Colicin M, a peptidoglycan lipid-II-degrading enzyme: potential use for antibacterial means?

Thierry Touzé*1, Hélène Barreteau*, Meriem El Ghachi*2, Ahmed Bouhss*, Aurélie Barnéoud-Arnoulet†, Delphine Patin*, Emmanuelle Sacco‡, Didier Blanot*, Michel Arthur‡, Denis Duché†, Roland Lloubès† and Dominique Mengin-Lecreulx*2

*Laboratoire des Enveloppes Bactériennes et Antibiotiques, IRBMC, Université Paris-Sud, UMR 8619 CNRS, 91405 Orsay, France, †Laboratoire d’Ingénierie des Systèmes Macromoléculaires, CNRS-Aix Marseille Université, 31 chemin Joseph Aiguier, 13402 Marseille Cedex 20, France, and ‡Laboratoire de Recherche Moléculaire sur les Antibiotiques, Centre de Recherche des Cordeliers, Université Pierre et Marie Curie, Université Paris Descartes, INSERM, UMR-S 872, 75006 Paris, France

Abstract
Colicins are proteins produced by some strains of Escherichia coli to kill competitors belonging to the same species. Among them, ColM (colicin M) is the only one that blocks the biosynthesis of peptidoglycan, a specific bacterial cell-wall polymer essential for cell integrity. ColM acts in the periplasm by hydrolysing the phosphoester bond of the peptidoglycan lipid intermediate (lipid II). ColM cytotoxicity is dependent on FkpA of the targeted cell, a chaperone with peptidylprolyl cis-trans isomerase activity. Dissection of ColM was used to delineate the catalytic domain and to identify the active-site residues. The in vitro activity of the isolated catalytic domain towards lipid II was 50-fold higher than that of the full-length bacteriocin. Moreover, this domain was bactericidal in the absence of FkpA under conditions that bypass the import mechanism (FhuA–TonB machinery). Thus ColM undergoes a maturation process driven by FkpA that is not required for the activity of the isolated catalytic domain. Genes encoding proteins with similarity to the catalytic domain of ColM were identified in pathogenic strains of Pseudomonas and other genera. ColM acts on several structures of lipid II representative of the diversity of peptidoglycan chemotypes. All together, these data open the way to the potential use of ColM-related bacteriocins as broad spectrum antibacterial agents.

Introduction
Peptidoglycan is a cell-wall polymer present on the outside of the cytoplasmic membrane of all bacteria and exclusively in these organisms. It consists of linear glycan chains alternating GlcNAc (N-acetylglucosamine) and MurNAc (N-acetylmuramic acid) [1]. The sugar strands are cross-linked via a peptide stem attached to the MurNAc moiety, thereby constituting a rigid three-dimensional network surrounding the entire cell. By withstanding the high internal osmotic pressure, peptidoglycan contributes to the integrity of the cell. Hydrolysis of the cell-wall polymer or blockage of its biosynthesis results in cell lysis. For these reasons, peptidoglycan is an attractive target for the search of new antibacterial agents.

Colicins are proteins released into their environment by certain strains of Escherichia coli to kill non-immune E. coli cells or closely related bacteria [2]. Approximately 20 colicins have been described to date; most of them proceed via the hydrolysis of nucleic acids or the depolarization of the plasma membrane. ColM (colicin M) displays many features that distinguish it from the other colicins [3]; for instance, ColM is the only one that targets cell-wall synthesis [4]. It was first described in the 1970s by Braun et al. [5], when it was shown to induce a lytic phenotype, which was attributed to the inhibition of peptidoglycan synthesis. Nevertheless, its precise mode of action remained unknown until 2006, when we demonstrated that ColM possesses a hydrolytic activity towards peptidoglycan lipid intermediate II, which is a unique mechanism among the lipid-II-targeting antibacterials [6]. This enzymatic feature makes ColM a very efficient drug as only a few molecules (fewer than ten) are sufficient to kill one single cell [7]. ColM characteristics have renewed interest for this toxin and for its potential use as a strategy to fight bacterial infections.

Peptidoglycan metabolism
The elucidation of the metabolic pathway of peptidoglycan synthesis was accomplished in the last three decades. The overall scheme of this pathway, common to all bacteria, can be divided in three steps in regard to the cell compartment in which the reactions occur. The cytoplasmic step ends up with the formation of the two nucleotide precursors: UDP-GlcNAc and UDP-MurNAc-pentapeptide [8]. The second
step, taking place in the cytoplasmic membrane, starts with the transfer of the phospho-MurNAc-peptide moiety from UDP to a polyisoprenoid carrier, undecaprenyl phosphate (C55-P) leading to lipid intermediate I (Figure 1). Thereafter, the GlcNAc moiety is attached to lipid I, yielding lipid intermediate II [9]. Finally, lipid II is translocated to the periplasmic side of the inner membrane to feed up in cell-wall building blocks the polymerization step catalysed by the penicillin-binding proteins [10]. During these reactions, the lipid carrier is released in its pyrophosphate form (C55-PP). It is then quickly recycled after being dephosphorylated and shuttled back to the inner face of the membrane [11]. It is also used in a similar way for the synthesis of other cell-wall polysaccharides [the LPS (lipopolysaccharide) O-antigen moiety in Gram-negative bacteria and the teichoic acids in Gram-positive bacteria]. It is important to mention that, besides recycling, C55-P is also generated by de novo synthesis, a process occurring on the cytoplasmic face of the plasma membrane.

Mode of action of ColM

The fact that the O-antigen synthesis was also inhibited by ColM suggested that the toxin was affecting the generation of the lipid carrier, which constitutes the branching point between the various cell-wall polymers [12]. Thus the potential inhibitory effect of ColM towards the different possible steps leading to a blockage of the C55-P cycle, i.e. C55-PP synthesis and dephosphorylation, lipid I and lipid II synthesis and peptidoglycan polymerization, was assayed in vitro [6]. It was clearly demonstrated that ColM did not inhibit any of the enzymes implicated, but was capable of degrading lipid I and lipid II molecules each into two dead-end products. These degradation products were characterized further and the site of cleavage was localized between the polyisoprenyl chain of the lipid and the pyrophosphate group, releasing undecaprenol and either 1-pyrophospho-MurNAc-peptide or 1-pyrophospho-MurNAc-(peptide)-GlcNAc. Cell labelling experiments with radioactive dianominopimelic acid, a specific marker of the peptidoglycan peptide stem, was used further to substantiate the effect of ColM in cellulo. Soon after ColM treatment and before lysis, sensitive E. coli cells accumulated the nucleotide precursors as well as lipid I and lipid II degradations products and concomitantly, they were depressed in the corresponding lipid intermediates. Therefore, as a mode of action, ColM displays a diphosphoric diester hydrolase activity raised against the cell-wall lipid intermediates [6] (Figure 1). This constitutes an unusual and very efficient way of killing bacteria with a dual mechanism that blocks both peptidoglycan synthesis and lipid-carrier regeneration.

Both lipid precursors are accessible from the inside of the cell where they are synthesized, but lipid II is also accessible from the periplasm after being translocated through the inner membrane, raising the question of the site of action of ColM. Several lines of evidence indicate that ColM exerts its toxic activity at the cytoplasmic face of the membrane: (i) ColM has to enter the cell from the external medium to be active since a ColM-producing cell is protected from the toxin it produces as long as it possesses the dedicated immunity protein (Cmi) or if the import machinery is inactivated [6,13]; (ii) the immunity protein is anchored in the inner membrane via a single transmembrane segment and its main structural body emerges in the periplasmic space, where it neutralizes ColM cytotoxicity by a yet unknown mechanism [14,15]; and (iii) when ColM is fused to the signal sequence of OmpA, it is excreted via the Sec apparatus into the periplasm, where it is toxic for the producer unless the immunity protein is co-expressed [16].

The ColM activity spectrum is strictly limited to bacteria that possess the complete import machinery [2]; in contrast the lipid II target is shared by all bacteria, except that its peptide stem presents some structural variations among the bacterial world [1]. For instance, dianaminopimelic acid present in the peptidoglycan of most Gram-negative bacteria is replaced by 1-lysine in Gram-positive bacteria. Additionally, the cross-link between two peptide stems is achieved either directly, such as in E. coli, or through the insertion of an additional side chain of different compositions and lengths. The 1-lysine-containing lipid II and several branched lipids II found in the main pathogenic bacteria were synthesized and the enzymatic activity of ColM towards these variants was determined in vitro, demonstrating that ColM activity was the same whatever the nature of the peptide stem and the presence or absence of an extra side chain [17]. Therefore, if ColM could cross the outer membrane of other Gram-negative bacteria or had access to the outer face of the plasma membrane of Gram-positive bacteria to meet its specific target, it should theoretically be capable of exerting a broad bactericidal activity.

Structural organization of ColM

Many colicins are co-expressed with their dedicated lysis protein, which is essential for their release into the external medium by a mechanism that is not yet well understood [2]. ColM export proceeds through a different and as yet unknown mechanism because such a committed lysis protein is missing. For their import through the outer membrane of the targeted cell, colicins parasitize specific outer-membrane receptors and energy-coupled transport systems [2]. ColM belongs to the class B colicins using the energy-coupled Ton system for translocation (TonB–ExbB–ExbD inner-membrane complex), and its outer-membrane receptor is the iron-siderophore transporter FhuA, through which ColM is thought to be internalized [2,18].

Mutagenesis studies revealed that ColM, like the other colicins, is composed of three functional domains: the N-terminal domain containing the TonB box and required for translocation across the outer membrane, the central domain dedicated for binding to FhuA and the C-terminal domain for the expression of the bactericidal activity [19,20]. The three-dimensional structure of ColM was determined, revealing a unique protein fold [21] (Figure 2). In contrast with the other known colicins that display three well distinct functional
Figure 1 | Synthesis of lipid II and mode of action of ColM

The enzymes MraY and MurG catalyse the successive transfers of MurNAc-pentapeptide (M·••••) and GlcNAc (G) motifs from the nucleotide precursors to the lipid carrier C55-P, generating the lipid I and lipid II intermediates respectively. Lipid II is then translocated to the periplasmic side of the membrane where polymerization reactions of peptidoglycan catalysed by the PBPs (penicillin-binding proteins) occur. The lipid carrier is released in its pyrophosphate form (C55-PP), which must be dephosphorylated by the UppPs (undecaprenyl pyrophosphate phosphatases) and shuttled back to the inner face of the membrane to be reused. ColM enters the periplasm of the cell via the FhuA/Ton system. Then, it requires the action of FkpA to exert its cytotoxic activity, which is the hydrolysis of lipid II. The FhuA/Ton system can be bypassed to internalize ColM into the periplasm, either by an osmotic shock that transiently permeabilize the outer membrane, or by the fusion of ColM to a signal peptide to be excreted via the Sec translocase. When ColM is sent in the periplasm by these bypass procedures, it still requires FkpA. In contrast, ColMter, which contains the activity domain, is totally FkpA-independent whatever the internalization process. The translocation domain, the receptor-binding domain and the toxicity domain of ColM are schematically represented in yellow, red and green respectively. I, catalytically inactive form of ColM; A, catalytically active form of ColM or ColMter; sp-ColMter, ColMter fused to OmpA signal peptide.

Noteworthily, ColM (29 kDa) is the smallest colicin as the others display a size of 50–70 kDa. A patch of a few surface-accessible residues located in the C-terminal part of ColM probably forms its active site as shown by mutagenesis studies [20] (Figure 2). Nevertheless, these residues form a relatively atypical active site and their side chains do not lie within short distances, suggesting that it may undergo a conformational change upon substrate binding and/or a maturation process.

ColM maturation

Many colicins, as long as their passage through the outer membrane is accomplished, can exert their cytotoxicity without the presence of any other cell component except for the target itself. The situation differs for ColM, which requires the presence of the periplasmic PPIase (peptidylprolyl cis-trans isomerase)/chaperone FkpA to acquire its toxicity since fkpA-null mutants are fully resistant to ColM [22]. The action of FkpA is not linked to the import, since ColM remains FkpA-dependent even though the FhuA/Ton import machinery is bypassed to internalize the toxin [20]. The bypass is achieved by two different ways: either the native ColM is sent to the periplasm of the targeted cell by an osmotic shock that transiently permeabilizes the outer membrane [23], or ColM is fused to the signal sequence of OmpA to be excreted via the Sec translocase directly from the inside of the cell [16]. Both the PPIase and chaperone activities of FkpA are required as shown by the use of different FkpA mutants deprived of one or the other activity [16]. It has been hypothesized that ColM unfolds during its import through FhuA and that FkpA is then required to support ColM refolding; however, when native ColM is sent to the periplasm by the osmotic shock procedure, it remains
FkpA-dependent, suggesting that ColM unfolding may not be such a prerequisite. One bond involving a proline residue (Phe175–Pro176) from ColM was shown to be the possible target for FkpA PPIase activity since the substitution of alanine for Pro176 results in a reduced cytotoxicity [24]. This change was not due to a problem of import and when the P176A mutant protein was sent into the periplasm by osmotic shock, its residual toxicity was independent from FkpA. No substitution of any other proline residue of ColM had such consequences. Nevertheless, when P176A penetrates the periplasm via FhuA or Sec, its residual activity is FkpA-dependent as observed for the wild-type, suggesting that the bond involving Pro176 is not the sole substrate for FkpA within ColM. The role of FkpA and its mode of action towards ColM are yet to be clarified.

ColM_{Cter} (C-terminal part of ColM) is FkpA-independent
From mutagenesis studies, it was clearly established that the C-terminal half of ColM contained the active site; however, the structure of the toxin did not allow the visualization of an independent catalytic domain as observed for other colicins. In an attempt to determine whether such a catalytic domain exists on its own or whether ColM works as a whole, dissection studies were performed [20]. When different variants, truncated at their N-terminus, were expressed in the cytoplasm of *E. coli* cells, they formed inclusion bodies. These variants could be readily solubilized and renatured in vitro. The 14-kDa C-terminal region of ColM that is conserved among ColM-like bacteriocins (see below) was precisely the minimal domain displaying lipid-II-degrading activity in vitro (Figure 2). Interestingly, this cytotoxic domain (ColM_{Cter}) exhibited 50-fold more in vitro activity than the full-length protein and of course it was non-toxic towards *E. coli* cells as being unable to be internalized. In contrast, when ColM_{Cter} was sent into the periplasm of a non-immune cell by the osmotic shock procedure, it was fully lethal (Figure 1). ColM_{Cter} was also fused to a signal sequence and it was then addressed in the periplasm via the Sec apparatus [16]. In this case, ColM_{Cter} was also fully lethal unless the immunity protein (Cmi) was co-expressed, showing the ability of this domain to fold in the periplasm without the aid of the rest of the protein and to exert its toxic activity.
Unexpectedly, whatever the means used to address ColM_{Cter} in the periplasm, its cytotoxicity was totally independent of the presence of FkpA (Figure 1). The fact that ColM_{Cter} was more active in vitro than the full-length protein and that it was no more FkpA-dependent strongly suggests that ColM_{Cter} reflects the conformation in which ColM meets lipid II. ColM must then undergo a maturation process that is independent of the import machinery (FhuA/Ton) and is probably achieved by the dual activity of FkpA. This mechanism of maturation is yet to be clarified. For instance, whether unfolding of ColM during its import is necessary remains unclear. Our attempts to reproduce this maturation process in vitro have failed to date. As a model of action, ColM is produced and released in an inactive form by the producing cell and it is matured once it has reached the periplasm of the sensitive cell (Figure 1). We can make the link with nuclease colicins that are produced in an inactivate form as a complex with their immunity protein, from which they are released only once they encounter a sensitive cell. The fact that ColM would be produced in an inactive form may explain why lipid intermediates are not substrates at the cytoplasmic side of the membrane, even when ColM is largely accumulated in the cytoplasm. The maturation process of ColM must lead to a conformational change that makes the active site operational, which is consistent with the atypical active site observed in ColM structure that would not be adapted for full expression of the phosphatase activity. The low level of in vitro activity of purified ColM could then arise from either an intrinsic low activity or from a minor population of ColM being in an active conformation. This latter population would have acquired this conformation spontaneously.

**ColM-like bacteriocins**

Genes encoding homologues of ColM have been identified in certain strains of human- and plant-pathogenic *Pseudomonas*, *Burkholderia* and *Pectobacterium* species [25,26]. The identity between ColM and its homologues was strictly limited to their C-terminal half, which contains the cytotoxic domain. These proteins must have evolved in order to parasitize specific import machinery while sharing the same target. The putative bacteriocins from *Pseudomonas* species (pyocins) were shown to exhibit a narrow range of antibacterial activity that could be attributed to the same action as ColM with regard to their capacity to degrade lipid II in vitro [25]. The bactericidal activity of ColM homologues from the phytopathogenic *Pectobacterium carotovorum* strains could also be confirmed towards strains of *P. carotovorum* and *Pectobacterium asterosepticum*. Interestingly, it was further shown that the translocation/binding domain sequences of these ColM-like bacteriocins (pectocins M1 and M2) are very similar to those of plant ferredoxins and that these domains are utilized to parasitize an iron-uptake system from the targeted bacteria [26].

**Conclusions**

Bacteriocins are produced by certain bacteria to acquire a privilege over others in order to proliferate in a given ecological niche. ColM and its orthologues form a novel and unique family of bacteriocins interfering with the cell-wall integrity by enzymatic degradation of the peptidoglycan lipid intermediate. They drive the bacteria to cell lysis and exert their bactericidal activity without crossing the cytoplasmic membrane. The only physical obstacle for the ColM-like bacteriocins is therefore the outer membrane or the peptidoglycan layers to reach lipid II. The fact that ColM_{Cter} exhibits higher activity compared with the wild-type protein and can bypass the requirements for maturation process, together with the activity of ColM towards lipid II found in the main pathogenic species, makes ColM an attractive bacteriocin in view of its potential use as an antibacterial agent endowed with a broad spectrum.

**Acknowledgements**

This work was supported by grants from the Agence Nationale de la Recherche PEPEGLYCOL project [grant number ANR-07-MIME-020], the European Community Framework Programme 6, COBRA project [grant number LSHM-CT-2003-503-335] and the Centre National de la Recherche Scientifique (UMR 8619).

**References**

13 Harkness, R.E. and Braun, V. (1990) Colicin M is only bactericidal when provided from outside the cell. Mol. Gen. Genet. 222, 37–40

Received 27 July 2012
doi:10.1042/BST20120189