holo and Nes, 1994). Like class IIb microcins, MccV is recognized by an outer membrane receptor for catecholate siderophores, and its uptake is TonB-dependent (Chehade and Braun, 1988). It is therefore likely taken up into sensitive cells at the expense of energy which is provided by the proton motive force of the inner membrane. However, while class IIb microcins are recognized by FepA, Cir and Fiu, Cir alone appears to be involved in MccV uptake (Chehade and Braun 1988). MccV activity also depends on the inner membrane protein SdaC, also involved in serine uptake (Gerard, Pradel and Wu, 2005). Although the mechanistic role of SdaC in MccV uptake and/or activity remains unknown, it could drive MccV to the inner membrane,

thereby promoting channel formation and subsequent disruption of membrane potential (Yang and Konisky, 1984).

**MccL** spectrum of activity covers a large number of Gram-negative species including *E. coli*, *Salmonella enterica*, *Klebsiella oxytoca*, *Shigella sonnei*, *Shigella flexneri*, *Pseudomonas aeruginosa*, and *Providencia stuartii* (Pons *et al.*, 2004). However, to date MccL has been poorly studied in terms of mechanism of action. Since it displays a 32-residue C-terminal sequence highly similar to that of MccV (87.5% identity), it could also have membrane-permeabilizing properties (Pons *et al.*, 2004). This conserved region could also be required for recognition and uptake, since like MccV, MccL is recognized by Cir and imported in a TonB-dependent manner (Sablé, personal communication).

**Mcc24** was reported to be active against the enterobacteria *E. coli* and *S. enterica* Typhimurium but not against *Listeria monocytogenes* or *Campylobacter jejuni* (Wooley, Gibbs and Shotts Jr, 1999). To date, nothing is known regarding its uptake and mechanism of action. It is actually the only class II microcin for which nothing is known in terms of recognition by catecholate siderophore receptors and their associated TonB-ExbB-ExbD complex. Mcc24 displays striking sequence similarities with MccE492 (Lagos *et al.*, 1999) but lacks the C-terminal region required for recognition by catecholate siderophore receptors. Based on their sequence identities, it was suggested that Mcc24 and MccE492 could have similar cellular targets and would both require ManYZ at the inner membrane (Bieler *et al.*, 2006).

#### 4.2.3.3 Class IIb microcins: the siderophore-microcins

Class IIb microcins are linear polypeptides carrying a **C-terminal siderophore as a post-translational modification** (MccE492, MccH47, MccM and presumably MccG492 and MccI47). These microcins are characterized by a highly conserved 10-amino acid C-terminal sequence, which is considered their signature (Vassiliadis *et al.*, 2010).

##### 4.2.3.3.1 Genetics

Contrary to class IIa microcins, which are all plasmid-encoded, class IIb microcins are **chromosome-encoded** and their gene clusters show a complex transcriptional organization (Fig. 2).

In addition to the four genes described in class IIa microcin gene clusters, genes encoding post-translational modification enzymes are encountered in class IIb microcin gene clusters.

The best characterized class IIb microcin is **MccE492**, which is secreted by *Klebsiella pneumoniae* RYC492 (de Lorenzo, 1984). The entire MccE492 gene cluster is contained within a 13-kb DNA fragment that has been cloned in *E. coli* (Wilkens *et al.*, 1997). Ten genes (*mceABCDEFGHIJ*) (Lagos *et al.*, 2001), organized at least in five transcription units, are necessary for MccE492 biosynthesis. The gene *mceA* encodes the 99-amino acid MccE492 precursor and *mceB* is involved in the self-immunity towards MccE492 (Lagos, Villanueva and Monasterio, 1999). Other genes are required for MccE492 post-translational modification. Those are *mceC*, *mceD* and *mceI*, which encode proteins homologous to a glycosyltransferase, an enterobactin esterase, and an acyltransferase, respectively, (Nolan *et al.*, 2007; Vassiliadis *et al.*, 2007), as well as *mceJ*, which does not display evident homologies with known genes (Corsini *et al.*, 2002; Nolan *et al.*, 2007). Two genes, *mceG* and *mceH*, are necessary for the export of MccE492. They encode an ABC transporter and an accessory protein, respectively. The *mceF* gene would also be involved in export (Lagos *et al.*, 2001) and the role of the last gene, *mceE*, remains unknown. The nucleotide sequence of this gene cluster has been recently corrected (GenBank accession number AF063590) showing that the orientation of *mceFGHIJ* is reverse to that previously described. The corrected sequence also unveiled six novel ORFs (*mceS2/mceS3/mceM/mceL/mceX/mceK*), among which *mceL* and *mceM* encode the 89-amino acid precursor of a new putative microcin termed **MccG492** and its self-immunity protein, respectively (Vassiliadis *et al.*, 2010).

As with *K. pneumoniae* RYC492, several microcins can be expressed by one single *E. coli* isolate. Thus, *E. coli* H47 was reported to express both **MccH47** and **MccI47** (Laviña, Gaggero and Moreno, 1990; Poey, Aspiroz and Laviña, 2006). Similarly, *E. coli* Nissle 1917 (also named Mutaflor), *E. coli* CA46, and *E. coli* CA58 strains express **MccM**, **MccH47** and possibly **MccI47** (Nissle 1925; Patzer *et al.*, 2003; Duquesne *et al.*, 2007; Vassiliadis *et al.*, 2010). The genetic determinants required for MccH47, MccI47 and MccM biosynthesis are all located within a 10.5-kb to 14-kb DNA fragment (Laviña, Gaggero and Moreno, 1990; Patzer *et al.*, 2003; Vassiliadis *et al.*, 2010). Analysis of the four microcin gene clusters showed that they share a common organization except for the 5' region located upstream of *mchX* (Fig. 2). Each microcin gene cluster is characterized by **common genes (homologues)** involved in microcin export. Those are *mchE*, *mchF* and *mcmM*, the later being

truncated in *E. coli* H47 (Gaggero, Moreno and Laviña, 1993; Azpiroz, Rodríguez and Laviña, 2001; Patzer *et al.*, 2003; Poey, Aspiroz and Laviña, 2006). Similarly, four genes required for the post-translational modification of class IIb microcins are common to the microcin gene clusters. Those genes are *mchA/mcmL*, *mchS1/mcmK*, *mchC* and *mchD*, which are homologous to *mceC*, *mceD*, *mceJ* and *mceI* from MccE492 gene cluster, respectively (Vassiliadis *et al.*, 2010). However *mcmL* and *mcmK* are lacking in *E. coli* Nissle 1917. Finally, two **specific genes** encode each microcin precursor and self-immunity protein. Thus, *mchB* encodes the 75-amino acid MccH47 precursor (Rodríguez, Gaggero and Laviña, 1999) and *mchI* confers the self-immunity towards MccH47 (Rodríguez and Laviña, 1998). The gene *mchS2* encodes the 77-amino acid MccI47 precursor and *mchS3* confers the specific self-immunity towards MccI47 (Poey, Aspiroz and Laviña, 2006). The *mcmA* (formerly *mcmC*) (Braun *et al.*, 2002), encodes the 92-amino acid MccM precursor, and *mcmI* encodes the MccM self-immunity protein (Patzer *et al.*, 2003). The role of *mchS4* and *mchX* located between *mchS3* and *mchI* remains unclear (Rodríguez and Laviña, 1998; Azpiroz and Laviña, 2004). In the 5' region of microcin gene clusters from *E. coli* CA58 and Nissle 1917 are located genes encoding transposase and insertion sequences, which strongly supports the hypothesis of an horizontal gene transfer of microcin gene clusters. Complete or partial MccM gene cluster are also encountered in the pathogenicity island of the uropathogenic *E. coli* strains (Dobrindt *et al.*, 2001; Welch *et al.*, 2002; Dezfulian, Tremblay and Harel, 2004).

#### 4.2.3.3.2 Structures

MccE492 was initially described as an 84-residue unmodified peptide (uMccE492) (Pons *et al.*, 2002) deriving from the MccE492 precursor (MceA). Culture conditions were found to be critical to obtain mature MccE492, which carries an original post-translational modification as revealed by mass spectrometry and nuclear magnetic resonance (Thomas *et al.*, 2004). This modification consists of a C-glucosylated linear trimer of *N*-(2,3 dihydroxybenzoyl)-L-serine (DHBS) linked to the C-terminal serine carboxylate via an *O*-glycosidic bond (Fig. 3) (Thomas *et al.*, 2004), a structure reminiscent of catecholate siderophores, and especially of salmochelin S4 (Hantke *et al.*, 2003). Thus, MccE492 was the first example of a natural siderophore-peptide. With the characterization of novel microcins carrying a siderophore post-translational modification, the name of siderophore-microcins was given to class IIb microcins. Indeed, MccM and MccH47 were recently shown to be 77 and 60 residue-peptides



(Table 1) deriving from the microcin precursors MccM and MccH47, and carrying a C-terminal post-translational modification similar to that previously described for MccE492 (Vassiliadis *et al.*, 2010). As such, they unambiguously belong to the siderophore-microcin family. However, when isolated from *E. coli* Nissle 1917, MccM and MccH47 did not carry any modification. The absence of post-translational modification was correlated to the absence of *mcmL* and *mcmK* in microcin gene clusters, two genes involved in the biosynthesis of the post-translational modification (Vassiliadis *et al.*, 2010). Two other putative members, MccG492 and MccI47, which remain to be fully characterized, would also belong to the siderophore-microcin family. After leader-peptide cleavage, putative MccG492 and MccI47 would contain 74 and 62 residues deriving from the microcin precursors MccL and MccS2, respectively (Table 1). Consequently, the class IIb microcins form the first homogeneous family amongst microcins, the siderophore-microcins.

#### 4.2.3.3.3 Biosynthesis and export

Several recent studies have shown that aside from the microcin gene clusters, genes involved in the biosynthesis of enterobactin and salmochelin are required for class IIb microcin biosynthesis, and that both siderophores are precursors for the post-translational modification (Azpiroz and Laviña, 2004; Nolan *et al.*, 2007; Vassiliadis *et al.*, 2007; Mercado *et al.*, 2008). This chapter describes the biosynthetic pathways that lead to siderophore-peptides, a unique combination of ribosomal and non-ribosomal biosynthetic steps (Nolan and Walsh 2008).

##### 4.2.3.3.3.1 Enterobactin and salmochelin biosynthesis

The maturation of class IIb microcins is dependent on catecholate siderophores. Catecholate-siderophores are named according to the chemical group that chelates iron and are one class of siderophores among the 500 siderophore structures described until now (Neilands, 1995; Crosa and Walsh, 2002; Miethke and Marahiel 2007). **Enterobactin** (also termed enterochelin) is a catecholate siderophore that was first isolated in *S. enterica* Thyphimurium (Pollack and Neilands, 1970) and *E. coli* (O'Brien, Cox and Gibson, 1970), and later in *Streptomyces* (Fiedler *et al.*, 2001), *Klebsiella* and *Shigella* species (Payne *et al.*, 1983; Podschun, Fischer and Ullmann, 1992). It consists of a cyclic trimer of DHBS that chelates iron through its three dihydroxybenzoate (also termed catechol) groups. The coordination between enterobactin and iron is of the hexadentate type. Enterobactin was

described as the best iron chelator isolated until now (Ecker, Matzanke and Kocher, 1986). More recently, a new class of siderophore, named **salmochelins**, was isolated from *Salmonella enterica* Thyphimurium (Hantke *et al.*, 2003). Also produced by several uropathogenic *E. coli* and *K. pneumoniae* strains, salmochelins derive from enterobactin by the addition of one or two glucose moieties on the enterobactin catechol group.

### **Enterobactin biosynthesis**

Most of the known siderophores are synthesized by the multienzymatic pathway and particularly by the nonribosomal peptide synthetases (NRPS) pathway (Miethke and Marahiel, 2007). Two main pathways lead to enterobactin biosynthesis.

The first pathway, which serves for the initial steps of enterobactin biosynthesis, is the **shikimate pathway**. From phosphoenol pyruvate and erythrose 4-phosphate, this pathway leads to chorismate, which is a common intermediate to enterobactin and aromatic amino acids biosynthesis. Genes involved in this pathway are *aroABCDEFGHKL*. Tyrosine, phenylalanine and tryptophan have been described as inhibitors of the shikimate pathway (Cobbett and Delbridge, 1987; Grove and Gunsalus, 1987; Heatwole and Somerville, 1992).

The second pathway, which serves for the last steps of enterobactin biosynthesis, involves an **NRPS** (Walsh *et al.*, 1990; Crosa and Walsh, 2002; Raymond, Dertz and Kim, 2003; ). Extensive studies have been performed on specific NRPS which lead from chorismate to enterobactin. All genes involved in this pathway are located in the enterobactin gene cluster. This 20 kb fragment, organized in six operons, contains 16 genes. Seven (*entABCDEFG* and *ybdB*) are involved in enterobactin synthesis, five (*fepABCDG*) in enterobactin uptake, one (*entS*) in enterobactin export, one (*fes*) in the release of iron from the siderophore, and two genes (*ybdZ* and *fepE*) still have unknown functions. The enterobactin gene cluster is regulated by two *fur* (ferric uptake regulator) boxes enabling the transcription regulation according to bacterial iron concentration (Brickman, Ozenberger and McIntosh, 1990). Thus, to achieve enterobactin biosynthesis, chorismate is subjected to three reactions leading to dihydroxybenzoate (DHB) (Sakaitani *et al.*, 1990; Gehring, Mori and Walsh, 1998). Afterwards, DHB is activated by adenylation and charged onto an aryl carrier protein. In parallel, a serine is also activated by adenylation and charged onto a peptidyl carrier protein (Reichert, Sakaitani and Walsh, 1992; Lambalot *et al.*, 1996; Gehring, Bradley and Walsh, 1997). DHB and serine are finally ligated by a condensation domain and transferred onto another peptidyl carrier protein. These steps are

repeated three times and the final trimer of DHB-serine is transferred onto a thioesterification domain which cyclizes and releases simultaneously the trimer to finally produce the enterobactin (Shaw-Reid *et al.*, 1999).

### **Salmochelin biosynthesis**

Conversion of enterobactin into salmochelins requires a 10 kb DNA fragment containing five genes, *iroBCDEN* (Bäumler *et al.*, 1996; Bäumler *et al.*, 1998) and two *fur* boxes (Bäumler *et al.*, 1996). The gene *iroB* is involved in salmochelins biosynthesis, *iroC* in salmochelins export, *iroN* in salmochelins import while *iroD* and *iroE* are involved in the release of iron chelated by salmochelins and degradation of these siderophores (Lin *et al.*, 2005; Zhu *et al.*, 2005). Enterobactin is diglucosylated by IroB on two catechol groups leading to salmochelin S4 formation (Bister *et al.*, 2004; Fischbach *et al.*, 2005). Salmochelin S2 is the linear form of salmochelin S4 while salmochelins S1, and SX are the monoglucosylated dimer and the monomer of DHBS, respectively. Salmochelins S2, S1 and SX are consequently degradation products of salmochelin S4.

#### **4.2.3.3.2 Siderophore microcin biosynthesis**

MccE492 was the first microcin found to carry a salmochelin-like siderophore as a post-translational modification (Fig. 3). As a consequence, it has been the most extensively investigated of the siderophore-microcins. The genes *mceC* and *mceD* from MccE492 gene cluster display strong identities sequences with *iroB* and *iroD*, which are involved in enterobactin glucosylation and linearization, respectively. *In vivo* and *in vitro* experiments showed that enterobactin and salmochelin are precursors for MccE492 post-translational modification (Vassiliadis *et al.*, 2007; Mercado *et al.*, 2008). Thus, while microcins use siderophore biosynthetic pathways for the initial steps of their post-translational modification, the last steps are encoded by the microcin gene clusters themselves. It was recently shown for MccE492 that MceC and MceD monoglucosylate and linearize enterobactin, respectively (Nolan *et al.*, 2007), and that MceI and MceJ are responsible for the O-glucosidic bond formation between the modified enterobactin and the peptide backbone (Nolan and Walsh, 2008).

The biosynthesis of MccM and MccH47, the two recently characterized siderophore-microcins, was also investigated (Vassiliadis *et al.*, 2010). Like MccE492, MccH47 post-translational modification is dependent on the enterobactin synthesis pathway (Azpiroz and Laviña, 2004). Interestingly, MchA and MchL display 85% identity with MceC, while MchS1 and MchK display 75% identity with MceD.

By coupling gene complementation to mass spectrometry analysis, our group recently showed that MchA, MchL, MceC, on the one hand, and MchS1, MchK, MceD, on the other hand, are required for siderophore-microcin post-translational modification, and that each group of genes displays the same function, *i.e.* enterobactin C-glucosylation and linearization, respectively (Vassiliadis *et al.*, 2007; Vassiliadis *et al.*, 2010). Similarly, MchC and MchD display 71% and 80% identity with MceJ and MceI, respectively, and complement each other (Nolan and Walsh, 2008). Therefore, MchC and MceJ, on the one hand, as well as MchD and MceI, on the other hand, most likely display the same function in O-glucosylation of the class II microcin C-terminal serine. Together with earlier heterologous functional complementation assays (Poey, Aspiroz and Laviña, 2006), this indicates that all genes involved in siderophore-microcin post-translational modification are probably interchangeable from one gene cluster to another.

MccI47 and the putative MccG492 have been very recently described, and as a consequence, little is known on their maturation. However, MccI47 structure and immunity genes are located within MccM/MccH47 gene cluster (Poey, Aspiroz and Laviña, 2006) while for MccG492 these genes are located within MccE492 gene cluster (Vassiliadis *et al.*, 2010). Because MccM and MccH47 use the same genes for their post-translational modification, one can speculate that those same genes modify MccI47 so that it carries a similar siderophore post-translational modification. Similarly, the genes required for MccE492 post-translational modification could modify MccG492.

A **model** is proposed for **siderophore-microcin biosynthesis** (Fig. 3) in which after biosynthesis, enterobactin is C-glucosylated by MceC/MchL/MchA and linearized by MceD/MchK/MchS1. The linearized glucosylated enterobactin (salmochelin) is then linked to the microcin precursor MceA (for MccE492), MchA (for MccM), MchB (for MccH47), MchS2 (for MccI47) or MceL (for MccG492) through O-glycosylation. This biosynthetic step is catalyzed by MceIJ or MchCD. Finally, cleavage of the leader peptide occurs simultaneous to the export by a dedicated ABC transporter encoded by the microcin gene cluster (see section 4.2.3.3.1). The existence of MccE492, MccM and MccH47 intermediates carrying a dimer and a monomer of glucosylated DHBS instead of the classical trimer (Vassiliadis *et al.*, 2007; Vassiliadis *et al.*, 2010) could result from the degradation of glucosylated enterobactin by MceD/MchK/MchS1, followed by linkage to the microcin precursor and cleavage of the leader peptide, as described above.

#### 4.2.3.3.3 Maturation and export

Analyses of class IIb microcin gene clusters led to several hypotheses on the cleavage of class IIb microcin leader peptide and associated export. The mechanism by which the microcins are matured and exported is supposed to be the same for all class II microcins (see section 4.2.3.2.2). The ABC transporters of MccE492/MccG492 and MccH47/MccM/MccI47 would be MceG and MchF, respectively. The accessory proteins of MccE492/MccG492 and MccH47/MccM/MccI47 would be MceH and MchE, respectively. Finally, TolC would be the outer membrane component of all class IIb microcin export machineries as shown for MccH47, whose antimicrobial activity is dependent on *tolC* (Gaggero *et al.*, 1993). An additional inner membrane protein could also be required for class IIb microcin export. Indeed, MccE492 export machinery seems to require MceF, a putative inner membrane protein that could interact with MceGH for processing or export (Lagos *et al.*, 2001). Similarly, MccM, which displays 62% identity with MceF, could have the same function in MccM/MccH47/MccI47 export.

#### 4.2.3.3.4 Recognition/uptake and mechanism of action

Among class II microcins, the siderophore-microcins (class IIb microcins) have been the best studied in terms of mechanism of action. While the mechanisms responsible for class IIb microcin antibacterial activity are still incompletely understood, it is now established that their conserved C-terminal domain as well as their siderophore moiety are key determinants for their recognition/uptake at the outer membrane of enterobacteria.

##### 4.2.3.3.4.1 Recognition/uptake by the receptors for catecholate siderophores

Spectra of antibacterial activity have been established very early for class IIb microcins. However, as presented in the genetics section, class IIb microcin-producing strains often express several class IIb microcins. The recent progresses in the purification of microcins have rendered possible the determination of reliable but non-exhaustive activity spectra. Thus, using pure microcin preparations or preparations that contain one single microcin, MccE492 was shown to be active against strains of *E. coli*, *Enterobacter cloacae*, *S. enterica*, and *K. pneumoniae*, MccM was active against *S. enterica* and *E. coli*, while MccH47 would to be active against *E. coli* only (Destoumieux-Garzón *et al.*, 2003; Thomas *et al.*, 2004 Vassiliadis *et al.*, 2010).

- **Determinants of class IIb microcin susceptibility in enterobacteria**

Two common features to all microcins are (i) an extremely potent activity, with minimum inhibitory concentrations (MICs) often below 0.1  $\mu$ M (Thomas *et al.*, 2004; Destoumieux-Garzón *et al.*, 2005), and (ii) a narrow spectrum of activity limited to few genera of enterobacteria, as illustrated above. The reason for that is a receptor-mediated recognition/uptake, which uses outer membrane proteins normally involved in nutrient uptake as receptors or docking molecules (Salomón and Farias, 1993; Laviña, Pugsley and Moreno, 1986; Pugsley, Moreno and de Lorenzo, 1986; Salomón and Farias, 1995; Trujillo, Rodríguez and Laviña, 2001). Those outer membrane proteins are also used by bacteriophages, antibiotics, and colicins for cell entry. They are considered an "Achilles' heel" for the bacterium.

- **The catecholate siderophore receptors FepA, Cir, Fiu and IroN**

Over the past years substantial knowledge has been acquired on microcin recognition showing that only enterobacteria that express catecholate siderophore receptors are susceptible to class IIb microcins. In *E. coli*, class IIb microcins parasitize the **FepA, Cir and Fiu receptors** involved in the uptake of catecholate siderophores (*e.g.* enterobactin) (Trujillo, Rodríguez and Laviña, 2001; Patzer *et al.*, 2003; Thomas *et al.*, 2004; Strahsburger *et al.*, 2005). The requirement of MccE492 for catecholate siderophore receptors is illustrated by a shift from a potent activity against *E. coli* H1443 (MIC = 40 nM) to a lack of activity against the isogenic *fepA cir fiu* triple mutant (MIC > 10  $\mu$ M) (Thomas *et al.*, 2004). In *Salmonella*, class IIb microcins would use **IroN**, FepA and Cir receptors (Patzer *et al.*, 2003). FepA is by far the best studied of the four receptors. Its three-dimensional structure is composed of a  $\beta$ -barrel embedded in the outer membrane with an N-terminal globular domain, either called the plug or the cork domain, folded inside the barrel (Buchanan *et al.*, 1999). This domain spans most of the interior of the barrel and occludes it. The globular domain is connected to the  $\beta$ -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 (Annamalai *et al.*, 2004). During the transport process, the globular domain of FepA would be expelled from the  $\beta$ -barrel (Ma *et al.*, 2007). Not only does FepA transport catecholate siderophores, but it also serves as a receptor/transporter for group B colicins and bacteriophages (Letellier and Santamaria, 2002; Rabsch *et al.*, 2007).

- **The energy-transduction system TonB/ExbB/ExbD**

The catecholate siderophore receptors use the **TonB-ExbB-ExbD inner membrane complex** as an associated energy-transduction system for the active uptake of iron. 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 where it drives the active transport by high-affinity outer membrane transporters (Postle and Kadner, 2003). ExbB and ExbD were proposed to influence the dimerization of TonB although they would not be strictly required (Sauter, Howard and Braun, 2003), and TonB-ExbD interaction was recently shown to be dependent on the proton motive force (Ollis *et al.*, 2009). TonB is required for the translocation across the outer membrane and the antibacterial activity of various microcins including class IIb microcins (Braun *et al.*, 2002; Pugsley *et al.*, 1986; Destoumieux-Garzón *et al.*, 2003; Thomas *et al.*, 2004; Trujillo, Rodríguez and Laviña *et al.*, 2001; Strahsburger *et al.*, 2005; Vassiliadis *et al.*, 2010). However, ExbB and ExbD are often dispensable, as evidenced for MccE492, MccM and MccH47 (Thomas *et al.*, 2004; Vassiliadis *et al.*, 2010). A similar ExbB-independent uptake has been reported for bacteriophage H8, which like class IIb microcins and group B colicins is a FepA ligand. Interestingly, when both ExbBD and TolQR systems are impaired, *E. coli* becomes resistant to bacteriophage H8 infection (Rabsch *et al.*, 2007). Similar functional complementation of ExbBD by TolQR may occur for the uptake of class IIb microcins.

- **Determinants of class IIb microcin recognition by catecholate siderophore receptors**

- **The salmochelin post-translational modification**

In a recent study, our group showed that the three class IIb microcins on which structural data have been acquired (*i.e.* MccE492, MccM and MccH47) possess a similar post-translational modification (see structure section) when expressed in a favourable genetic background (Vassiliadis *et al.*, 2010). Since this modification mimics the catecholate siderophore salmochelin and behaves as a siderophore (Thomas *et al.*, 2004), it was hypothesized that it could play a major role in class IIb microcin recognition. MccE492 is to date the only class IIb microcin for which modified and unmodified forms have been separated and purified in sufficient amounts to measure minimal inhibitory concentrations (MICs). Interestingly, the activity of the unmodified MccE492 (u-MccE492) against *E. coli* H1443 is 4 to 8-fold lower than that of the mature microcin (Thomas *et al.*, 2004) indicating that the

salmochelin post-translational modification significantly enhances MccE492 recognition at the outer membrane of *E. coli*. As shown recently, the same post-translational modification is observed in MccM, MccH47, and MccE492 (Vassiliadis *et al.*, 2010), and is likely to occur in the recently discovered MccI47 (Poey, Aspiroz and Laviña, 2006; Nolan and Walsh, 2008) and MccG492 (Vassiliadis *et al.*, 2010). Therefore, as shown for MccE492, the salmochelin post-translational modification is expected to increase the antibacterial activity of all class IIb microcins by mimicking the natural ligands of iron-siderophore receptors.

- **The serine-rich C-terminal region**

The unmodified MccE492 (u-MccE492) lacking the salmochelin post-translational modification was shown to be active against *E. coli* but, as the mature microcin, it was inactive against the isogenic *fepA cir fiu* triple mutant (Destoumieux-Garzón *et al.*, 2006). This showed that molecular motives other than the sole post-translational modification are involved in MccE492 recognition by the catecholate siderophore receptors. Later, Bieler *et al.* showed that the serine-rich C-terminal sequence of MccE492 was essential for the activity of extracellular but not intracellular MccE492 (Bieler *et al.*, 2006). Therefore, the C-terminal sequence is required for receptor recognition and/or translocation across the outer membrane. Because this C-terminal sequence is highly conserved among class IIb microcins, being considered as a signature for the family, it is very likely that, as demonstrated for MccE492, all class IIb microcins are recognized by catecholate-siderophore receptors through their serine-rich C-terminal sequence.

#### **4.2.3.3.4.2 Antibacterial activity of class IIb microcins**

While class IIb microcins exhibit a conserved C-terminal domain required for recognition, the N-terminal domain is very diverse in terms of sequence (Table 1). Interestingly, the N-terminal domains of class IIb microcins display homologies with those of class IIa microcins. Therefore, class IIb N-terminal domains likely endow the antibacterial activity of the microcins. This is largely supported by the finding that a C-terminally truncated MccE492 remains active provided it is expressed intracellularly (Bieler *et al.*, 2006). Among class IIb microcins, only MccE492 and MccH47 have been investigated in terms of mechanism of action, certainly due to the recent identification of MccI47, MccM and MccG492. Data on those last three microcins are limited to their recognition at the outer membrane of *E. coli* (see the above section).



MccE492 membrane-disruption properties have been identified very early, well before the peptide was isolated and characterized. Thus, *in vivo*, culture supernatants containing MccE492 depolarised the inner membrane of *E. coli* (de Lorenzo and Pugsley, 1985). This was later confirmed with homogenous peptide preparations, showing that the MccE492 activity was energy- and TonB-dependent (Destoumieux-Garzón *et al.*, 2003; Destoumieux-Garzón *et al.*, 2006). Interestingly, the damages to the inner membrane were not responsible by themselves for the lethal effect of neither u-MccE492 nor MccE492, and did not result in cell lysis even at lethal concentrations (Destoumieux-Garzón *et al.*, 2003; Destoumieux-Garzón *et al.*, 2006). *In vitro*, the microcin was able to form ion-channels in planar lipid bilayers (Lagos *et al.*, 1993). The u-MccE492 pore-forming activity could be observed at concentrations as low as  $2 \times 10^{-10}$  M, and the insertion was shown to be voltage-independent (Destoumieux-Garzón *et al.*, 2003). Altogether, this strongly suggests that the damages to *E. coli* inner membrane rely on a pore-forming activity.

Although the mechanistic details of MccE492 bactericidal activity are still incompletely understood, Bieler *et al.*, evidenced that MccE492 targets **the mannose permease**, *manY* and *manZ* being critical genes for MccE492 antibacterial activity against *E. coli* (Bieler *et al.*, 2006). 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 (Erni *et al.*, 1987; Williams *et al.*, 1986). The *manYZ* mutants resistant to MccE492 were unable to metabolize mannose and became insensitive to the inner membrane depolarisation mediated by periplasmic MccE492 (Bieler *et al.*, 2006). Therefore, the mannose permease is required for MccE492 antibacterial activity but may not be the only intracellular target of the microcin.

Unlike MccE492, MccH47 has never purified to homogeneity (Vassiliadis *et al.*, 2010). Nonetheless, several studies by Laviña and collaborators have documented the mechanism of action of MccH47. The authors initially showed that the *atp* operon conferred susceptibility to MccH47 (Rodríguez, Gaggero and Laviña, 1999). Later, they found that MccH47 targets the F<sub>0</sub>F<sub>1</sub> **ATP synthase**, and particularly its F<sub>0</sub> membrane component, which serves as a proton channel, while the F<sub>1</sub> catalytic unit was dispensable (Trujillo, Rodríguez and Laviña, 2001; Rodríguez and Laviña, 2003). We recently found that contrary to MccE492, MccM and MccH47 are active against *manXYZ* mutants (Peduzzi and Vandervennet, unpublished results). Therefore the mannose permease is not required for all siderophore-microcins antibacterial activity. Consequently, while the conserved C-terminal

sequence of class IIb microcins is responsible for their recognition, this reinforces our hypothesis that their non-conserved N-terminal sequence is responsible for the interference with specific cellular functions.

## **Concluding remarks**

The recent progress on class II microcins gathered in this review have shown that their mechanism of action relies on the recognition by iron-siderophore receptors and that one subclass (class IIb) uses molecular mimicry to improve recognition by iron receptors. Interestingly, the siderophores, which serve as precursors for the post-translational modification of class IIb microcins, are recognized as important virulence factors involved in the battle for iron (Demir and Kaleli, 2004; Lawlor, O'Connor and Miller, 2007; Caza *et al.*, 2008). Moreover, the siderophore-encoding gene clusters are usually located in genomic islands as also found for class IIb microcins in *E. coli* Nissle 1917 (Grozdanov *et al.*, 2004). Together with the modular structure of their gene cluster, this strongly suggests that class II microcins are subject to extensive horizontal gene transfer, in agreement with an important role in microbial competition and/or virulence. The recent studies on colicins, large antimicrobial proteins expressed by enterobacteria that use similar iron receptors for recognition, indicate that their expression favours bacterial persistence in the gastrointestinal tract (Kirkup and Riley, 2004; Gillor *et al.*, 2009). Together with a recent study on uropathogenic class II microcins (Azpiroz, Poey and Laviña, 2009), this strongly supports the role of class II microcins in enterobacterial virulence and *in vivo* fitness.

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Table 1. Multiple amino acid sequence alignment of class II microcin precursors

Class			Leader peptide :	mature microcin
IIa	CvaC <sup>a</sup>	(MccV) <sup>b</sup>	MRITLTNELDSVSGG :	ASGR--DIAMIGT LSGQFVAGGIGAAAGGVAGGAIYDYASTHKPNPAMSPSGLGGTIKQKPEGIPSEAWNYAAGRLCNWSPNNLSDVCL
	MclC <sup>a</sup>	(MccL) <sup>b</sup>	MREITLNEYNNVSGA :	GDVNWVDVGKTVAI NGAGVIGGAFGAGLCGPVCAGAFVGSAAVAALYDAAGNSNAKQKPEGLPPEAWNYAEGRM CNWSPNNLSDVCL
	MtfS <sup>a</sup>	(Mcc24) <sup>b</sup>	MRELDREELNCVGGG :	GDPL-ADPNSQIVRQIMSNAANGPPL-VPERFRMAVGAAGGVTQTVLQGA AHMPVNVPIPKVPMGPSWN-GS-KG
IIb	MceA <sup>a</sup>	(MccE492) <sup>b</sup>	MREISQKDLNLAFGA :	GE---TDPNTQLLNDLGNNMANGAALGAPGGLGSAALGAAGGALQTVGQGLIDHGPVNVPIP-VLIGPSWN-GSGSGYNSATSSSGSGS
	McmA <sup>a</sup>	(MccM) <sup>b</sup>	MRILSENEIKQISGG :	DG---NDGQAEILIAIGS--LA-GT-FISPG-FGSIAGAYIGDKVHSWATT-ATVSPSMSPSGIGL-SSQF--GSGRGTSSASSSAGSGS
	MceL <sup>a</sup>	(MccG492) <sup>b</sup>	MRALTENDFFAVSGA :	DR---GDAAVAGAVAG--GTA-GAAAGGWAGCAQMGATVGSAGPVGTVVGFVAGAAAGA--YGGA----F---IYDSFSSPSNSSSGS
	MchB <sup>a</sup>	(MccH47) <sup>b</sup>	MREITESQLRYISGA :	GG---APATSA-----NAA-GAAATVGALAGIPGGPLGVVGVAVSAG--LTTA-----I--GSTVSGSASSSAGGGS
	MchS2 <sup>a</sup>	(MccI47) <sup>b</sup>	MREISDNMLDSVKGG :	MN---LNGLPA-----STNVIDLRK KDMG--TYIDANGACWA----PDTPSIIMYPGGS-GPSY-----SMSSSTSSANS GS

<sup>a</sup> Name of the microcin precursors. <sup>b</sup> Name of the mature microcins (known or putative).

A vertical dash indicates the known or putative cleavage site of microcin precursors. Dashes in the amino acid sequence indicate gaps. Amino acids conserved in at least 50% of both class IIa and IIb sequences are highlighted in black. Amino acids conserved in at least 66% of class IIa sequences or 60% of class IIb sequences are highlighted in grey.

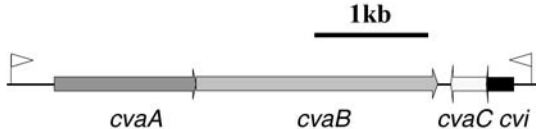
## Captions to figures

**Fig. 1 Genetic organization of class IIa microcin biosynthetic gene clusters.** A colour code identifies genes with similar functions. Genes encoding microcin precursors are shown in white. Genes required for self-immunity and microcin export are shown in black and grey, respectively. Promoters are indicated by flags. The truncated gene in MccL gene cluster is crossed through.

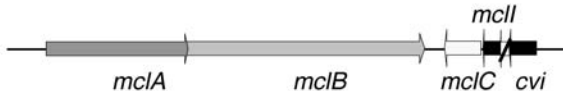
**Fig. 2 Genetic organization of class IIb microcin biosynthetic gene clusters.** A colour code identifies genes with similar functions. Genes encoding microcin precursors are shown in white. Genes required for self-immunity and microcin export are shown in black and grey, respectively. Genes involved in the post-translational modification are indicated with right- and left-inclined hatching. Genes encoding transposases and proteins of unknown function are indicated with horizontal hatching and vertical hatching, respectively. Truncated genes are crossed through.

**Fig. 3 Model for the class IIb biosynthesis.** u-microcin corresponds to unmodified microcin while u-microcin-Glc-DHBS and u-microcin-Glc-DHBS<sub>2</sub> correspond to microcin intermediate forms. Ent, Glc and DHBS stand for enterobactin, glucose, and *N*-(2,3 dihydroxybenzoyl)-L-serine, respectively.

**MccV**



**MccL**



**Mcc24**



## 1 kb

