Are specialised ABC transporters responsible for the circularisation of type I circular bacteriocins?

Authors

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Abstract

Circular bacteriocins are relatively stable, antimicrobial proteins produced by a range of Gram-positive bacteria and are circularised by a peptide bond between the N and C termini. They are compact, basic, α-helical proteins which are hydrophobic in nature and assemble to form Na⁺ or H⁺ membrane pores. Circularisation contributes to a stable protein structure with enhanced thermostability, pH tolerance, and proteolytic stability. Secretion of active bacteriocin requires a multi-gene cluster than enables production, self-immunity and secretion with a putative ATP-binding cassette (ABC) transporter playing a major role. However the mechanism of circularisation is not known.

By analysing sequence alignments and structural predictions for the specialised ABC transporters of known circular bacteriocins and comparing them with more conventional ABC transporters, a mechanism for bacteriocin circularisation can be proposed. The additional conserved proteolytic domains of these ABC transporters are likely to be sedolisins or serine-carboxyl endopeptidases which firstly remove the signal sequence before coupling this directly to ligation of the N and C termini prior, probably via an enzyme bound acyl intermediate, prior to an ATP dependent translocation which ensures thermodynamic feasibility. We propose that circular bacteriocins are processed and circularised in this way, via their own specialised ABC transporters.
Circular bacteriocins are secreted antimicrobial proteins [2] found thus far in Gram-positive bacteria only [1, 3, 4], which are ligated at the N and C termini by a peptide bond [5, 6]. Circularisation gives them a number of advantageous traits such as thermostability, pH tolerance, proteolytic resistance and temporal stability [7-9]. This also renders them resistant to exopeptidases. The circularity does not have to directly contribute to antimicrobial activity per se, as shown in AS-48 [10], but rather contributes to the three-dimensional stability of the molecule which is required for antimicrobial activity [11]. This enhanced stability for a relatively small molecule is important for functional efficacy. After binding to the target cell membrane through conserved basic residues, they assemble to form pores in the target bacteria which form non-selective ion channels, killing the target bacteria [12-15]. In general, their antimicrobial activity functions via binding to the cell membrane and forming pores. Garvicin ML is an exception however as it has been shown to bind selectively to the maltose ATP-binding cassette (ABC) transporter [16]. Phylogenetic analysis shows two distinct families of circular bacteriocins [1, 3, 4], and despite considerable sequence differences, they appear to demonstrate functional and structural convergence [15, 17-20], even when the precise N to C terminal ligation point is offset within the structure [1]. There are 21 circular bacteriocins reported thus far, including aureocyclicin 4185 [21], enterocin NKR-5-3B[22], amylocyclicin [23], amylocyclicin CMW1 [24], enterocin AS-48 [6], bacA [25], carnocyclin A [26], circularin A [27], thermocin 458 [28], garvicin ML [29], lactocyclicin Q [30], leucocyclicin Q [31], Pumilarin [4] uberolysin [32], acidocin B [33], butyrivibriocin AR10 [34], paracyclicin [35], gassericin A/reutericin 6 [5], plantaricyclin A [9], plantacyclin B21AG [36-38] and cerecyclin [3].
However, it is currently unknown how circular bacteriocins are processed from the linear form to a fully ligated form. The enzyme(s) involve in the cleavage of leader peptide and the subsequent circularisation and/or extracellular transportation is likely a sequence-specific peptidase with a specific sequence recognition motif, although there is variation of terminal residues. In most cases of lantibiotic bacteriocins, ABC transporters have an N-terminal ATP-dependant proteolytic domain, usually within the first 150 residues [39-41]. Similarly, within each circular bacteriocin cluster, an ABC transporter (circABC transporter) sequence is always found [1, 3, 4]. This is putatively involved with extracellular export of these proteins and likely forms a protein-protein complex with the membrane proteins such as DUF95 found within the bacteriocin clusters. When the membrane-spanning DUF95 superfamily protein was knocked out in the leucocyclicin Q gene cluster, mature, circularised bacteriocin was found within the cell [42], indicating post-translational circularisation occurs intracellularly before export. This is consistent with the ABC transporter circularising the bacteriocin, as it was not anchored in the membrane and could not export the circularised bacteriocin extracellularly.

In this analysis, based on the sequence alignment and structural analysis of ABC transporters found within circular bacteriocin clusters, we propose that ABC transporters cleave the signal sequence with direct coupling to ligation, probably through an enzyme bound acyl intermediate resulting in a closed circular molecule that is then subject to translocation across the membrane.

**Materials and methods**

**ABC transporter sequence analysis**
HMMER [43] (https://www.ebi.ac.uk/Tools/hmmer/, date accessed: 2/9/19) was used to identify the N-terminal ABC transporter domain from the gene clusters of the circular bacteriocins (Data S1). This region (starting at residue 1) was aligned against known peptidase-domains from ABC transporters to determine any putative peptidase domains using BLAST [44] (https://blast.ncbi.nlm.nih.gov/Blast.cgi, date accessed: 2/9/19). Once confirmed, sequences of the N-terminal domains of these circular bacteriocin associated-ABC transporters were aligned using Clustal Omega and a phylogenetic tree was built using FigTree.

**Hydrophobicity profiles**

Hydrophobicity profiles were generated using the protscale website https://web.expasy.org/protscale/ with a sliding window of 9 [45]. Data was visualised in R version 4.0.2, with the following packages: viridis (version 0.5.1), reshape2 (version 1.4.4), ggplot2 (version 3.3.2), grid (version 4.0.2), RColorBrewer (version 1.1-2). The local regression line drawn was computed using the loess method from ggplot2.

**Structural analysis**

I-TASSER [46] (https://zhanglab.ccmb.med.umich.edu/I-TASSER/, date accessed 1/9/19) was used to predict the 3D structure of the plantacyclin B21AG ABC transporter. Sequences used can be found in Data S1. The structure of the ABC transporter ATP-binding protein from *Thermotoga maritima* (Protein Data Bank ID: 4YER) was also utilised for analysis.

**Results and discussion**

**Analysis of the circular bacteriocin-associated ABC transporters**

Each circABC transporter was aligned against known ABC transporter N-terminal peptidase domains which have been demonstrated to cleave signal peptides. They showed high levels
of similarity to known N-terminal peptidase domains from EpiF and SpaT (Table S1), which allow signal peptide cleavage and translocation of post-translationally modified bacteriocins epidermin and subtilin, respectively [47-49]. We hypothesize that circular bacteriocin-associated ABC transporters are not only performing the cleavage of the signal peptide, but also the subsequent ligation and translocation across the cell membrane. Recently, it has been shown that ABC transporter EnkT from Enterococcus faecium NKR-5-3 could cleave signal sequences and export several non-circular bacteriocins [50].

Alignment of the proteolytic domain at the N-terminal region of the circABC transporters (Figure 1) showed conserved residues at Gly$^{37}$, Gly$^{40}$, Gly$^{42}$, Lys$^{43}$, Thr$^{45}$, Ser$^{121}$, Gly$^{123}$, Asp$^{144}$, and Glu$^{145}$ (numbered based off plantacyclin B21AG circABC transporter sequence) across every sequence. The ubiquitously conserved Gly-X-X-Gly-X-Gly-Lys-Thr/Ser motif forms a mononucleotide or ATP/GTP binding site [51, 52]. The conserved Ser$^{121}$, Asp$^{144}$ and Glu$^{145}$ indicate these proteolytic domains are likely serine-carboxyl peptidases or sedolisins, relying on a SED catalytic triad [53].

Sedolisins are peptidases with activity at low pH [54], which matches the general activity and low pH local environment of L. plantarum species growing fermentatively [55]. Ser$^{121}$ is the nucleophile involved in amide bond breakage, while Glu$^{145}$ acts as a general base for proton transfer from serine during nucleophilic attack, then acts as an acid to protonate the leaving group. Asp$^{144}$ acts as a general acid forming the oxyanion hole to protonate the glutamate as well as forming the acyl intermediate [53, 56].
Figure 1: Multiple sequence alignment of the peptidase domains of the ABC transporters (from all experimentally verified circular bacteriocins) downstream of the circular bacteriocin structural genes, along with an ABC transporter from Thermotoga maritima MSB8. Bolded in blue shows the conserved ATP binding motif Gly-X-X-Gly-X-Gly-Lys-Thr/Ser. Bolded in red shows the predicted peptidase Ser-Asp-Glu catalytic triad, indicating these peptidases are serine-carboxyl peptidases. Bolded in black show other conserved residues without predicted functions. Alignment performed with ClustalW output.
To further corroborate this hypothesis, the 3D structure of the plantacyclin B21AG circABC transporter was predicted to see if these residues would be predicted to be within potential catalytic proximity (Figure 2). The ABC transporter ATP-binding protein from *Thermotoga maritima* (PDB accession 4YER) had the closest structural homology (TM score of 0.891, RMSD of 1.42) and was used for this analysis. This protein was also included in the multiple sequence alignment (Figure S1) and is also likely a sedolisin due to the conserved SED triad. This also showed ADP molecules bound to the Gly-X-X-Gly-X-Gly-Lys-Thr/Ser ATP/GTP binding motif. These ABC transporters exist functionally as dimers [57, 58] as seen in Figure 2. Existing as a dimer may allow the SED triad to be in close enough proximity, given they are all found within the main groove of the structure. It is possible the catalytic Asp and Glu from chain A may form the triad with Ser from chain B, and vice versa.
Figure 2: Protein structure of ABC transporter dimer from *T. maritima* (PDB accession 4YER) visualised in PyMOL. Chain A is shown in red. Chain B is shown in blue. A shows a side on view of the protein. B shows the active site pocket formed between the ABC transporter dimer. C shows a closeup of the active site. The proposed catalytic triad of Ser, Asp and Glu are shown as stick models. ADP is shown in yellow.
We hypothesise that this peptidase also performs circularisation after cleavage. As the circABC transporters contain a SED motif allowing amide bond cleavage, the opposite reaction (amide bond formation) should also be occurring. Similarly to a ribosome, the peptidase likely shields the catalytic active site from water [59], encouraging the hydrophobic bacteriocin pre-peptide (containing the leader signal sequence) to fit and bind for subsequent cleavage of the signal sequence. Energy from the hydrolysis of 2 ATP at the ATP binding domain of the ABC transporter produces ~30.5 kJ/mol -ΔG [60], allowing this reaction to take place. Cleavage of the amide bond between the C terminus of the signal sequence and the N terminus of the mature peptide results in the addition of a single water molecule and 8 – 16 kJ/mol -ΔG are produced via hydrolysis of the amide bond [61, 62].

The equilibrium constant for peptide bond formation in water is extremely small [63], which may explain why both termini of circular bacteriocins are hydrophobic [1], as they will favour low-water environments located in the SED active site within the circABC transporter. Circular bacteriocins are unique in this aspect, as hydrophobicity profile comparisons to other non-circular bacteriocins do not have dual hydrophobic termini (Figure 3, Data S2).
Figure 3: Facet graphs showing the Kyte-Doolittle hydrophobicity of a range of bacteriocin subclasses. Circular bacteriocins are divided into both main families (i and ii) [1]. Hydrophobicity is shown ranging from high (red), to neutral (white), to hydrophilic (blue). Black dots show individual points. The different subclasses of bacteriocins are shown in different colours, labelled at the top of each graph. Data used can be found in Data S2. Graphs produced using the ggplot R package.
Prior to ligation/circularisation, the hydrophobic C terminal region of the linear bacteriocin protein would then move into close proximity to the N terminus of the bacteriocin within the water-minimal active site.

This is also likely due to the α-helix conformation found at both termini and where the mid-α-helix ligation point is usually found in circular bacteriocins. It is proposed that the terminal ligation occurs via condensation, likely using 8 – 16 kJ/mol -ΔG from the hydrolysis reaction along with any available free energy from the 2 ATP hydrolysis reactions. The ejected water molecule could be used to stabilize the oxyanion of the tetrahedral intermediate through hydrogen bond donation, as it does in ribosomial peptide bond catalysis [59], or is otherwise donated to the acylated aspartate, deacylating it and releasing the carboxylic acid of the newly ligated residue and producing a free bacteriocin. The SED catalytic triad may be favourable for circularisation as the weaker basicity of glutamate as compared to the usual histidine in Ser-His-Asp (SHD) catalytic triads of classical serine proteases may allow greater opportunity for N and C termini ligation though the increased hydrophobicity and weaker basicity of the site. Substitution of the histidine with glutamine eliminates and slows hydrolysis of esters in meta-cleavage product (MCP) hydrolases [64].
Figure 4: Schematic demonstrating the proposed mechanism of circularisation. The ABC transporter dimer is shown in red and blue, with the catalytic triad residues shown by their corresponding structure. The signal sequence of the bacteriocin is shown in grey lettering, while the mature bacteriocin sequence is shown in black. **A** shows the prepeptide before binding to the ABC transporter. **B** shows the hydrophobic prepeptide binding to the peptidase domain of the ABC transporter. **C** shows the signal peptide cleavage at the cost of 2 ATP. The yellow arrow shows nucleophilic attack of the peptide bond between the C terminal signal sequence and N terminal mature peptide. Black arrows show protonation direction. **D** shows the hydrolysis of the signal peptide and $8 - 16 - \Delta G$ produced from amide bond cleavage. **E** shows the hydrophobic C terminus moving into and binding to the peptidase active site and subsequent condensation reaction, resulting in amide bond formation and the loss of $8 - 16 - \Delta G$. Ejection of the water molecule is also shown. **F** shows the released peptide release from the active site, ready for extracellular export.
The likely explanation for the direction reversal of the peptidase from hydrolysis to condensation lies in the substrate: product equilibrium. It is possible that linear bacteriocin is simply not preferentially exported from the cell, as seen in mutants of AS-48 which impact ligation residues [65]. In the Met1Ala mutant, no bacteriocin was detected externally, however in the Trp70Ala mutant, a small amount of linear bacteriocin was found in supernatant. This indicates linear circular bacteriocins can be exported, though not efficiently. A build-up of non-mutant linear bacteriocin may push the cleavage equilibrium towards terminal bond formation although no such internal accumulation has been noted, and the process appears tightly coupled. Overall, this process would be energetically favourable, if the product is removed quickly through ATP/GTP dependant translocation to allow efficient recycling of resources and energy-efficient circularisation of circular bacteriocins.

**Conclusions**

We propose that the bacteriocin circularisation mechanism involves the cleavage of a signal peptide by the peptidase domain of the bacteriocin-associated ABC transporter followed by subsequent amide bond formation with the nascent N terminus, driven by an SED catalytic triad in a uniquely hydrophobic environment. This is coupled to rapid ATP/GTP dependent translocation for extracellular export to ensure overall thermodynamic feasibility.
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Declaration of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

Analysis and interpretation performed by BV, BHAR and ATS. All authors read and approved the final manuscript.

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Data availability

All data utilised in this analysis is provided in Table S1, Table S2, Data S1, and accessible data from public databases including the structure of the ABC transporter ATP-binding protein from Thermotoga maritima (Protein Data Bank ID: 4YER).

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