# Supporting Information

## Leader- and terminal residue requirements for circularin A biosynthesis probed by systematic mutational analyses

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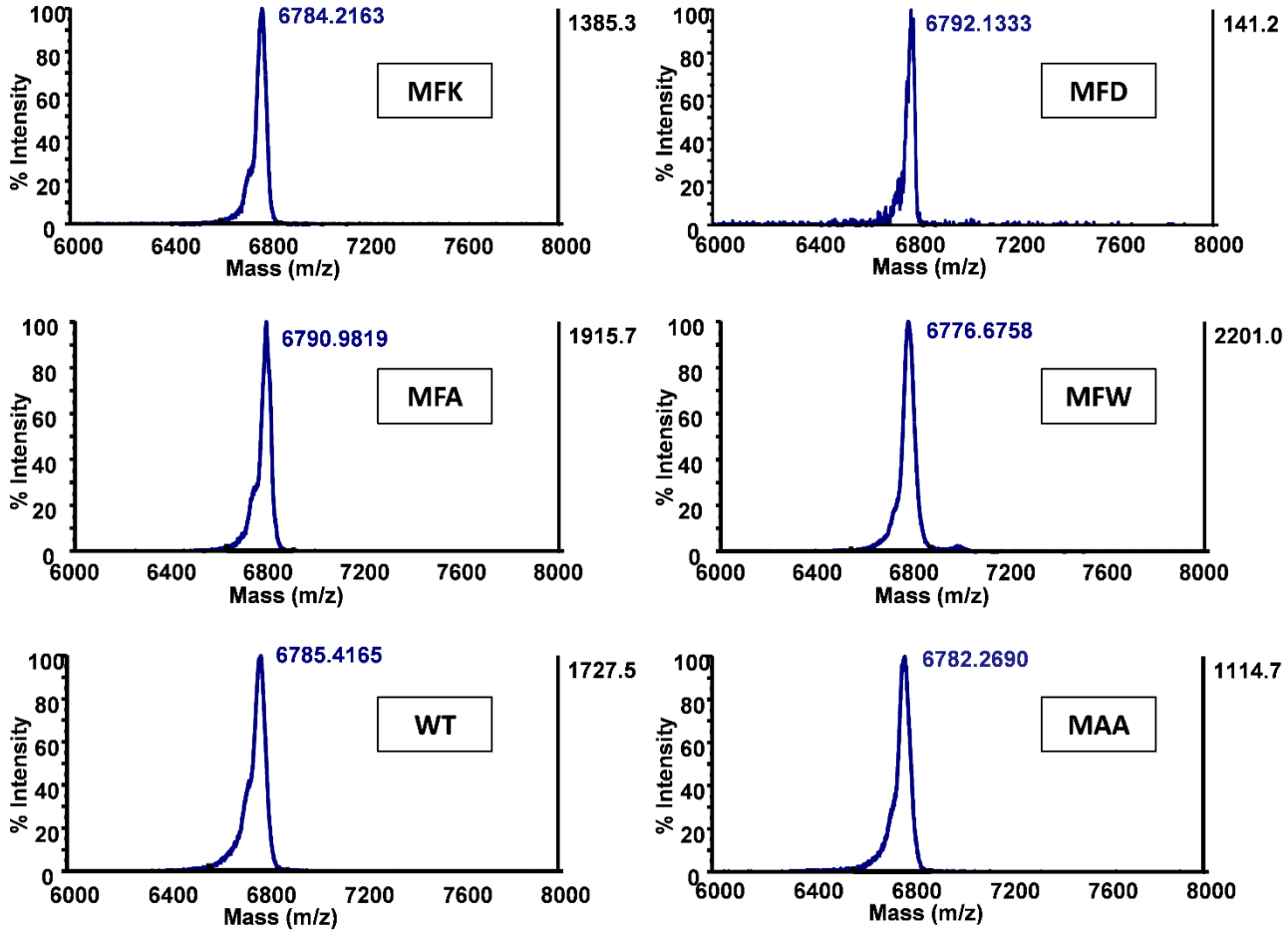
**S1. Amino acid sequence alignment of the characterized subgroup I circular bacteriocins.**

The alignment was performed using Clustal Omega with default settings. The color coding was shown in Jalview by setting conservation with percentage identity above 30%.

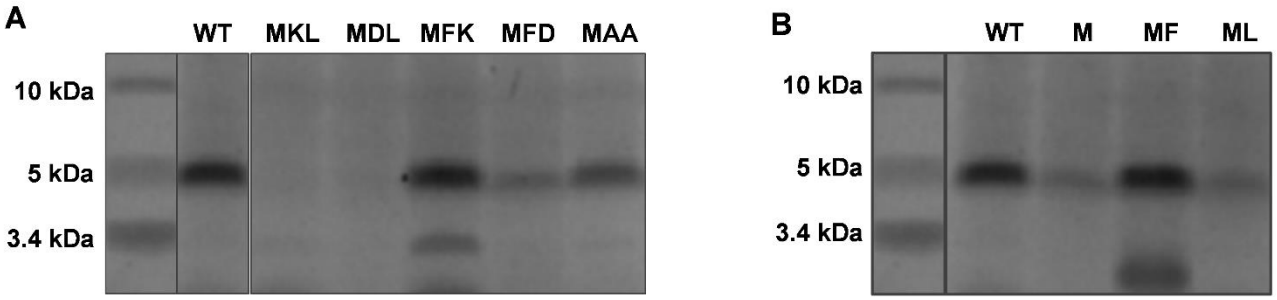
**S2. MALDI-TOF spectra of circularin A leader variants with site-directed substitutions.**

All these active leader variants produce the fully modified circularin A: the leader cleavage site of these leader variants is always in front of Val1, and the mass difference of a few Daltons is likely because the sample contains a mixture of WT bacteriocin and bacteriocin with 1x (or 2x) oxidation of the Met residue(s).



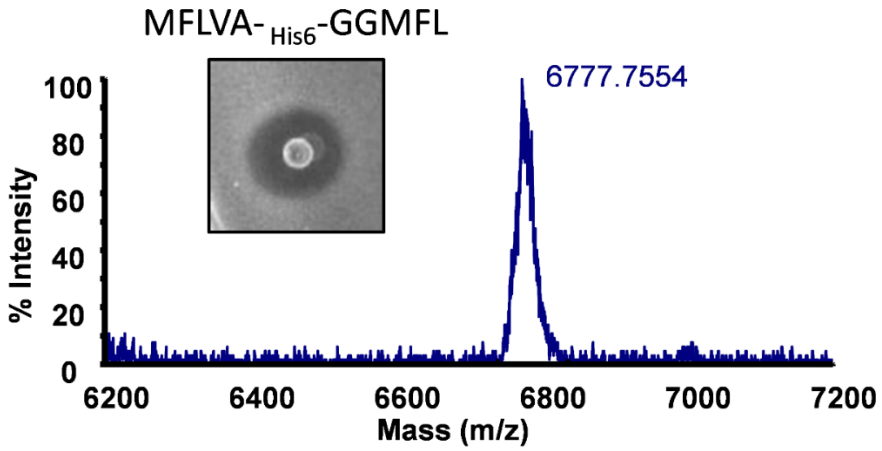
**S3. Tricine-SDS-PAGE to determine the production levels of circularin A in various leader mutants.**

The wild-type peptide showed a peptide band corresponding to a size close to 5K Dalton (Da). MFK seemed to have better yield relative to its activity level. However, the sample of MFK was also more prone to degradation (shorter peptides that run below the mature peptide were observed), which might explain the lower activity detected for MFK.

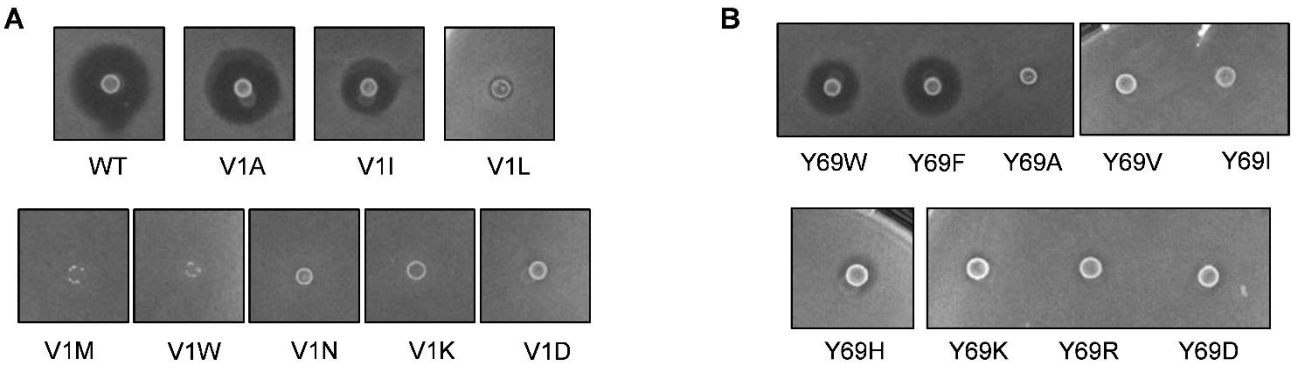


**S4. MALDI-TOF spectrum of peptide purified from the 16-aa leader variant (MFLVA-$_{His6}$-GGMFL).**

The theoretical mass of the mature circularized circularin A (WT) is 6771.05 Da. The mass difference between peptides produced from this 16-aa leader variant and the 3-aa leader WT is less than 7 Dalton, suggesting the correctly circularized bacteriocin production for this leader variant.



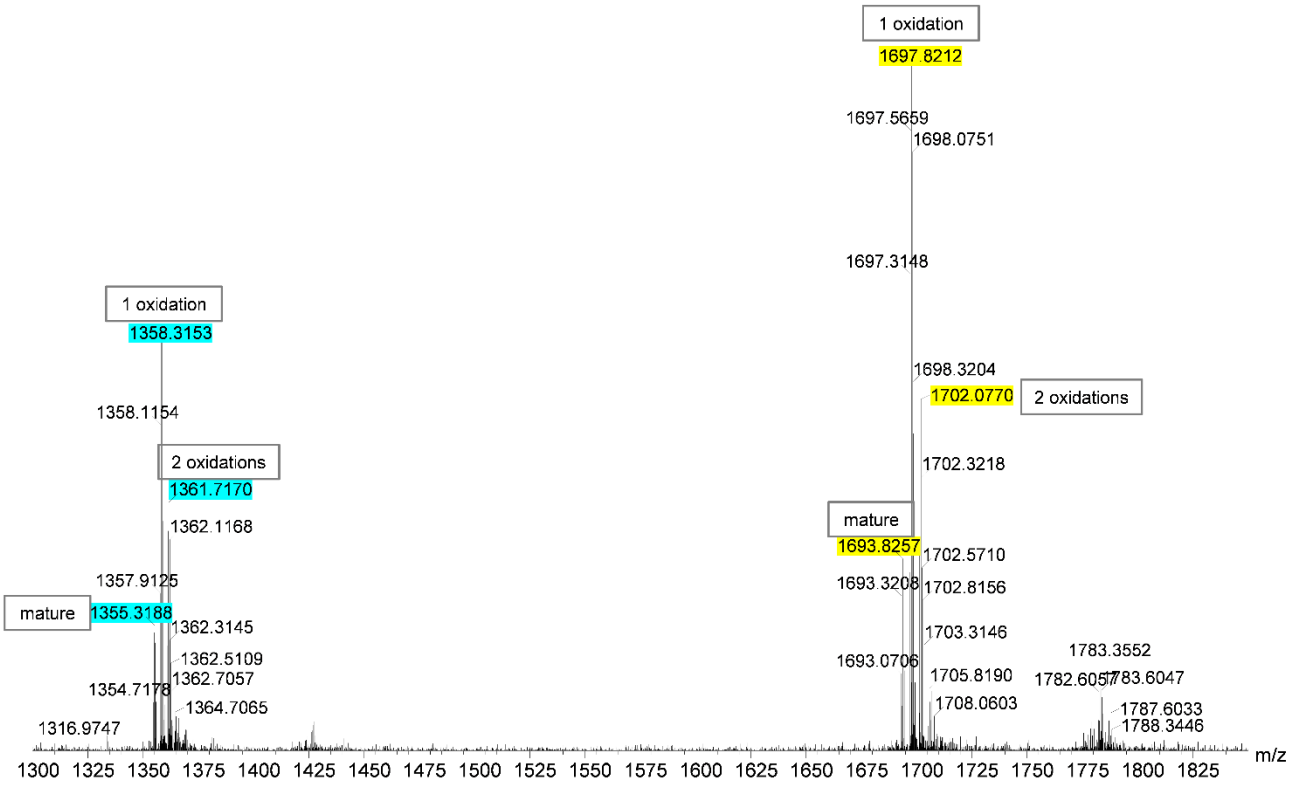
**S5. Antimicrobial activity of Val1 (or Tyr69) mutants with site-directed substitutions in circularin A.** The indicator strain: *Lactobacillus sake* ATCC 15521.



**S6. Detected masses from the LC-MS analysis of the wild-type circularin A.**

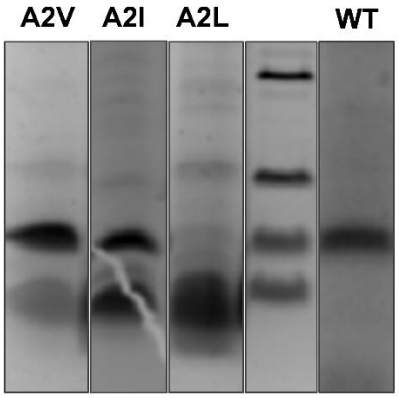
Three forms of fully modified circularin A were identified: the circular mature bacteriocin (WT), WT with 1 oxidation and WT with 2 oxidations.

| Variant form | Mw | M/Z | | |
|---|---|---|---|---|
| | | $+3H^+$ | $+4H^+$ | $+5H^+$ |
| **Circular/** mature | WT 6771 | 2258 | 1693,75 | 1355,2 |
| **Circular/** 1 oxidation | WT+16 6787 | 2263,3 | 1697,75 | 1358,4 |
| **Circular/** 2 oxidations | WT+32 6803 | 2268,67 | 1701,75 | 1361,6 |
| **Linear** No leader | WT+18 6789 | 2264 | 1698,25 | 1358,8 |
| **Linear** With leader | 7180.58 | 2394,52 | 1796,14 | 1437,11 |



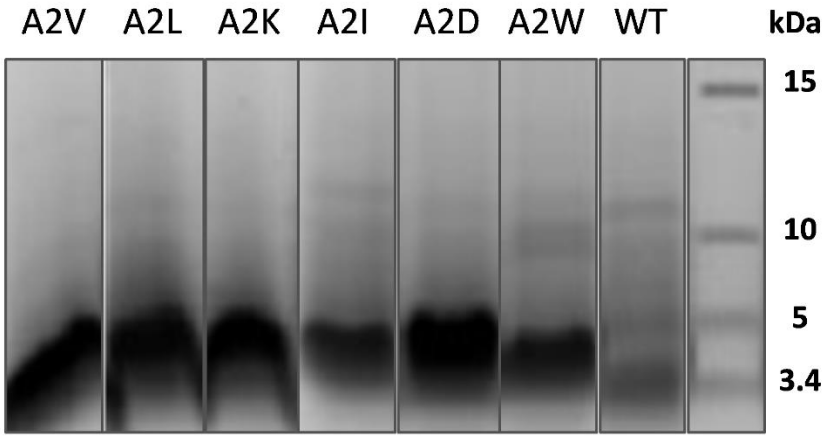
**S7. Tricine-SDS-PAGE to determine the production levels of mature circularin A derivatives in Ala2 mutants.**

The wild type showed a single band of the target peptide (corresponding to a size close to 5K Dalton, the second lowest band of the protein ladder).



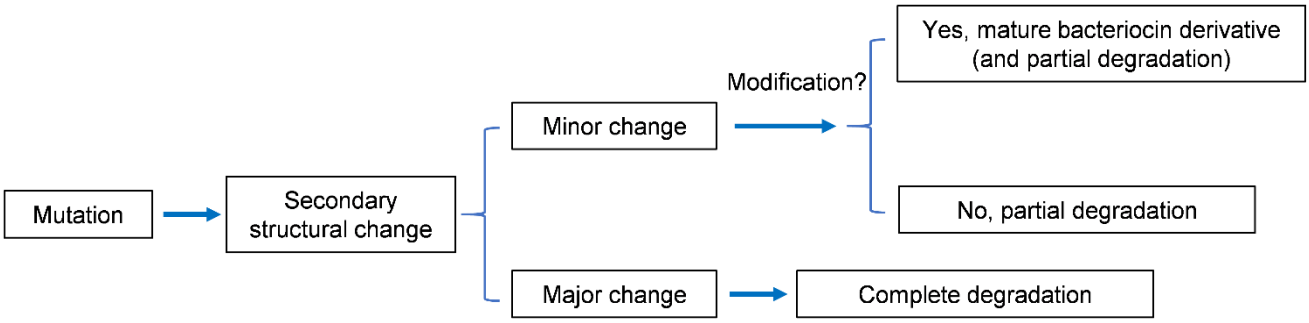
**S8. Peptide degradation of various Ala2 mutants**

These peptide fragments were eluted in 50% solvent fraction from C18 purification and they ran slightly below 5K Dalton (Da). The mature circularin A was often eluted in 80% solvent fraction and showed a peptide band corresponding to a size close to 5K Dalton (Da). Compared to Ala2 mutants, the wild type (WT) was less degraded.



**S9. Proposed scheme of possible biosynthetic processing of circularin A derivatives.**

In this theory, the maintaining of the intrinsic structure of the precursor in mutants is critical for biosynthetic processing of the derivatives with the cognate enzyme(s).



**S10 (Table S1). Short-leader circular bacteriocins and their leader sequences**

| **Circular bacteriocin** | **Leader sequence** [a] | | | | | |
|---|---|---|---|---|---|---|
| | -6 | -5 | -4 | -3 | -2 | -1 |
| Circularin_A | | | | M | F | L |
| Cerecyclin | | | M | L | F | N |
| Uberolysin | M | D | I | L | L | E |
| Lactocyclicin_Q | | | | | M | K |
| Leucocyclicin_Q | | | | | M | F |
| Garvicin_ML | | | | M | F | D |
| Carnocyclin_A | | | M | L | Y | E |
| Bacicyclicin XIN-1 | | | M | L | F | E |
| Aureocyclicin 4185 | | | M | L | L | E |

[a] aromatic residues are highlighted in yellow and charged residues in green.

**S11 (Table S2). Oligonucleotides used in this study**

| Variant | Primer | Sequence (5'→3') |
|---|---|---|
| Leader variant | | |
| M | P01 | ACTCACCAUGGTTGCAGGAGCACTAGG |
| | P02 | ATGGTGAGUGCCTCCTT |
| ML | P03 | ACTCACCAUGTTAGTTGCAGGAGCACTAGGC |
| | P04 | ATGGTGAGUGCCTCCTT |
| MF | P05 | ACTCACCAUGTTTGTTGCAGGAGCACTAGGC |
| | P04 | ATGGTGAGUGCCTCCTT |
| $M_{His6}$ | P06 | ACCATCACCAUCATGTTGCAGGAGCACTAGG |
| | P07 | ATGGTGATGGUGATGCATGGTGAGTGCCTCCTT |
| $M_{His6}$MFL | P08 | ACCATCACCAUCATATGTTTTTAGTTGCAGGAGCAC |
| | P09 | ATGGTGATGGUGATGCATGGTGAGTGCCTCCTT |
| MFLVA-$_{His6}$-GGMFL | P10 | CATCATCACCACGGTGGTATGTTTTTAGTTGCAGGAGCACTAG |
| | P11 | GTGATGAGCAACAAGGAACATTTTGAGTGCCTCCTTATAATTTATTTTGTAGTTCC |
| MDL | P12 | AGGAGCACUAGGCGTGCAA |
| | P13 | AGTGCTCCUGCAACTAAATCCATAATTAATCACC |
| MFD | P12 | AGGAGCACUAGGCGTGCAA |
| | P14 | AGTGCTCCUGCAACATCAAACATAATTAATCACC |
| MKL | P12 | AGGAGCACUAGGCGTGCAA |
| | P15 | AGTGCTCCUGCAACTAATTTCATAATTAATCACC |
| MFK | P12 | AGGAGCACUAGGCGTGCAA |
| | P16 | AGTGCTCCUGCAACTTTAAACATAATTAATCACC |
| MAA | P12 | AGGAGCACUAGGCGTGCAA |
| | P17 | AGTGCTCCUGCAACTGCAGCCATAATTAATCACC |
| MFA | P45 | GGAGCACTAGGCGTGCAA |
| | P50 | TGCAACAGCAAACATGGTGAGTGCCTCCTT |
| MFW | P45 | GGAGCACTAGGCGTGCAA |
| | P51 | TGCAACCCAAAACATGGTGAGTGCCTCCTT |
| Bacteriocin derivative | | |
| V1I | P18 | TAAAAACATGGTGAGTGCCTCC |
| | P19 | ATTGCAGGAGCACTAGGCG |
| V1L | P18 | TAAAAACATGGTGAGTGCCTCC |
| | P20 | TTAGCAGGAGCACTAGGCG |
| V1M | P18 | TAAAAACATGGTGAGTGCCTCC |
| | P21 | ATGGCAGGAGCACTAGGCG |
| V1N | P18 | TAAAAACATGGTGAGTGCCTCC |
| | P22 | AATGCAGGAGCACTAGGCG |
| A2K | P23 | GTTTTTAGTTAAAGGAGCACTAGGCGTGCAA |
| | P24 | CACGCCTAGTGCTCCTTTAACTAAAAACATGG |
| A2V | P25 | GTTTTTAGTTGTTGGAGCACTAGGCGTGCA |
| | P26 | ACGCCTAGTGCTCCTAAAACTAAAAACATGG |
| A68L | P27 | TATGGCAAGAGCTATATTATACTAAGCTTTCTTTGAACCAA |
| | P28 | GTTCAAAGAAAGCTTAGTATAATATAGCTCTTGCCATACTT |

| | | |
|---|---|---|
| A68V | P29 | TATGGCAAGAGCTATAGTTTACTAAGCTTTCTTTGAACCAA |
| | P30 | GTTCAAAGAAAGCTTAGTAAACTATAGCTCTTGCCATACTT |
| A68K | P31 | AAGTATGGCAAGAGCTATAAAATACTAAGCTTTCTTTGAACC |
| | P32 | TTCAAAGAAAGCTTAGTATTTTATAGCTCTTGCCATACTTTGC |
| A68W | P33 | GTATGGCAAGAGCTATATGGTACTAAGCTTTCTTTGAACC |
| | P34 | GTACCATATAGCTCTTGCCATACTTTGCTTAG |
| Y69H | P35 | TAAGCTTTCTTTGAACCAAAATTAGAAAAC |
| | P36 | TTTGGTTCAAAGAAAGCTTAATGAGCTATAGCTCTTG |
| Y69I | P35 | TAAGCTTTCTTTGAACCAAAATTAGAAAAC |
| | P37 | TTTGGTTCAAAGAAAGCTTATATAGCTATAGCTCTTGCC |
| Y69R | P35 | TAAGCTTTCTTTGAACCAAAATTAGAAAAC |
| | P38 | TTTGGTTCAAAGAAAGCTTATCTAGCTATAGCTCTTGCC |
| V1A | P39 | AGGAGCACUAGGCGTGCAA |
| | P40 | AGTGCTCCUGCAGCTAAAAAC |
| Y72F | P41 | AGCTATAGCUCTTGCCATAC |
| | P42 | AGCTATAGCUTTCTAATCAAAATTTATG |
| Y72W | P41 | AGCTATAGCUCTTGCCATAC |
| | P43 | AGCTATAGCUTGGTAATCAAAATTTATG |
| Y72A | P41 | AGCTATAGCUCTTGCCATAC |
| | P44 | AGCTATAGCUGCATAATCAAAATTTATG |
| A2D | P45 | GGAGCACTAGGCGTGCAA |
| | P47 | ATCAACTAAAAACATGGTGAGTGCC |
| A2I | P45 | GGAGCACTAGGCGTGCAA |
| | P48 | AATAACTAAAAACATGGTGAGTGCC |
| A2W | P45 | GGAGCACTAGGCGTGCAA |
| | P49 | CCAAACTAAAAACATGGTGAGTGCC |
| V1D | P45 | GGAGCACTAGGCGTGCAA |
| | P52 | TGCATCTAAAAACATGGTGAGTGCCTC |
| V1K | P45 | GGAGCACTAGGCGTGCAA |
| | P53 | TGCTTTTAAAAACATGGTGAGTGCCTCC |
| V1W | P45 | GGAGCACTAGGCGTGCAA |
| | P54 | TGCCCATAAAAACATGGTGAGTGCCTCC |
| Y69D | P56 | TAAGCTTTCTTTGAACCAAAATTAGAAAACCAAG |
| | P57 | ATCAGCTATAGCTCTTGCCATACTTTG |
| Y69K | P56 | TAAGCTTTCTTTGAACCAAAATTAGAAAACCAAG |
| | P58 | TTTAGCTATAGCTCTTGCCATACTTTG |
| Y69V | P56 | TAAGCTTTCTTTGAACCAAAATTAGAAAACCAAG |
| | P59 | AACAGCTATAGCTCTTGCCATACTTTG |
| A68D | P56 | TAAGCTTTCTTTGAACCAAAATTAGAAAACCAAG |
| | P60 | GTAATCTATAGCTCTTGCCATACTTTGCT |
| A68I | P56 | TAAGCTTTCTTTGAACCAAAATTAGAAAACCAAG |
| | P61 | GTAAATTATAGCTCTTGCCATACTTTGCT |

**S12 (Table S3). Amino acid sequences of the characterized subgroup I circular bacteriocins**

| Bacteriocin | Amino acid sequence |
|---|---|
| **Circularin_A** | VAGALGVQTAAATTIVNVILNAGTLVTVLGIIASIASGGAGTLMTIGWATFKATVQKLAKQSMARAIAY |
| **Cerecyclin** | VVSKLGWTGINIGTANALIGALMTGSDIWTAISVAGLAFGGGIGTAISTIGRKAIMEMVEKVGKKKAAQW |
| **Lactocyclicin_Q** | LIDHLGAPRWAVDTILGAIAVGNLASWVLALVPGPGWAVKAGLATAAAIVKHQGKAAAAAW |
| **Leucocyclicin_Q** | LVNQLGISKSLANTILGAIAVGNLASWLLALVPGPGWATKAALATAETIVKHEGKAAAIAW |
| **Garvicin_ML** | LVATGMAAGVAKTIVNAVSAGMDIATALSLFSGAFTAAGGIMALIKKYAQKKLWKQLIAA |
| **Uberolysin** | LAGYTGIASGTAKKVVDAIDKGAAAFVIISIISTVISAGALGAVSASADFIILTVKNYISRNLKAQAVIW |
| **Carnocyclin_A** | LVAYGIAQGTAEKVVSLINAGLTVGSIISILGGVTVGLSGVFTAVKAAIAKQGIKKAIQL |
| **Bacicyclicin XIN-1** | LTGIGIGSGTAATIVNWIMWGMSAATILSLISGVASGGAWILAGAREALKAGGKKAAIAW |
| **Amylocyclicin** | LASTLGISTAAAKKAIDIIDAASTIASIISLIGIVTGAGAISYAIVATAKTMIKKYGKKYAAAW |
| **Amylocyclicin_CMW1** | LASTLGISAAAAKKAIDIIDAASTIASIISLIGIVTGAGAISYAIVATAKTMIKKYGKKYAAAW |
| **AS-48** | MAKEFGIPAAVAGTVLNVVEAGGWVTTIVSILTAVGSGGLSLLAAAGRESIKAYLKKEIKKKGKRAVIAW |
| **BacA** | MAKEFGIPAAVAGTVLNVVEAGGWVTTIVSILTAVGSGGLSLLAAAGRESIKAYLKKEIKKKGKRAVIAW |
| **Aureocyclicin_4185** | LTGLGIGTGMAATIINAISVGLSAATILSLISGVASGGAWVLAGAKQALKEGGKKAGIAF |
| **Enterocin_NKR-5-3B** | LTANLGISSYAAKKVIDIINTGSAVATIIALVTAVVGGGLITAGIVATAKSLIKKYGAKYAAAW |
| **Pumilarin** | LAKEFGIPGSVAAVVLNVVEAGGAVTTIVSILTAVGSGGLSLIAAAGKETIRQYLKNEIKKKGRKAVIAW |