Biochemical characterization of a bacteriocin-like inhibitory substance produced by Enterococcus faecium MXVK29, isolated from Mexican traditional sausage

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Abstract

BACKGROUND: Enterococci are lactic acid bacteria that can produce bacteriocins, which may offer an additional hurdle to control the growth of food-borne pathogens; moreover, these bacteriocins may have great potential as natural biopreservatives. The aim of this work was to characterize a bacteriocin-like inhibitory substance (BLIS) with antilisterial activity produced by an enterococcal strain.

RESULTS: The bacteriogenic strain was isolated from Mexican fermented sausages and identified as Enterococcus faecium with 99% sequence similarity. Maximal activity was detected at 16 h, where bacterial growth was in middle of the stationary phase. The producer strain was not inhibited by its own antimicrobial peptide. BLIS showed a strong anti-Listeria activity and was inactivated by proteinase K. Heating (121 °C for 15 min) induced some inactivation, but thermotolerance was higher at acid pH values. The yield obtained with a pH-mediated purification process was 32.7%, showing a band with an estimated molecular weight of 3.5 kDa. Automated N-terminal Edman degradation showed the following sequence: YYGNGVTCGSHHCSVD.

CONCLUSION: Biochemical characteristics of BLIS produced by E. faecium MXVK29 suggested that it belongs to Class IIa of the Klaenhammer classification and could be considered as a natural food preservative, although further studies need to be performed.

Keywords: bacteriocin; Enterococcus; food preservative; lactic acid bacteria

INTRODUCTION

Enterococci are lactic acid bacteria (LAB) widely distributed in nature; they occur as part of the natural microbiota of many fermented foods and are often used as components of starter cultures.\(^1\) One special benefit of the presence of enterococci in foods is that such strains may also produce bacteriocins, called enterocins. Bacteriocins are usually defined as antimicrobial peptides that are synthesized ribosomally by bacteria and secreted to the extracellular milieu, where they act against closely related species without affecting the producer strain.\(^4,5\) Bacteriocins are divided into three classes.\(^4,5\) Class I consists of the lantibiotics: membrane-active peptides that contain the unusual amino acids lanthionine and methylanthione. Class II bacteriocins are heat-stable, non-lanthionine-containing peptides, and are subdivided into three subgroups: (a) Listeria-active peptides that are ‘pediocin-like’ and are characterized by a well-conserved YGNGVXC consensus motif at their N-terminal ends; (b) two-peptide bacteriocins; and (c) bacteriocins that are secreted by the bacterial pre-protein translocase (sec pathway). Class III are heat-labile bacteriocins with proteins (>30 kDa). Most enterococcal bacteriocins belong to class II of the Klaenhammer classification.

Enterocins have become attractive in recent years as natural additives for food preservation, because they present a broad spectrum of activity against many Gram-positive bacteria and food-borne pathogens, such as Listeria monocytogenes, Staphylococcus aureus, Bacillus cereus, and vegetative cells and spores of Clostridium botulinum,\(^8,9\) and they are being extensively investigated for possible use as probiotics and food additives in dairy and...
Enterococci normally are found in fermented foods including such as meat and dairy products. The production of bacteriocins may play a role for enterococci to colonize and to become a major component of the microbial communities associated with these foods. Therefore, much interest has arisen for the biochemical and genetic characterization of the bacteriocinogenic Enterococci. Some of the more recent enterococci characterized with a high potential for use in foods are produced by Enterococcus faecalis WHE 96 and N1-333 (enterocin 96 and MR10A, respectively), Enterococcus faecium IT62 (enterocin IT), Enterococcus durans QU 49 (durancin TW-49M) and Enterococcus mundtii CUGF08 (mundtcin L).

This study was aimed at characterizing the properties of a bacteriocin-like inhibitory substance (BLIS) produced by Enterococcus faecium MXVK29 isolated from Mexican traditional sausage (chorizo), which showed antimicrobial activity against Listeria monocytogenes.

### EXPERIMENTAL

#### Bacterial strain and growth conditions

The bacteriocin producer Enterococcus faecium MXVK29 was isolated from Mexican fermented sausages by Dr M Collins’ research group at the Queen’s University of Belfast, Northern Ireland, UK. Bacteriocin-sensitive strains were obtained from various sources (Table 1). All cultures were stored at −80 °C in stocks with glycerol (500 µL mL⁻¹). All bacteria were grown in trypticase soy broth (TSB; Difco Laboratories, Detroit, MI, USA) at 37 °C, with the exception of Enterococcus faecium MXVK29, Pedicoccus parvulus MXVK133, and Enterococcus faecalis MXVK22, which were grown in casein–glucose broth (CGB: 5 g L⁻¹) yeast extract, 20 g L⁻¹ Bacto Tryptone, 10 g L⁻¹ glucose, 1 mL L⁻¹ Tween 80, 0.1 g L⁻¹ magnesium sulfate, 0.05 g L⁻¹ manganese sulfate, 2 g L⁻¹ ammonium citrate, 2 g L⁻¹ disodium phosphate) at 37 °C.

#### Strain identification

Bacterial DNA of E. faecium MXVK29 was extracted from an overnight culture by the method of Lawson et al. Amplification of the 16S rDNA gene by polymerase chain reaction (PCR) was performed using the following universal primers: 5′-GAGGTGTGACCTTGAGCTCAG-3′ (E9F-forward) and 5′-CTTGGCGGGCCGCTAATT-3′ (E939R-reverse), as previously reported by Forney et al. Amplified 16S rDNA fragments were visualized using agarose gel electrophoresis (0.8 g 100 mL⁻¹). The amplicon product was purified using a commercial Miniprep Wizard SV Gel and PCR clean-up kit (Promega Corp., Madison, WI, USA). Fragments were sequenced, and sequences were compared using a BLAST homology search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) in a database contained in NCBI.

#### Kinetics of BLIS production

The profile of BLIS production was determined by inoculating CGB broth with a fresh culture of E. faecium MXVK29 (1 mL 100 mL⁻¹). Ten-milliliter samples were taken at different time intervals and analyzed for cell growth, pH, and antimicrobial activity. The fermented broth was heated for 30 min at 70 °C to inactivate proteases, followed by centrifugation at 3100 × g for 10 min at 37 °C. The cell-free culture supernatant was adjusted to pH 6.5 with 0.1 mol L⁻¹ NaOH, followed by membrane sterilization (0.22 µm diameter pore, Millipore, Durapore® Membrane Filters, GV, Ireland). The filtrate was labeled as crude extract (CE) and was used to determine antimicrobial activity.

#### Antimicrobial activity

Bacteriocin activity was measured by well diffusion assay. Plates containing a bottom layer of trypticase soy agar (TSA) were overlaid with a soft layer of TSA (0.8 g agar 100 mL⁻¹) and inoculated with 70 µL of a culture of Listeria innocua ATCC33090 (10⁵ – 10⁷ CFU mL⁻¹), as indicator strain. Wells of 7 mm in diameter were cut into these agar plates, and serial twofold dilutions of extract (30 µL) were placed into each well. The plates were incubated for 24 h at 37 °C and examined for growth inhibition zones. The reciprocal of the highest dilution showing clear zones of inhibition, of at least 1 mm, was expressed as arbitrary units (AU) per milliliter. Each assay was conducted in triplicate.

#### Inhibitory spectrum and auto-immunity assays

Antagonistic activity was determined by well diffusion assays, as described previously. Gram-positive and Gram-negative strains were used as indicator strains using the appropriate medium for each one (CGB or TSB), and 30 µL of CE were placed into each well. The presence of a distinct inhibition zone around the wells was considered a positive antagonistic effect. Autoimmunity assays were performed using E. faecium MXVK29 as sensitive strain.

#### Bacteriocin purification

A modified pH-mediated adsorption–desorption method was used, with minor modifications. A 16 h culture of E. faecium

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**Table 1.** Bacterial strains, inhibitory spectrum, and autoimmunity assays of BLIS 29

<table>
<thead>
<tr>
<th>Tested microorganisms</th>
<th>Growth inhibition halo (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterococcus faecium MXVK29</td>
<td>n.d.</td>
</tr>
<tr>
<td>Enterococcus faecalis MXVK22</td>
<td>8</td>
</tr>
<tr>
<td>Pedicoccus parvulus MXVK133</td>
<td>9</td>
</tr>
<tr>
<td>Brochothrix thermosphacta NCIB-10018</td>
<td>n.d.</td>
</tr>
<tr>
<td>Listeria innocua ATCC33090</td>
<td>10</td>
</tr>
<tr>
<td>Listeria monocytogenes Scott A</td>
<td>7</td>
</tr>
<tr>
<td>Listeria monocytogenes LM 82</td>
<td>n.d.</td>
</tr>
<tr>
<td>Listeria monocytogenes LMB 92000/48</td>
<td>8</td>
</tr>
<tr>
<td>Listeria monocytogenes LMB 911204/47</td>
<td>7</td>
</tr>
<tr>
<td>Staphylococcus aureus NCTC8325</td>
<td>5</td>
</tr>
<tr>
<td>Staphylococcus camosus MC-102055</td>
<td>n.d.</td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td>6</td>
</tr>
<tr>
<td>Staphylococcus agalactiae</td>
<td>5</td>
</tr>
<tr>
<td>Lactococcus lactis ATCC11454</td>
<td>n.d.</td>
</tr>
<tr>
<td>Lactobacillus casei sp. rhamnosus NRRL B-445</td>
<td>7</td>
</tr>
<tr>
<td>Lactobacillus plantarum NRRL B-813</td>
<td>n.d.</td>
</tr>
<tr>
<td>Escherichia coli JM P101</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

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**Notes:**

- a Donated by Dr M. Collins’ research group at Queen’s University, Belfast, Northern Ireland, UK.
- b Dr Urruburu, Universidad de Valencia, Spain.
- c Dr Iniesta, Universidad de Murcia, Spain.
- d Dr Angel Alpuche, IPICYT, San Luis Potosi, Mexico.
- e Christian Hansen, Horsholm, Denmark.
- f Bacteriocin producer strain.
- n.d., not detected.
MXVK29 was heated for 30 min at 70 °C. Bacteriocin adsorption to producer cells was carried out for 4 h at pH 5.5, and desorption was conducted for 10 h at pH 2. Finally, the extract was ultra-filtered through 10,000 and then through 5000 kDa cut-off membranes (YM 10/YM 5 Centricon, Millipore, Bedford, MA, USA) following the manufacturer's instructions. The concentrated extract was filter-sterilized and lyophilized. The bacteriocin extract (BE) was stored at −80 °C until use.

The BE was analyzed with a tris–tricine–sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) system. One half of the gel was stained with Coomasie R-250 dye (Bio-Rad, Hercules, CA, USA), while the other was used to test antimicrobial activity by washing with sterile water and overlaying with soft agar inoculated with *Listeria innocua* ATCC33090 (10⁶ – 10⁷ CFU mL⁻¹), and incubating for 18 h at 37 °C. This gel was compared with the stained gel to locate the active band.

Amino acid sequence of the bacteriocin
Proteins in the SDS-PAGE gel were transferred to a polyvinylidene fluoride (PVDF) membrane (Sequi-Blot, Bio-Rad, Hercules, CA, USA) in a Mini Trans-Blot Cell (Bio-Rad) at 90 V for 40 min at 5 °C, using Towbin buffer (192 mmol L⁻¹ glycine, 25 mmol L⁻¹ Tris and 20 mL 100 mL⁻¹ methanol, pH 8.3). After blotting, the membrane was stained with Coomassie R-250 dye (0.025 g 100 mL⁻¹ of 400 mL L⁻¹ aqueous methanol) for 30 min at room temperature, and destained with aqueous methanol (50 mL 100 mL⁻¹). The PVDF membrane fragment corresponding to the protein band of interest was excised, cut into small pieces, and applied to a Procise 491 protein-sequence system (Applied Biosystems, Inc., Foster City, CA, USA) using the blot cartridge. The pulsed-liquid blot method was employed. After sequencing, the amino acid sequence was compared with the Protein NCBI database to determine similarities with previous reported sequences.

Effect of enzymes, pH, and heat on antimicrobial activity
The sensitivity of the bacteriocin to proteinase K (Fermentas, 18 mg mL⁻¹) was determined. Aliquots of CE and BE were incubated with the enzyme at a final concentration of 1 mg mL⁻¹. Samples were then incubated for 30 min at 65 °C and finally the reaction mixtures were heated at 90 °C for 10 min to inactivate the enzyme before assessing the remaining bacteriocin activity. The effect of heat and pH on the bacteriocin activity was determined. The CE was divided into three fractions and adjusted to pH values ranging from 4 to 8 with NaOH solution (1 mol L⁻¹). Each fraction was subdivided into three aliquots. The first was used as control, while the second and third were subjected to pasteurization (63 °C for 30 min) and sterilization (121 °C for 15 min), respectively. After thermal treatments, samples were cooled at room temperature, adjusted to neutral pH (phosphoric acid, 5 g 100 mL⁻¹, and NaOH), and assayed for bacteriocin residual activity.

RESULTS AND DISCUSSION

Strain identification
A 750 bp fragment of 16S rDNA was obtained. BLAST search homology identified the sequence as *Enterococcus faecium* strain CSI35MX (accession no. FJ538584; Fig. 1) with 100% similarity, 98% query coverage, and 0.0 E-score. The result allowed identification of the isolated strain 29 as *Enterococcus faecium*.

Kinetics of BLIS production
The antimicrobial activity of BLIS was detected in the CE after 6 h of fermentation, close to the end of the logarithmic growth phase. Maximum activity (1100 AU mL⁻¹) was observed at 16 h, which corresponded to the middle of the stationary phase, and it decreased after 24 h of incubation (820 AU mL⁻¹), remaining almost constant until the end of the incubation time (30 h). During bacterial growth, the pH of the medium decreased steadily from 6.4 to 3.7, and BLIS production was detected at a pH of 4.6 (Fig. 2). Results are similar to those described in the literature, which show that production of the bacteriocin decreases after an unspecified time. This reduction in bacteriocin activity may be attributed to the readsoption on the producer cells (BLIS is adsorbed to the cell at the beginning of the fermentation, and is desorbed while the pH decreases), to a bacteriocin aggregation, or to degradation of BLIS by extracellular proteases.

Figure 1. Distance tree of strain 29 with *Enterococcus faecium* strains. This was produced using BLAST pairwise alignments by the neighbor joining method.
Figure 2. Batch fermentation profile of Enterococcus faecium MXVK29 grown in CGB broth at 37 °C. Means of three replicates with standard deviation.

Table 2. Recovery of BLIS at different stages of purification

<table>
<thead>
<tr>
<th>Bacteriocin recovered</th>
<th>Activity (AU mL⁻¹)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture supernatant</td>
<td>2.6 × 10⁵</td>
<td>100</td>
</tr>
<tr>
<td>Adsorption of the bacteriocin</td>
<td>6.6 × 10⁴</td>
<td>25.4</td>
</tr>
<tr>
<td>Desorption of the bacteriocin</td>
<td>1.6 × 10⁵</td>
<td>61.5</td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>9.7 × 10⁴</td>
<td>37.3</td>
</tr>
<tr>
<td>In completely dried preparation</td>
<td>8.5 × 10⁴</td>
<td>32.7</td>
</tr>
<tr>
<td>AU g⁻¹ of dry matter</td>
<td>4.0 × 10⁵</td>
<td></td>
</tr>
<tr>
<td>AU g⁻¹ of protein</td>
<td>1.7 × 10⁹</td>
<td></td>
</tr>
</tbody>
</table>

inhibitory spectrum and autoimmunity assays

Results are shown in Table 1. The producer strain was not inhibited by its own antimicrobial peptide, thus proving autoimmunity.⁹ The BLIS showed a relatively narrow inhibitory spectrum towards Gram-positive strains, including Listeria strains, Pediococcus acidilactici MXVK133, Enterococcus faecalis MXVK22, Lactobacillus casei subsp. rhamnosus, and Staphylococcus spp. These results agree with those found by other authors reporting that bacteriocins inhibit phylogenetically related microorganisms⁴,⁶ and for class Ila bacteriocin-producing Enterococcus strains, which have shown a strong activity toward Listeria strains and limited antagonistic activity towards dairy starter cultures, such as Lactococcus.⁴⁰,⁴¹

Some authors have shown that class Ila bacteriocins have a wide spectrum against food-borne pathogens because they have two cysteine residues linked by an S–S bridge.⁴²,⁴³ Eijsink et al.⁴⁴ showed that bacteriocins containing two cystine bridges (PA-1 and some enterocins) are more active and have a broader inhibitory spectrum (especially with high antilisterial activity) than those with only one cystine bridge. However, one out of five Listeria spp. strains (L. monocytogenes LM82) was not sensitive to the BLIS, and was previously reported as resistant to streptomycin (Marín-Iniesta F., personal communication 2002).⁴⁵ This two resistance behaviors could be related, as antibiotics also target the cell membrane, while the majority of class II bacteriocins induce membrane permeabilization and the subsequent leakage of molecules from the target bacteria. Some mechanisms of resistance generated by microorganisms to bacteriocins and antibiotics include decreased permeability of the cell membrane, alteration of cell receptors, and modification or destruction of antimicrobial compounds by enzymes.⁴⁶ E. coli was not sensitive to the BLIS as Gram-negative bacteria are usually considered to be resistant to the bacteriocin of lactic acid bacteria.³⁵

Bacteriocin purification

Purification of BLIS was performed by a pH-dependent method, ultrafiltration, and lyophilization. The overall purification procedure is summarized in Table 2. It is noteworthy that, after the adsorption–desorption method, 61.5% of total bacteriocin activity was recovered, but ultrafiltration did not serve as a concentration method because it decreased the yield of activity by 24.2%, while other concentration methods increase the yield almost 50%.⁴⁸ The yield obtained with our protocol of purification (32.7%) is similar to other more expensive protocols that include precipitation with ammonium sulfate and chromatography.⁴⁷–⁴⁹ The purified extract (BE) showed a main band with antimicrobial activity after SDS-PAGE, with an estimated molecular weight of 3.5 kDa (Fig. 3), which is similar to other enterocins previously reported.¹⁷,²⁰–⁵²
was observed at acid pH values. Abriouel and 64.6% of activity in acid and basic conditions, respectively. Under pasteurization, the BLIS lost 44.0% residual activity was 54.0% at pH 4, and decreased by 13.0% at pH 8.

The antimicrobial activity of CE against E. faecium MXVK29 was explored. The effect of enzymes, pH, and heat on antimicrobial activity was assessed.

**Table 3.** Sequence alignment of BLIS 29 with class Ila bacteriocins.

<table>
<thead>
<tr>
<th>Bacteriocin</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLIS 29 sequence (this study)</td>
<td>XYYGNGVTCGSHHCSVD</td>
</tr>
<tr>
<td>Enterocin A 4829</td>
<td>TYNGNVYCTKNKCTVD</td>
</tr>
<tr>
<td>Enterocin CRL 35 4289</td>
<td>KYNGNVYCNKQCSVD</td>
</tr>
<tr>
<td>Enterocin SE-K4 5356</td>
<td>KYNGNVYCNKQCSVD</td>
</tr>
<tr>
<td>Enterocin P</td>
<td>RSYNGNVYCNQKWWVN</td>
</tr>
<tr>
<td>Pediocin PA-1</td>
<td>KYNGNVYCNKQCSVD</td>
</tr>
</tbody>
</table>

Pediocin-box motif indicated by a gray shadow.

**Table 4.** Effect of proteinase K on the BLIS activity.

<table>
<thead>
<tr>
<th>Assays</th>
<th>Inhibition halo (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crude extract (CE)</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
</tr>
<tr>
<td>Proteinase K (1 mg mL⁻¹)</td>
<td>n.d.</td>
</tr>
<tr>
<td>% Residual activity</td>
<td>0%</td>
</tr>
</tbody>
</table>

*Residual activity = ((inhibition halo in presence of proteinase K (mm) × 100%)/inhibition halo of control (mm)).

n.d., not detected.

**Amino acid sequence of the bacteriocin**

Automated N-terminal Edman degradation (16 cycles) revealed the sequence YYGNGVTCGSHHCSVD and BLAST similarity search (NCBI database) demonstrated that BLIS has a significant similarity to other bacteriocins, and belongs to Class Ila of the Klaenhammer classification (pediocin-like bacteriocins; Table 3). They contain a hydrophilic cationic region with the conserved YGNGV(X)C(X)₄C region suggest that BLIS 29 belongs to Class IIa of the Klaenhammer classification. These properties could grant BLIS 29 great potential for use as a preservative, but the optimal use of this bacteriocin as an additive to extend the shelf-life of food products requires more research.

The modified pH-mediated adsorption–desorption method produced good results, because the yield obtained was similar to that obtained with other more expensive protocols. However, it is necessary to perform more studies to minimize the cost of purification and the recovery of large amounts of bacteriocin.

This work is relevant to food microbiology, because the BLIS produced by E. faecium MXVK29 could be used as an antimicrobial substance, either as a purified additive or by direct inoculation of the producer strain as a starter or protective culture. However, further studies need to be performed such as detection of strain virulence factors, and to understand the whole mechanism of BLIS production, including identification of the bacteriocin structural gene, as well as to develop applications for use of the producer strain or its BLIS as a hurdle technology.

**CONCLUSIONS**

This is the first report for E. faecium MXVK29, isolated from Mexican fermented sausages, producing a BLIS. The biochemical characteristics, such as autoimmunity, proteinaceous nature, thermostolerance, low molecular weight (3.5 kDa), inhibitory activity against Listeria spp., and the presence of a conserved YGNGV(X)C(X)₄C region suggest that BLIS 29 belongs to Class Ila of the Klaenhammer classification. These properties could grant BLIS 29 great potential for use as a preservative, but the optimal use of this bacteriocin as an additive to extend the shelf-life of food products requires more research.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


