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## REVIEW

### **Multifaceted ABC transporters associated to microcin and bacteriocin export**

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## **Abstract**

Microcins and bacteriocins are ribosomally-synthesized defence peptides produced by Gram-negative and -positive bacteria to target competitors in their niche. Some of them carry posttranslational modifications established by dedicated enzymes. To protect themselves from their own toxic peptides, bacteria use dedicated immunity proteins or expel the toxin using ATP-binding cassette (ABC) transporters. In this last case, this immunity function is associated to export of the antimicrobial peptide out of the producing cells for targeting their competitors. Here we review the characteristics of these ABC-exporters and the mechanisms they use that unexpectedly cover from high promiscuity to high specificity or ensure another function concomitantly.

## **Key words**

Antimicrobial peptides; bacteriocins; microcins; ATP-binding cassette; ABC exporter; AMS/PCAT exporter

## 1. Introduction

A relatively universal strategy used by both eukaryotic and prokaryotic organisms to survive, develop and grow at the expense of their rivals is the production of an arsenal of antagonistic antibiotic molecules, including antimicrobial peptides (AMP) that play a key role. This production is particularly crucial in hostile or highly competitive environments. In the bacterial world, AMPs are termed bacteriocins, or microcins more specifically in Gram-negative bacteria. They result from a maturation process that transforms a ribosomally-synthesized and generally inactive or poorly active precursor peptide into a potent antibacterial peptide, upon a specific protease cleavage. The precursor peptides usually contain an N-terminal leader region and a C-terminal core region that harbours in some cases post-translational modifications established by dedicated enzymes encoded in the AMP gene cluster. These AMPs are termed ribosomally-synthesized and post-translationally modified peptides (RiPPs) [1,2]. Secretion of the matured peptide is then ensured by ATP-binding cassette (ABC) transporters (exporters), which in some cases also bear the proteolytic activity [2].

It has now been well established that bacteriocins and microcins are used by bacteria to outcompete congeners in a given niche [3-5] and are able to kill pathogens in complex environments, such as the gut microbiota [6,7]. To exert their potent activity against competitors, bacteria utilise transporters to both export their toxic peptides out of the producing cells and to protect themselves against their own internally produced toxins, i.e. to provide self-immunity. In other cases, the two functions are distinct and a specific immunity protein is produced to 'neutralise' the toxin and prevent it from killing the producer [8,9]. In a few cases, full immunity requires the action of both the exporter and the immunity protein [10].

Although Gram-negative and -positive bacteria can utilise several secretion systems to export toxic molecules out of cells, microcins and bacteriocins are usually exported only by ABC exporters, which harness the energy from ATP hydrolysis as a source of energy. This review specifically focuses on the different classes of ABC transporters that are involved in either export and/or maturation of microcins and bacteriocins in Gram-negative and -positive bacteria, respectively.

## 2. Overall architecture and mechanism of ABC exporters

ABC exporters are multidomain membrane proteins that utilise the energy from ATP binding and hydrolysis to export their substrates. Their common modular architecture is composed of two cytoplasmic nucleotide-binding domains (NBD), which are the site of ATP binding and hydrolysis, and two transmembrane domains (TMD), which bind and transport the substrates. The TMD consensus organization for bacterial ABC exporters consists of multiple transmembrane  $\alpha$ -helices, most often two sets of six hydrophobic helices, although exceptions occur (Figure 1A). Coupling helices, which are  $\alpha$ -helical regions of the TMD intracellular loop that interact directly with the NBD surface and form a transmission interface between TMDs and NBDs, ensure coupling of ATP binding and hydrolysis to ligand transport. NBDs are highly conserved in both their sequences and three-dimensional structures; they contain specific motifs for ATP binding and hydrolysis (consensus signature sequence LSGGQ or C-motif, Walker A and Walker B motifs, A, H, and Q-loops) [11, 12]. In contrast, the TMDs which contain the substrate-binding site, have diverse sequences, sizes and three-dimensional structures [13], which provide the transporter either with polyspecificity or high selectivity. Increasing the number of available ABC transporter structures together with functional studies afforded evidence that mechanistic diversity

occurs among ABC transporters [14]. Most ABC exporters utilise the alternating access mechanism where the transporter switches between inward- and outward-facing states, with domain intertwining, thus exposing the ligand binding site alternatively to the inside or the outside of the membrane [15]. A critical step in ABC transporter function is NBD dimerisation; in brief, binding of ATP at the two NBDs induces dimerization and subsequent conformational changes that are transferred to the TMDs via the coupling helices and triggers the transporter adopting the outward-facing conformation.

ABC exporters ensure the transport of a vast spectrum of molecules, including fatty acids, cholesterol, proteolytic enzymes, S-layer proteins, but also drugs which are taken in charge by MDR ABC-exporters, as well as microcins, bacteriocins and toxins [16]. Some of them are bifunctional ABC transporters that ensure both maturation and export [17-19].

### **3. The landscape of bacteriocin and microcin ABC transporters**

To achieve their maturation and acquire their activity, bacteriocins and microcin precursor proteins must undergo a proteolytic cleavage to remove the N-terminal leader region and give rise to a mature and active modified or unmodified peptide. The leader regions are cleaved off concomitant with or after secretion of the bacteriocins. This maturation step is generally ensured inside or at the membrane of producer cells, either by dedicated proteases that can be or not encoded in the peptide gene cluster, or by other cellular proteases that can fulfill this role [9, 19-22]. Rarely, the precursor processing occurs inside the target bacteria, as in the case of microcin C; the uncleaved and inactive precursor is exported out of the producer cells and when imported in susceptible bacteria, it is further processed by endogenous peptidases [23].

Export of the mature peptide or its precursor can be ensured by different types of transporters reviewed in [19,24]. Two major types of ABC-exporters are involved in self-immunity: transporters that exclusively export AMPs such as those of the NisT-or McjD-type, and bifunctional transporters that ensure both maturation of the AMP by cleavage of the leader region of the precursor and secretion of the mature peptide [17-19]. These dual function proteins belong to the ABC-transporter maturation secretion (AMS) superfamily, or Peptidase-Containing ATP-binding Transporters (PCAT), also called the SunT-type transporter family [18,19] after the peptidase characterized for the glycopeptide sublancin system in *Bacillus subtilis* strain 168 [25] (Figure 1B and C). Database searches for NisT-type transporters in Firmicutes, the main group of Gram-positive bacteria among which lactic acid bacteria, show they are quite rare, whereas AMS/PCAT/SunT transporters are quite common [24].

#### **4. Bacteriocin and microcin ABC transporters without extra-transport function: from tolerance to high specificity**

##### *4.1. LanT-type ABC transporters in Gram-positive bacteria*

In many cases, newly synthesized Gram-positive bacteriocins, including many lantibiotics, are exported by NisT-type ABC exporters [24]. Lantibiotics are highly modified AMPs which belong to a broader group of RiPPs (lantipeptides) [1]. These lantipeptides, which are not all antimicrobials, are defined as peptides containing lanthionine rings formed by successive dehydration and thioether ring formation catalysed by LanB dehydratases and LanC cyclases (Figure 2A) [26] (the general LanX term is used collectively to call the proteins involved in any lantibiotic biosynthesis and export). Nisin produced by *Lactococcus lactis* is the first studied lantibiotic and is classified as type I [22,26]. It is used as a food preservative (Nisaplin<sup>R</sup>, Niprosin<sup>R</sup>) in more than eighty countries for more than fifty years. Its mechanism

of action involves binding to and sequestering of lipid II, the essential precursor of cell wall biosynthesis, which results in both blocking of peptidoglycan biosynthesis and formation of lipid II - nisin heteromolecular pores in the membrane bilayer.

The export of nisin is ensured by the NisT ABC exporter, which is the prototype of this type of bacteriocin transporters. NisT brings the modified full-length precursor peptide NisA (or prenisin) that contains the five lanthionine rings, and not the mature nisin, outside the producer cells where it is cleaved by the extracellular subtilisin-like serine-protease NisP localized at the membrane [19,26]. NisT shows relaxed substrate specificity, as it can similarly export the unmodified precursor, as well as unrelated peptides fused to the nisin leader [27]. By contrast, cleavage of the NisA precursor by NisP can only occur if the precursor has undergone the posttranslational modifications, showing specificity of the protease; the two processes, leader cleavage and transport, are independent. It has been shown that the action of the modification enzymes NisB and NisC and the transporter NisT affect the level of production of modified NisA [28] and that the export of modified NisA by NisT occurs before proteolysis by the NisP protease.

Other lantibiotics such as Pep5, subtilin, aureocin A70 are similarly exported by NisT-type ABC transporters, collectively termed LanT, before protease processing [24]. Two highly similar transporters have been also identified in *Staphylococcus gallinarum* and *S. epidermidis* for export of the lantibiotics gallidermin and epidermin, but they were shown to possess accessory proteins of unknown role in transport. Formation of a complex between LanT transporters and their dedicated modification enzymes LanB-LanC has been demonstrated but it is not mandatorily required for transport [24].

#### 4.2. McjD-type ABC transporters in Gram-negative bacteria



Export of microcins in Gram-negative bacteria is poorly documented. Microcins are essentially secreted by Enterobacteria and form a diverse although restricted group of AMPs that can be of low (below 5 kDa) or higher (until 10 kDa) molecular masses and are postrationally modified or not [9,22]. The most extensively studied export system is the McjD ABC exporter, which ensures export of microcin J25 (MccJ25) and simultaneously provides producing cells with self-immunity. Indeed, MccJ25 is produced by *Escherichia coli* and principally active against *E. coli* and *Salmonella* strains. This narrow spectrum of activity, essentially directed against bacteria closely related to the producing strain, is prevalent for microcins and bacteriocins and reflects their role in competitions in a given ecological niche.

This microcin belongs to the class I of posttranslationally modified microcins and has a particularly original [1]-rotaxane structure termed the lasso topology (Figure 2B). This typical fold is formed via an uncommon isopeptide bond established between the N-terminal amino group and the side chain carboxylate of a Glu or Asp residue at position 7 to 9. This posttranslational modification generates a macrocycle through which the C-terminal tail is threaded and locked by bulky side chains that act as plugs or/and disulfide bridges, thus preventing the tail slipping out of the ring. This fascinating lasso topology results from the activity of two enzymes McjB and McjC encoded in the *mcjABCD* MccJ25 gene cluster. McjB and McjC cleave the leader region and close the ring wrapped around the tail via formation of the isopeptide bond, while simultaneously ensuring that the precursor McjA adopts the appropriate topology [9,22,29]. McjC is a macrolactam synthetase, which catalyses macrocyclization and McjB is an ATP-dependent cysteine protease that cleaves the leader region. The functions of the two enzymes are distinct but interdependent, and it was proposed they form a complex that was called lasso-synthetase [29,30]. Comparison of the sequences of different lasso peptide precursors shows that the only conserved feature in the

leader is the presence of a Thr residue at the penultimate position and that a Gly, often present at the cleavage site (P1 position), is not mandatory. Therefore, cleavage of the leader in MccJ25 and other lasso peptide RiPP systems does not require the presence of a double-Gly motif, in contrast to AMS/PCAT systems (see section 5 below). Moreover, using another lasso peptide machinery it was shown that the McjC enzymes could be exchanged between the two systems and that overall specificity of the lasso synthetase is determined by the ATP-dependent protease McjB [29].

The third partner of the MccJ25 biosynthetic machinery is the ABC transporter McjD, which exports MccJ25 from the cytoplasm to the periplasmic space. The final export step out of the cells is provided by the outer-membrane protein TolC that forms a channel and transports it out of the cell. McjD has been proposed to be localized at the inner-membrane in close proximity to McjB and McjC [for reviews see 29,31]. The McjD structure and function have been extensively studied in our groups. The crystal structure of the full-length McjD has been determined in absence of MccJ25 but in the presence or absence of various nucleotides, and together with functional studies allowed deciphering the mechanism of transport by McjD and its specificity [32-36] (Figure 1A). The presence of lipids is essential for the McjD structure, stability and activity, with a synergistic role played by phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) that act for the structure stabilisation and the function, respectively [33]. The crystal structure of McjD revealed an occluded TMD that contains a large binding cavity, around 5900 Å<sup>3</sup>, and MccJ25 could be modeled within the cavity; F86, N134, N302 were found to be important for recognition [32]. The interaction of McjD with MccJ25 was further probed by NMR, and it revealed that of the 21-amino acids forming MccJ25 only three, which are located in the lasso peptide loop, are involved in the interaction with McjD [36]. A combination of functional assays,

crystal structures in different conformations, pulsed electron-electron double resonance (PELDOR) measurements, and single molecule Förster resonance energy transfer (sm-FRET) to probe the conformational changes and the dynamics of the transporter along the transport cycle [32,34,35], has led us to propose the “occluded-mechanism with transient opening” for substrate transport and selectivity. In brief, in the absence of MccJ25, McjD exists in an occluded conformation, where the TMD remains shielded to both the cytoplasmic and periplasmic sides of the membrane, regardless of the absence or presence of nucleotides. As MccJ25 is produced by the synthetase, McjD samples an inward-open conformation and its binding cavity becomes accessible to the peptide. MccJ25 and ATP binding induce a transient outward-open conformation for its release to the periplasm and subsequently the TMD adopts an occluded conformation. ATP hydrolysis resets the transporter to its inward conformation. Unlike MDR ABC transporters that can adopt the outward-open conformation in the absence of substrate, McjD requires both MccJ25 and ATP to open. The specificity of the McjD ABC exporter was found to be extremely high, as all other drugs or AMPs, or even tightly close lasso peptides were not taken in charge by McjD, while conversely another ABC transporter responsible for the export of another lasso peptide was unable to transport MccJ25 [36].

The ABC transporter YojI, whose actual function is unknown, is also involved in MccJ25 secretion. YojI is chromosomally encoded and is under the control of the leucine-response protein Lrp. Similar to McjD, YojI requires the action of TolC at the outer-membrane to export MccJ25 outside the cells [22,29]. Other microcins from the two classes, such as the class IIa siderophore-microcins or the class I microcins B17 or C7 also use ABC transporters for export, but they were only poorly explored [9,22].

## 5. Bifunctional ABC-transporters of the AMS/PCAT type

While in many cases the proteolysis and export functions are ensured by distinct proteins, these two functions can also be carried both by the same protein. Håvarstein *et al.* described the cleavage of the two partners of lactococcin G, a two-peptide unmodified bacteriocin, by the N-terminal domain of the ABC-transporter LagD and proposed that all precursor peptides containing leader peptides of the double-glycine type, such as those of the type IIa bacteriocin pediocin PA-1, or of microcin V, were processed by their dedicated ABC transporter concomitant with export [37]. Involvement of AMS/PCAT transporters have also been described in both Gram-positive and Gram-negative bacteria for a large variety of bacteriocins and microcins, bearing or not post-translational modifications [2,18,19,24,38,39] as well as for peptides involved in bacterial communication [17, 38]. Such extracellular signaling peptides (called autoinducer peptides or peptide pheromones) are produced by bacteria to communicate between them and coordinate their activities. In particular, the pheromones can sense the cell density and mediate the bacterial cell-cell signaling system called quorum sensing that alters the gene expression to adequately respond to environmental changes. ABC transporters from this sub-family share a common organization that includes an additional N-terminal peptidase domain upstream of the TMDs and NBDs, which ensures removal of the leader peptide concomitant with export of the mature and active bacteriocin/microcin. Cleavage of the leader occurs systematically at the C-terminal side of a double-Gly type motif (Gly-Gly, Gly-Ala, Gly-Ser), a hallmark of papain-like cysteine proteases.

### 5.1. AMS/PCAT ABC transporters in Gram-negative bacteria

In Gram-negative bacteria, microcin V (also frequently termed colicin V (ColV) as initially described as a colicin [40]) is a class IIa microcin produced by *E. coli*, which was reported to require a two-component system CvaA-CvaB, with CvaB identified as an ABC transporter and CvaA as an accessory protein of the membrane fusion protein (MFP) type, and TolC [41] for export. Such an organization is reminiscent of type 1 secretion systems (T1SS) [42], but the transported substrates do not exhibit the characteristics shared by proteins transported via T1SS, i.e. the specific Gly/Asp-rich repeat motifs at the C-terminus (see below). Furthermore, mutational studies have shown that CvaB is an AMS/PCAT, which possesses Ca<sup>2+</sup>-dependent proteolytic activity, and that Cys32, His105 and Asp121 form the catalytic triad of the protease domain [43,44], which belongs to the C39 class of cysteine proteases [38,39]. Pro122 and other adjacent residues surrounding the catalytic triad were also shown to play a critical role in the secretion of ColV [44]. It has been proposed that other microcins of class II [9,22], i.e. unmodified class IIa microcins (microcins L and 24) and siderophore-microcins (class IIb microcins E492, H47 and M), which contain an N-terminal leader region cleaved by a C39 peptidase domain of the ABC transporter prior to secretion would use such transport systems [22,45].

## 5.2. Structural features of AMS/PCAT ABC transporters

The first crystal structure of a C39 peptidase domain to be determined was that of the ABC transporter ComA, which ensures maturation and translocation of the autoinducer bacteriocin ComC in *Streptococcus* species [17]. It provided the first general scheme of the recognition rules and catalytic mechanism applying to the AMS/PCAT ABC-transporters. The study revealed the arrangement of the Cys-His-Asp catalytic triad in the global architecture of the C39 peptidase domain, and identified the residues in the leader region important for

recognition. The consensus sequence  $^{-12}\text{LSXXELXXIX}^{-3}$  (where X stands for any amino acid) located between positions -12 and -3 upstream to the double-Gly motif at the cleavage site was identified in a docking model generated for the C39 domain / precursor peptide complex and was validated by site-directed mutagenesis [17].

Although most structural studies of AMS/PCAT have been limited to isolated peptidase domains, the first crystal structure of full-length AMS/PCAT, PCAT1 from *Clostridium thermocellum*, was determined in two different conformations (Figure 1B). It supports that substrate processing and translocation/secretion ensured by the bifunctional ABC transporter are coupled, which is a clearly different model from that applying for the *sec*-dependent pathway [46]. Interestingly, the overall structure of PCAT1 is very similar to McjD-type transporters, suggesting that the transport step is likely very similar (Figure 1A and B). The TMD contains a cavity that can accommodate small or partially folded proteins and the active transport could operate by the alternating access mechanism [46].

### 5.3. AMS/PCAT ABC transporters in the Gram-negative T1SS

T1SS are three-component systems widespread in Gram-negative bacteria that ensure translocation and export of various substrate proteins, including adhesion proteins, proteases, lipases, surface layer proteins and toxins, from the cytoplasm across both the inner- and outer-membrane [42,47]. The T1SS transported proteins belong to the family of RTX (repeats-in toxins) proteins, which contain numerous RTX domains containing repeated highly conserved Gly- and Asp-rich nonapeptide motifs, with the typical consensus sequence GGXGDXUX (where X and U are any amino acid and a large hydrophobic one, respectively) [42]. They share a common characteristic that the information triggering secretion is located within the 50/60-amino acid extreme C-terminal region, which contains these motifs,

contrary to many other transport systems, where the information is a signal sequence located at the N-terminus. Therefore, based on this definition, T1SS systems do not apply to ColV or many Gram-positive bacteriocin transport [42].

The first characterized T1SS is that of haemolysin A (HlyA), the 108 kDa haemolytic toxin which is found in most uropathogenic *E. coli* strains [48] and was discovered in the 1980's. The HlyA T1SS serves as a paradigm for the family. It is composed of three components: two inner-membrane proteins, an ABC transporter (HlyB) and an MFP (HlyD), which work together with the endogenously expressed outer-membrane protein TolC [49,50]. An additional component HlyC, a cytosolic acetyltransferase that acetylates two HlyA lysines before secretion, was shown to be essential for HlyA activation, but not for secretion [51]. This ABC transporter - MFP protein - outer-membrane protein three-component organization represents the basic structure of the T1SS secretion system, which forms a continuous tripartite "channel-tunnel" from the cytoplasm to the extracellular space in presence of the substrate [52]. While the ABC transporter and the MFP always form a complex in the inner-membrane, TolC is not permanently associated and its recruitment to make the channel-tunnel is triggered upon interaction of the HlyA substrate with HlyB and/or HlyD [53,54]. However, the supramolecular assembly of the complex remains controversial [42], although the crystal structure of a soluble fragment of HlyD was obtained [55] and directionality of the one-step transport was demonstrated and shown to involve the secretion of the C-terminus of T1SS substrates first [47]. A certain degree of promiscuity is found in such systems, as the Hly system was found to be able to secrete toxins in other Gram-negative genera, such as *Neisseria*, *Pasteurella* or *Bordetella* [51 and references herein].

Interestingly, some T1SS ABC transporters, such as HlyB, contain an additional N-terminal cytosolic region [45], which shows high similarities to C39 peptidase domains in terms of amino acid sequences and three-dimensional structures, but are devoid of proteolytic activity [37,43,56]. Those are termed C39 peptidase-like domains (CLD) [56,57]. They appear degenerated, as they do not possess the typical catalytic triad involved in the proteolytic activity. Despite this, the model of interaction between a C39 peptidase domain and its substrate first proposed for ComA was confirmed for the HlyB-CLD complex. Although the solution NMR three-dimensional structure [56] appeared highly similar to the crystal structure of the ComA-C39 peptidase complex [57], a tyrosine has replaced the catalytic cysteine and the histidine has been flipped outside, while the right position of the aspartate in the catalytic centre was maintained, which is responsible for the absence of protease activity of CLDs. These divergent specific arrangements of CLDs and C39 peptidase domains clearly distinguish between the two ABC transporter types, leading to a diagnostic tool currently used [45,56]. Further functional studies showed another role was ensured by the CLD. In the Hly system, Lecher *et al.* showed that the CLD specifically interacts with the unfolded state of HlyA, which was suggested to be transported before the folding step that is triggered by the Ca<sup>2+</sup> ions present in higher concentration out of the cells [56]. It was proposed that the GG repeats in HlyB would be involved in the interaction eliciting the transport, and more broadly that the CLDs would specifically recognize the highly conserved GG repeats which are a hallmark of RTX proteins [56].

#### 5.4. AMS/PCAT ABC transporters in Gram-positive bacteria

In Gram-positive bacteria, AMS/PCAT have been shown to cleave and export a very broad range of bacteriocins [22], from unmodified ones such as the pediocin-like class IIa



bacteriocins, to highly modified ones, typically lantibiotics but also several classes of RiPPs [1], as well as peptides having quorum-sensing activity [18,58]. The hallmark of this specific pathway ensuring concomitantly processing and export is the presence in the leader region of the precursor peptides of the double-Gly type motif required for processing by papain-like cysteine proteases. As the first historical example, the two partners of the two-peptide unmodified bacteriocin lactococcin G were shown to be both matured and exported by the AMS/PCAT LagD [37]. Furthermore, several one- and two-peptide lantibiotics were shown to be matured by AMS/PCAT transporters [19]. Nukacin ISK-1 from *Staphylococcus warnerii* is transported and cleaved by the bifunctional transporter NukT. The peptidase activity was reconstituted in inside-out membrane vesicles and cooperativity between peptidase activity and ATP hydrolysis was evidenced, the leader peptide cleavage by the peptidase region significantly enhancing the ATPase activity of the NBD domain [19,59,60].

### 5.5. The specificity of AMS/PCAT ABC transporters

The specificity of AMS/PCAT transporters has been recently examined. The *Enterococcus faecium* AMS/PCAT EnkT, which secretes several different types of bacteriocins (a pediocin-like bacteriocin (class IIa), a two-peptide unmodified bacteriocin (class IIb) and a circular bacteriocin (class IIc)), was shown to have broad tolerance to the length and amino acid sequence of the leader peptide [61]. It was suggested that a specific conformation, and in particular the amphipathic  $\alpha$ -helix proposed to be involved in recognition [62], is critical for the recognition rather than a specific sequence [61]. Recently, the N-terminal peptidase C39 domain LahT150 of the full-length AMS/PCAT LahT of the Gram-positive bacterium *Lachnospiraceae* bacterium C6A11, which transports a lantipeptide, was functionally characterized and its structure determined (Figure 1C) [18]. It was shown that the

AMS/PCAT has promiscuity to the core peptide region of the precursor and most of the leader region, and to identify a minimal C-terminal 13 amino acid sequence of the leader ( $^{-13}\text{NLS}\text{DDELEGVAGG}^{-1}$ ; numbering from the double-Gly motif upstream the cleavage site) as important for recognition and further processing, in agreement with the general consensus sequence previously identified [17]. Furthermore, mutational data identified the critical roles of V<sup>-4</sup>, L<sup>-7</sup> and L<sup>-12</sup> (shown in bold in the 13 amino acid sequence above) and the crystal structure of LahT150 in complex with a covalently bound leader peptide analogue (inhibitor 1) afforded rationale for the role of these three hydrophobic residues in the substrate recognition. The recognition occurs about 20 Å away from the double-Gly motif and an  $\alpha$ -helix formed in this region positions V<sup>-4</sup>, L<sup>-7</sup> and L<sup>-12</sup> of the substrate into a hydrophobic groove of the C39 protease domain located at the junction with the NBD. This localization would direct the substrate and drive the translocation process. Such a model would apply not only to all AMS/PCAT but also to T1SS CLDs devoid of protease activity [18].

Bioinformatics studies have shown that AMS/PCAT would be much more widespread than previously expected and particularly inspection of bacterial genomes has revealed the concomitant presence of such AMS/PCAT ABC transporters together with double-Gly leader peptide sequences in Gram-negative bacteria [18,45,56]. Taken together the data described above support the idea that AMS/PCAT would not prevail exclusively in Gram-positive bacteria as previously thought and shown in Firmicutes [24], but would be involved extensively in both Gram-positive and Gram-negative bacteria. Such transporters, together with the closely related Gram-negative T1SS, would operate in maturation and transport of a broad range of compounds, not only of many bacteriocins, microcins and other toxins bearing or not posttranslational modifications, but also of a large panel of RiPPs

acting as defence, or communication and quorum sensing molecules, and playing critical roles in symbiosis [18,38,39].

## 6. Immunity and resistance to bacteriocins and microcins

Bacteria that produce bacteriocins or microcins to prevail in their ecological niche need to develop self-immunity strategies to abrogate them from the lethal effects of their own toxic peptide. This is ensured by two principal ways that are the action of export systems and the production of dedicated immunity proteins [63]. However, several bacteria have also evolved resistance systems against nisin and other lantibiotics that involve other transporters or strategies. It is therefore necessary to clearly distinguish between the two processes and the transporters associated.

Immunity to nisin is provided by a specific immunity protein NisI, a lipoprotein which is covalently anchored to the extracellular surface of the cell membrane and oriented to the cytoplasm. NisI binds mature nisin with high specificity, preventing it to reach its target molecules, and particularly lipid II, thus making it inactive. The NMR and X-ray structures of NisI have been determined, showing it is a two-domain protein and the C-terminal region is critical for nisin binding [64,65]. LanI immunity proteins form a family that ensures immunity of strains producing different other lantibiotics [66], including two-peptide lantibiotics [63].

However, to get full immunity to nisin, strains need expressing the NisFEG ABC transporter that is distinct from the NisT export system (see section 4.1.). The NisFEG system is composed of NisF, which dimerizes to form the cytoplasmic NBDs that bind and hydrolyse ATP, and NisE/NisG that form a heterodimeric membrane complex, i.e. the TMDs [66]. The three proteins assemble to form a functional ABC transporter which expells nisin from the membrane before or during pore formation. Moreover, it has been shown that NisFEG

expells intact nisin molecules, which have not undergone modification or degradation [66].

Similarly, for providing full immunity to circular bacteriocins the action of ABC transporters is required to complement cognate immunity proteins [67]. The unmodified class IIa bacteriocins of the pediocin type also use immunity proteins, that involve a specific recognition of the C-terminal region of the cognate bacteriocin [68]. Based on microcin gene cluster analyses, Gram-negative bacteria appear to have also evolved complementary and specific systems involving both an immunity protein and an ABC transporter to achieve full immunity. This is the case for instance of the modified class I microcin B17 [69], or the class II microcins, either unmodified (ColV, 24, L) or modified (siderophore microcins E492, H47) [22]. By contrast, immunity to MccJ25 is only ensured by a single mechanism using the same ABC exporter MccJ which is used by the strain for exporting the microcin for competition purpose.

Resistances against lantibiotics have been described in various bacterial genera [70-72]. They result from different mechanisms, among which some are common to many classes of antibiotics and antimicrobials (modifications in the peptidoglycan or other cellular membrane components, assembly of biofilms, inhibition of spore formation, action of two-component systems (TCS) that regulate the transcription of resistance-associated genes). Other mechanisms are specific to lantibiotics, such as the nisin lytic protein nisinase (a dehydropeptide reductase), the nisin resistance protein NSR, which cleaves the C-terminal region of nisin, or the immune-mimicry strategy, which involves the production of proteins homologous to lantibiotic immunity proteins. Furthermore, ABC transporters which work together with TolC for substrate transport and are generally found associated with TCS, are responsible for many of the resistances developed to lantibiotics. They can belong to the CprABC-type or the BceAB-type [72], the last ones being members of the MacB ABC

transporters which use mechanotransmission, i.e. conformational change, to couple substrate transport to ATP hydrolysis [71]. Among such resistance systems of major importance that are well-studied are the CprABC-KR system from *Clostridium difficile* in the first group, and the BceRS-AB from *Bacillus subtilis* and VraDE-BraRS systems from *Staphylococcus aureus* in the second one [see 72 for a review on the ABC transporters involved in lantibiotic resistance].

Resistance mechanisms to microcins have been poorly explored. In the case of MccC, which is ultimately processed in non-producing target bacteria, resistance has been shown to involve two different mechanisms using an acetyl-CoA-dependent acetyl transferase and a specific protease, but none of them involves an ABC transporter [10]. The ABC transporter YojI also involved in MccJ25 secretion [9,22] could presumably contribute to self-immunity of the producing strains but also to resistance to MccJ25 in non-producing strains.

## 7. Concluding remarks

This review provides for the first time an overview of the various roles and degrees of specificity endorsed by ABC exporters involved in translocation and excretion of bacteriocins and microcins. A main conclusion is that there is no evident rule that could emerge from the different cases we browsed among Gram-positive and Gram-negative systems. In some cases, independent of the Gram-positive or -negative classification, protease cleavage of the leader peptide and exporter functions are tightly connected. This is the case of AMS/PCAT ABC transporters that occur both in Gram-positive (class II lantibiotics) and -negative (haemolysin, microcin V) bacteria and are also frequently found in various RiPP machineries [1,2]. In other cases, the two functions are independent, but the protease activity can occur either outside or inside the producer and the exporter can take in charge either the

precursor or the mature AMP. As an example, in class I lantibiotics the exporter has a sole role of transporting the modified precursor and not the modified mature peptide and cleavage is ensured outside the cell by an independent and specific protease of high specificity, whereas the transporter specificity is broad. In contrast, in the MccJ25 lasso system, the protease activity is associated with the posttranslational modification machinery and not with the ABC transporter that exports the mature peptide. In this regard, the role of the MccJ25 lasso peptide ABC exporter also clearly distinguishes from those of other RiPP biosynthesis machineries using AMS/PCAT ABC transporters. Furthermore, the specificity of the ABC transporters is largely variable, from almost high promiscuity observed in Gram-positive AMS/PCAT and Gram-negative T1SS to high or total specificity in the case of *E. coli* McjD. Finally, the translocation process taken in charge by the ABC transporter can be associated with another function than the proteolytic activity, such as a role of recognition and binding pointed for the CLD of the haemolysin ABC transporter HlyB.

Another distinction is the role of the partners associated to the ABC transporter in the translocation and export event, and the mechanism used to cross the two membranes of Gram-negative bacteria. Export of MccJ25 across the two membranes occurs in two steps: McjD is not bound to the outer-membrane TolC and ensures transport only from the cytoplasm to the periplasmic space, while in the T1SS system the ABC transporter and TolC form a continuous channel-tunnel from the cytoplasm to the exterior of the cell and the transport is ensured via a single step.

Although, most bacterial ABC exporters have been linked with multidrug resistance, it is unclear how such multisubstrate “recognition” was acquired. As discussed earlier, the sequences of the TMDs is not well conserved but why are bacteriocin and microcin ABC exporters very specific to their substrate? For the ABC exporters that have been

characterised as multidrug, in many cases, their natural substrate is unknown. It is evident from this review that bacteriocin and microcin ABC exporters can provide very useful insights on how certain families of ABC exporters have become multidrug. We cannot exclude that the close proximity of the maturation enzymes to the ABC exporters provides such selectivity; these exporters are usually transcribed under the same promoter as the maturation enzymes and therefore they are not under the pressure of drug selection. Our current understanding on the function, mechanism and structure of AMP ABC exporters is very limited, within a couple of families. Therefore, more detailed understanding of these complex machineries is still necessary.

### **Conflict of interest**

The authors declare no conflict of interest.

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## Figure legends

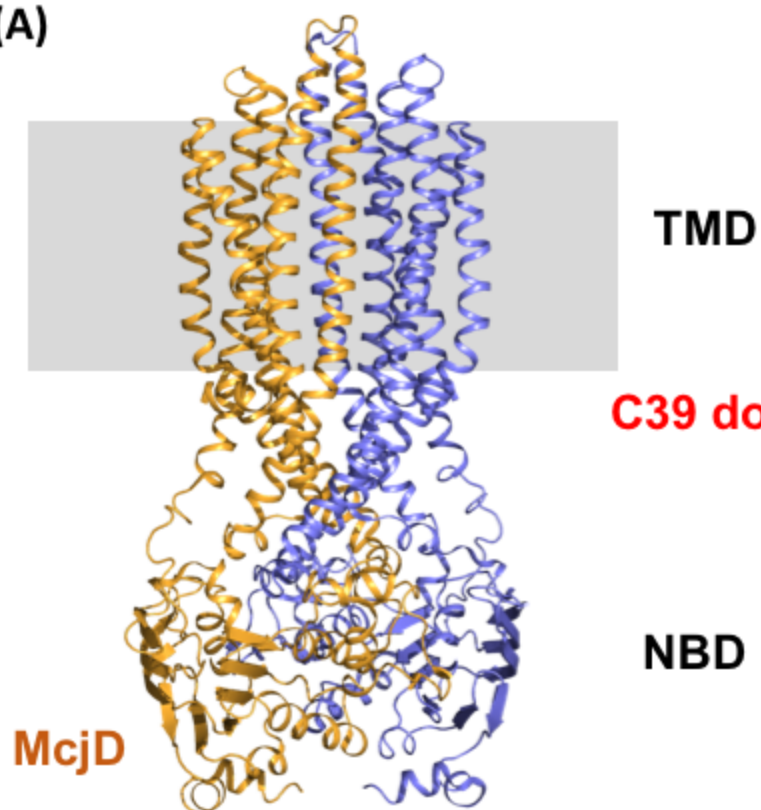
**Figure 1.** Crystal structures of ABC transporters involved in microcin and bacteriocin export.

(A) Crystal structure of the apo McjD from *E.coli* (PDB ID: 5OFP) that is involved in the export of and self-immunity to the lasso peptide MccJ25. The structure is shown in cartoons and each monomer is coloured in orange and blue. The positions of the TMDs and NBDs are indicated. The membrane is shown as grey box. (B) Crystal structure of the full length PCAT1 from *C. thermocellum* (PDB ID: 4RY2). It belongs to the AMS/PCAT family of ABC exporters. The C39 peptidase domain is located at the N-terminus and is shown as red cartoons. The colour scheme for the transporter is the same as in panel A. (C) Crystal structure of the isolated C39 peptidase domain, LahT147, from the LahT AMS/PCAT transporter (PDB ID: 6MPZ) from *L. bacterium* C6A11 in complex with inhibitor 1. LahT147 is shown in blue cartoon and the catalytic residues **C27, H101 and D117 in stick format**. The peptide inhibitor 1 is shown in pink cartoon.

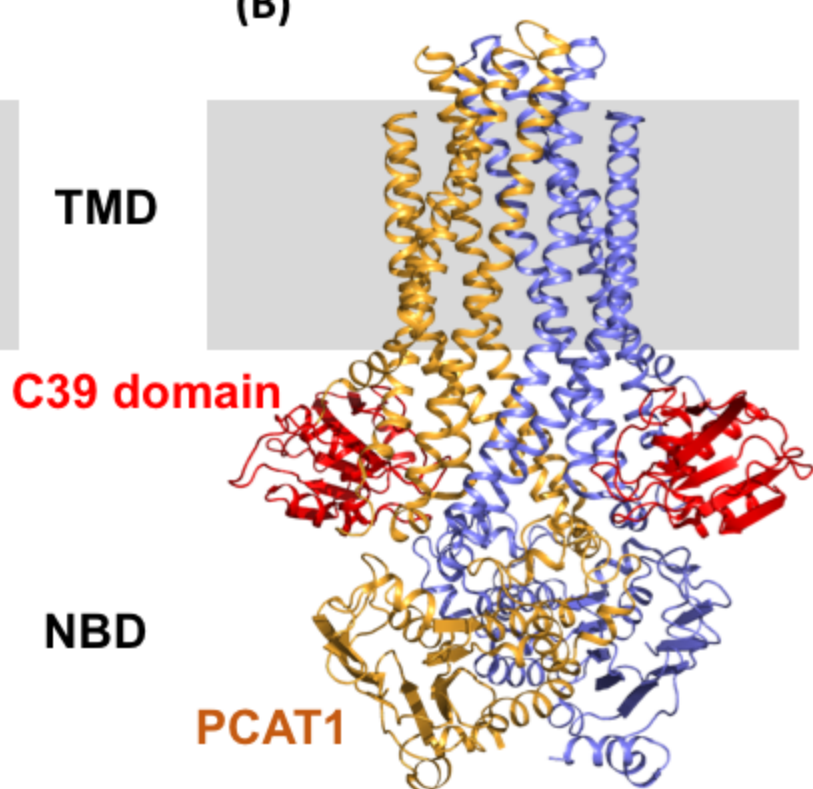
**Figure 2.** Structures of a lantipeptide and a lasso microcin. (A) Sequence of the mature and posttransitionally modified lantipeptide nisin. The first and last amino acids are indicated with subscript numbers. Residues that are involved in disulfides are shown in red or orange colour and indicated with lines. The structure of nisin is shown in stick format (PDB ID: 1WCO). Dha = dehydroalanine, Dhb = dehydrobutyrine, Abu = aminobutyryne. (B) The mature lasso peptide MccJ25 consists of 21 amino acids. The isopeptide bond is indicated by a green line in the sequence. The NMR structure of MccJ25 (PDB ID: 1Q71) shows that the lasso topology is maintained by two bulky amino acids, Phe19 and Tyr20, on either side of the macrolactam ring.

**Figure 1**

**(A)**



**(B)**



**(C)**

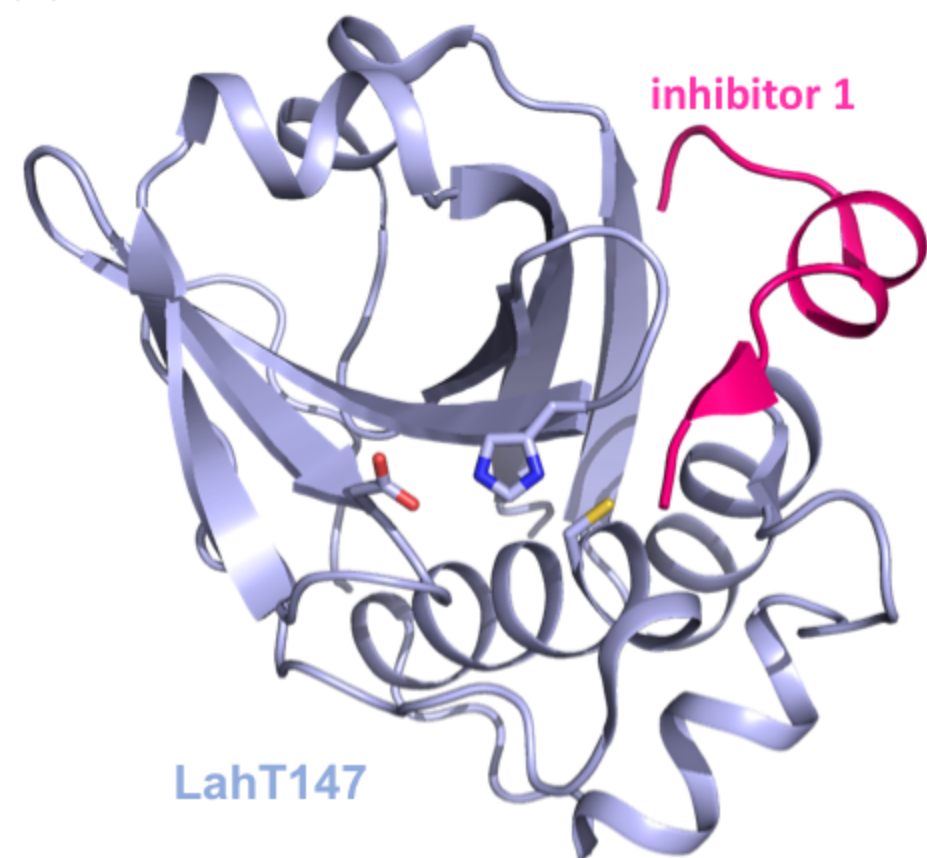
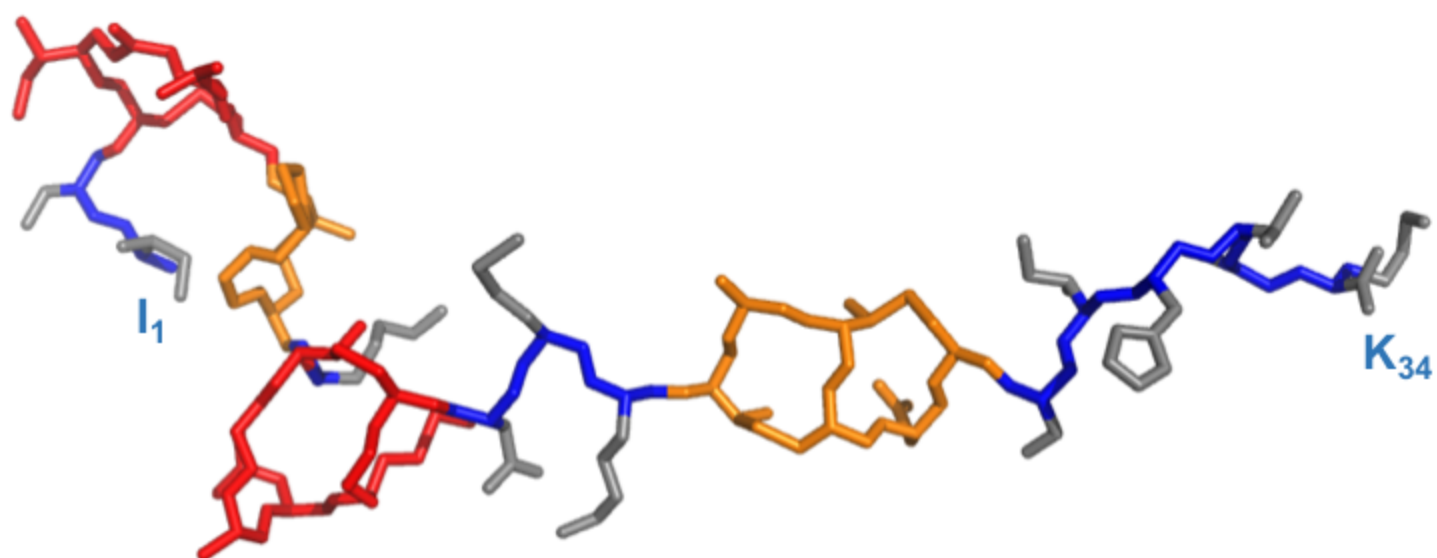


Figure 2

(A)

Nisin

I<sub>1</sub>DhbAIDhaLAAbuPGA**K**AbuGALMG**A**NMKAbuAAbuANASIHVDhaK<sub>34</sub>



(B)

MccJ25

G<sub>1</sub>-G-A-H-V-P-E<sub>8</sub>-Y-F-V-G-I-G-T-P-I-S-F<sub>19</sub>-Y<sub>20</sub>-G<sub>21</sub>

