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Identification and Heterologous Expression of the *sec*-Dependent Bacteriocin Faerocin MK from *Enterococcus faecium* M3K31

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Abstract
Genome sequencing of *Enterococcus faecium* M3K31, a strain isolated from griffon vultures, previously revealed the presence of three sequences encoding for bacteriocins, namely enterocin P, enterocin HF, and a SRCAM 602-like bacteriocin. In this work, we describe the SRCAM 602-like bacteriocin that we named faerocin MK. Mature faerocin MK consists of 43 residues and contains an YGNGV-motif and two cysteine residues at positions 10 and 15, consistent with other class IIa bacteriocins. Faerocin MK and SRCAM 602 were chemically synthesized and their scope of activity was tested. Faerocin MK is active against a wide range of Gram-positive organisms while SRCAM 602 was not active against any tested organism. Although both peptides are more structured in trifluoroethanol, faerocin MK has an α-helical character nearly twice that of SRCAM 602. Nucleotide sequence analysis revealed that the faerocin MK precursor is produced with a 28-amino acid signal peptide and that an immunity gene follows the structural faerocin MK gene. Heterologous expression of the faerocin MK operon showed that faerocin MK and its immunity protein were successfully expressed in other organisms. This indicates that the bacteriocin is secreted through the *sec* pathway.

Keywords Bacteriocins · Enterococci · Probiotics · *sec*-dependent · SRCAM peptides

Introduction

Enterococcus species are widespread among plants, insects, animal products, and also within the human gastrointestinal tract. Their abundancy is an advantage that can be exploited, particularly as they possess beneficial attributes, such as the potential to produce a great variety of bacteriocins [1]. The diverse nature of these antimicrobial peptides allows enterococci to be active against a broad scope of organisms; of particular interest is the activity against food-borne pathogens and food spoilage organisms [1, 2]. In addition to their use in food products, enterococci have also been investigated as potential probiotics. However, the presence of virulence factors in many strains of enterococci raises the issue of safety when used in food products or as probiotics [2, 3]. Often cited as an opportunistic pathogen, enterococci are among the most prevalent nosocomial pathogens and are responsible for endocarditis, bacteremia, and urinary tract infections, among others [2, 4]. With the advent of whole genome sequencing, bacteriocinogenic enterococci can be discovered that lack virulence factors and might have potential as probiotics [5]. Analysis of the recently sequenced genome of *Enterococcus faecium* M3K31, isolated from griffon vultures, *Gyps fulvus*, indicates that this particular strain does not contain virulence factor genes [6]. Further genome mining revealed the presence of genes encoding enterocin P, enterocin HF, and a SRCAM 602-like bacteriocin. Enterocin P is a *sec*-dependent class IIa bacteriocin that contains an YGNGV-motif [7]. Enterocin HF also belongs to class IIa bacteriocins and is produced as a precursor with an N-terminal double-glycine leader peptide. The 3D structure of enterocin HF was recently elucidated [8]. The SRCAM 602-like peptide resembles multiple SRCAM peptides that were claimed to have a wide scope of antimicrobial activity against both Gram-positive and Gram-negative bacteria [9]. However, subsequent studies were unable to detect the production of several of these peptides and their genetic determinants were not found in the corresponding bacterial genomes [10, 11]. It was shown that the inhibitory activity towards Gram-negative bacteria by *Paenibacillus polymyxa* NRRL B-30509, that was claimed to produce SRCAM 602, could be attributed to the production of lipopeptides [12]. This

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prompted us to investigate the activity and structure of this SRCAM-like bacteriocin from *E. faecium* M3K31, which we named faerocin MK (FaeMK), and compare it to that of the reported SRCAM 602 peptide. In this study, we show that FaeMK is a 43-residue class IIa bacteriocin that is active against Gram-positive organisms and is likely to be structurally similar to enterocin HF. The FaeMK precursor contains a signal peptide that enables the bacteriocin to access the general secretion pathway. The FaeMK operon, containing the structural bacteriocin gene and the immunity gene, was cloned and successfully expressed in heterologous strains, which confirms the *sec*-dependent nature of this bacteriocin.

Materials and Methods

Bacterial Cultures

Gram-positive strains were grown using All Purpose Tween medium (Difco, Sparks, MD) at 25 °C, except for *Staphylococcus aureus* ATCC 25923 that was grown on tryptic soy broth medium (Difco). Gram-negative strains were grown using Luria Broth Base, Miller medium (Difco) at 37 °C. If required, chloramphenicol was added to the media at 5 μg/mL for *Carnobacterium maltaromaticum* UAL26 and 10 μg/mL for *Enterococcus faecium* BFE 900.

Bacteriocin Assays

FaeMK and SRCAM 602 were chemically synthesized and acquired in 98% purity from GenScript USA Inc. (Piscataway, NJ). Prior to use, the sequence and purity of the peptides were verified with mass spectrometry in-house. The minimum inhibitory concentrations (MICs) were tested using a spot-on-lawn assay. The peptides were tested against the indicator strains listed in Table 1. Using the growth conditions discussed above, the strains were grown overnight and 100 μL of each indicator strain was used to inoculate soft agar (0.75% *w*/*v*) and overlaid on solid agar plates using the appropriate media. A 2-fold series dilution of the peptides was prepared from a 64 μM stock solution and 10 μL of each concentration was spotted onto the agar plate. After an overnight incubation, the plates were examined for zones of inhibition and MICs were determined.

Circular Dichroism Spectroscopy

An OLIS DSM 17 CD spectrophotometer with a thermally controlled quartz cell with a 0.2-mm path length was used. Data were collected during five scans from 185 to 260 nm in 1 nm increments at 20 °C. FaeMK and SRCAM 602 solutions were prepared in 0, 25, and 50% 2,2,2-trifluoroethanol (TFE) in water at concentrations of 0.83 and 1.52 mg/mL, respectively. Results are reported in molar ellipticity units (deg × cm^2 / dmol) and the percent α-helicity of the two peptides was calculated using: $\frac{3000-\theta_{222}}{39000} \times 100\%$ [17].

Sequence Alignment and Homology Modeling

Clustal Omega was used to align the sequences of FaeMK with SRCAM 602, durancin GL, and hiracin JM79 [18]. A 3D model was created using SWISS-MODEL and enterocin HF as the model template [19].

Construction of Plasmids

A 552-bp DNA fragment containing the FaeMK operon was obtained from BioBasic Inc. (Markham, ON, Canada). The DNA fragment was designed with two different restriction sites, *Sac*I and *Xba*I, to enable the cloning of this DNA fragment into the multiple cloning site of the broad host range shuttle vector pMG36c [20]. The ligation mixture was electroporated into *C. maltaromaticum* UAL26 according to the method described previously [21]. The resulting plasmid, pMG36c-faeMKI, was isolated from UAL26 transformants using the GeneJET Plasmid Miniprep Kit (Fermentas Canada Inc., Burlington, ON, Canada). To facilitate the isolation of the DNA, UAL26 cells were first treated with 5 mg/mL lysozyme for 30 min at 37 °C before cell lysis. The isolated plasmid DNA was sequenced to confirm the accurate sequence of pMG36c-faeMKI. Plasmid pMG36c-faeMKI was then electroporated into *E. faecium* BFE 900 as described above.

Results and Discussion

DNA Sequence of Faerocin MK Operon

A gene encoding a bacteriocin that is highly similar (85% identity) to that of the putative peptide SRCAM 602 was detected on the genome of *E. faecium* M3K31 [6]. Analysis of the nucleotide sequence reveals that this bacteriocin, which we termed faerocin MK (FaeMK), consists of 43 amino acids and is produced as a precursor with a 28-amino acid leader peptide (Fig. 1). FaeMK contains the YGNGV-motif that is characteristic of class IIa bacteriocins [23]. The leader peptide of FaeMK precursor resembles a *sec*-dependent signal peptide with a positively charged N-terminus, a stretch of hydrophobic amino acids, and cleavage site following an alanine residue. Bacteriocins produced with this type of leader peptide can access the general secretion pathway of the cell and do not require a dedicated transport machinery for secretion [14]. Antimicrobial peptides that belong to this class kill sensitive cells by membrane permeabilization [24]. Immediately

Table 1 Antimicrobial activity of FaeMK and SRCAM 602

Indicator strain	MIC (μM)		Source
	FaeMK	SRCAM 602	
Escherichia coli DH5α	–	–	Invitrogen
Salmonella typhimurium ATCC 13311	–	–	ATCC
S. typhimurium ATCC 23564	–	–	ATCC
Lactococcus lactis cremoris ATCC 19257	16	–	ATCC
Enterococcus faecalis ATCC 7080	0.5	–	ATCC
Listeria monocytogenes ATCC 15313	0.031	–	ATCC
L. monocytogenes UAFM1	0.125	–	Laboratory collection
Carnobacterium maltaromaticum UAL26	0.031	–	[13]
Carnobacterium divergens LV13	0.125	–	[14]
Lactobacillus sakei 706	2	–	[15]
Enterococcus faecium BFE 900	1	–	[16]
Brochothrix thermosphacta ATCC 11509	–	–	ATCC
Staphylococcus aureus ATCC 25923	–	–	ATCC

–, no inhibition at highest concentration tested (64 μM)

ATCC American Type Culture Collection

downstream of the structural gene for FaeMK, *faeMK*, a putative immunity gene, *faeI*, is located that could encode a peptide of 94 amino acids. Both *faeMK* and *faeI* are preceded by a putative ribosomal binding site (RBS). A putative promoter, with TTGTCA as the -35 region and TTTTAT as the -10 region, was identified upstream of the FaeMK structural gene. In addition, a 13-bp inverted repeat is located between the -10 region and the RBS of *faeMK*. The function of this repeat is unclear but it might suggest that the expression of the FaeMK operon is regulated. A BLAST search for the FaeMK peptide showed high homology with durancin GL (98% identity), and hiracin JM79 (86% identity) (Fig. 2). Durancin GL and hiracin JM79 are *sec*-dependent bacteriocins produced by *Enterococcus durans* 41D and *Enterococcus hirae* DCH5, respectively [22, 25]. Comparison between the DNA sequence of the FaeMK operon and that of durancin GL showed

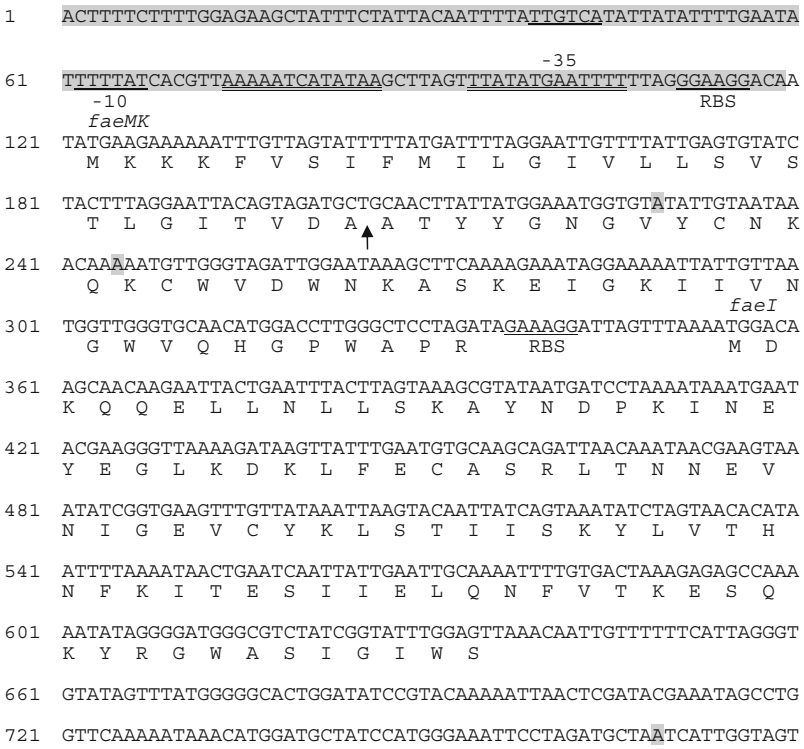

Fig. 1 Nucleotide sequence of the faerocin MK operon. The deduced amino acid sequences for *faeMK* and *faeI* are shown below the nucleotide sequence. The putative -35 and -10 promoter regions, as well as putative RBSs, are underlined. An inverted repeat identified upstream of *faeMK* is double underlined. Nucleotides that are different from those reported for the durancin GL operon [22] are highlighted in gray. The vertical arrow indicates the cleavage site of the faerocin MK precursor

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SRCAM 602          ATYYGNGLYCNKQKHYTWVDWNKASREIGKITVNGWVQH-------
Hiracin JM79       ATYYGNGLYCNKEK--CWVDWNQAKGEIGKIIVNGWVNHGPWAPRR
Faerocin MK        ATYYGNGVYCNKQK--CWVDWNKASKEIGKIIVNGWVQHGPWAPR-
Durancin GL        ATYYGNGVYCNKQE--CWVDWNKASKEIGKIIVNGWVQHGPWAPR-
                   *******:****::   *****:*. ***** *****:*
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Fig. 2 Sequence alignment of FaeMK, SRCAM 602, hiracin JM79, and durancin GL. Asterisks, colons, and periods indicate conserved, conservative, and semiconservative substitutions, respectively. Cysteine residues are highlighted in gray

99% identity. The structural gene *faeMK* showed two mutations compared to the structural gene for durancin GL: one is a silent mutation and the other is a mutation that results in a substitution at position 14 of the FaeMK peptide (Figs. 1 and 2). Remarkably, the DNA sequence immediately upstream of the ATG start codon of *faeMK* is different and shows no sequence homology with the DNA sequence upstream of the structural gene for durancin GL (Fig. 1). In contrast to the DNA sequence upstream of *faeMK*, no inverted repeat was reported between the promoter and RBS of the durancin GL structural gene [22]. Another major difference is the fact that the durancin GL operon is situated on a plasmid whereas the FaeMK operon is located on the chromosome.

Characterization of Faerocin MK

The peptide sequence for FaeMK was compared to the reported SRCAM 602 sequence, hiracin MJ79, and durancin GL (Fig. 2). This homologous sequence alignment highlights the conserved YGNGV-motif, with a single conserved mutation in the SRCAM 602 and hiracin MJ79 peptides at position 8 yielding an YGNGL-motif instead. A consequential inconsistency in the SRCAM 602 sequence is the presence of a single cysteine residue at position 10. In contrast, the other three peptides contain two cysteine residues at positions 10 and 15. This allows for the formation of a disulfide bridge, a feature found on all other class IIa bacteriocins that is attributed to providing structural stability at the N-terminus [23].

The FaeMK and the reported SRCAM 602 peptides were chemically synthesized for this study. Activity assays showed that FaeMK inhibits many of the Gram-positive bacteria tested and is particularly potent against *Listeria* and *Carnobacterium* spp., showing a MIC for strains of these genera at the low nanomolar range (Table 1). FaeMK was not active against the Gram-negative strains tested. The inhibition of Gram-positive bacteria and lack of activity against Gram-negative bacteria are consistent with other class IIa bacteriocins [23]. However, the observed activity of SRCAM 602 was not consistent with that reported in literature [9]. SRCAM 602 showed no activity against Gram-positive or Gram-negative organisms (Table 1).

The relative structures of the two peptides were analyzed using circular dichroism spectroscopy (Fig. 3). The α-helicity of both peptides was determined as a percentage using a structure inducing solvent, trifluoroethanol. The averaged results indicate a 28 and 16% α-helical character for FaeMK and SRCAM 602, respectively, in a 1:1 solution of water:trifluoroethanol. This result prompted us to create a model of FaeMK based on the published 3D structure of enterocin HF as a template, which is the most closely matched class IIa bacteriocin with a known structure, having a 46% identity with FaeMK [8]. The resulting 3D model showcases the

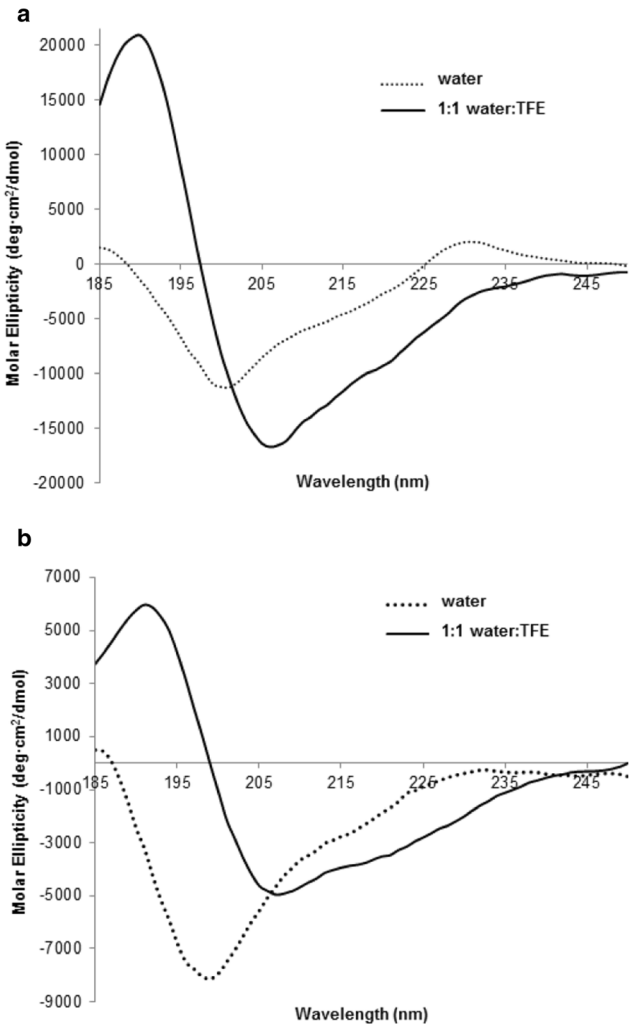

Fig. 3 CD spectra of FaeMK (**a**) and SRCAM 602 (**b**) in water as a dotted line and 1:1 water:trifluoroethanol as a solid line

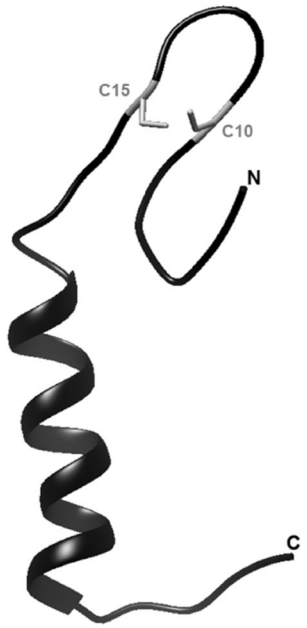

Fig. 4 Model of FaeMK structure derived using SWISS-MODEL with enterocin HF as a template; emphasized (gray) are the two cysteine residues responsible for the disulfide bridge

expected hairpin at the N-terminus, with the cysteine residues responsible for the disulfide bridge formation highlighted in gray, and a single α-helix structure observed in typical class IIa bacteriocins (Fig. 4) [23].

Heterologous Expression of Faerocin MK

To investigate whether we could achieve FaeMK production in heterologous hosts, a 552-bp fragment containing the *faeMKI* operon that includes a 16-bp nucleotide sequence immediately upstream of the ATG start codon for *faeMK* was cloned behind the constitutive P32 promoter of the shuttle vector pMG36c. The DNA ligation mixture was transformed into *C. maltaromaticum* UAL26 giving pMG36c-faeMKI in the resulting UAL26 transformant. UAL26 containing pMG36c-faeMKI was used in a deferred inhibition test with UAL26 as the indicator strain and UAL26 containing pMG36c without an insert was used as a negative control. A small, but clear inhibition zone produced by UAL26 containing pMG36c-faeMKI could be detected whereas the negative control showed no inhibition (Fig. 5a). To test heterologous FaeMK production in enterococci, plasmid pMG36c-faeMKI was isolated from the UAL26 transformant and introduced into *E. faecium* BFE 900. BFE 900 containing pMG36c without an insert was again used as a negative control. Deferred inhibition test using BFE 900 as the indicator strain showed that FaeMK production could also be achieved in *E. faecium* BFE 900 (Fig. 5b). To determine whether UAL26 and BFE 900 containing pMG36c-faeMKI showed a decrease in sensitivity to FaeMK, a spot-on-lawn test was performed using a 2-fold serial dilution of FaeMK. UAL26 and BFE 900 containing pMG36c-faeMKI were 8-fold and 32-fold less sensitive to FaeMK, respectively, compared to the control strains that contained pMG36c. These results indicate that the *faeMKI* operon is also responsible for immunity and that *faeI* is the immunity gene. This is in agreement with the identification of the immunity gene for durancin GL, which is identical to that for FaeMK [22]. The finding that heterologous production of FaeMK can be achieved without the additional cloning of a dedicated bacteriocin transport machinery confirms the proposal that FaeMK is a *sec*-dependent bacteriocin.

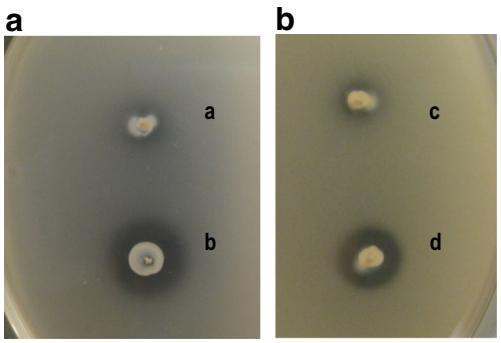

Fig. 5 Antagonistic activity of *C. maltaromaticum* UAL26 containing pMG36c (a) or pMG36c-faeMKI (b), and *E. faecium* BFE 900 containing pMG36c (c) or pMG36c-faeMKI (d), respectively; indicator strains were *C. maltaromaticum* UAL26 (**a**) and *E. faecium* BFE 900 (**b**), respectively

Concluding Remarks

We demonstrated that faerocin MK is a class IIa bacteriocin, matching both its activity and structure to previously reported bacteriocins in this class. Our heterologous expression studies indicate that this operon, containing only *faeMK* and *faeI*, is sufficient for the expression and secretion of FaeMK as well as providing immunity for the host organism. In addition, we conclude that the previously reported SRCAM 602 peptide sequence would yield an inactive and loosely structured product [9]. This confirms our previous conclusion that SRCAM 602 is not a bacteriocin produced by *P. polymyxa* NRRL B-30509 [12]. It is interesting to note that *E. faecium* M3K31 encodes three bacteriocins with an YGNGV-motif, two of which are *sec*-dependent bacteriocins. Future studies could investigate the methods of production of these bacteriocins in *E. faecium* M3K31, a strain that has great potential to be used as a probiotic.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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