Lactococcin MMFII, a novel class IIa bacteriocin produced by *Lactococcus lactis* MMFII, isolated from a Tunisian dairy product

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Abstract

A novel bacteriocin, lactococcin MMFII, produced by *Lactococcus lactis* MMFII isolated from a Tunisian dairy product had been identified. The bacteriocin was purified to homogeneity from fresh overnight M17 broth culture by sulfate ammonium precipitation, cation-exchange chromatography, *sep-pack* chromatography and two steps of reverse-phase chromatography. The purified bacteriocin was heat stable, pH resistant and protease sensitive. Its amino acid sequence, obtained by Edman degradation, revealed a 37-amino acid peptide with two cysteine residues in positions 9 and 14 and a calculated mass of 4144.6 Da. Laser desorption mass spectrometry analysis gave a molecular mass of 4142.6, suggesting the presence of a disulfide bond within the purified bacteriocin. Lactococcin MMFII contains the N-terminal YGNGV consensus motif and is active against *Listeria*. Thus, it belongs to the class IIa bacteriocins figuring the first example of such a bacteriocin produced by a lactococcal strain. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Class IIa bacteriocins; Lactic acid bacteria; Lactococcin MMFII; Anti-Listeria; *Lactococcus lactis*

1. Introduction

Numerous bacterial species produce one or more antimicrobial substances called bacteriocins, that enhance their competitiveness toward other related bacterial species [1]. By secreting multiple bacteriocins, many lactic acid bacteria (LABs) had attracted increasing interest in recent years [2–6]. They are extensively used because of their bactericidal activity and their new potential applications not only to preserve and develop flavor in food industry but also in human health [7]. The LAB bacteriocins which have been described and characterized to date share a number of common traits which justify their classification into at least four classes [8–10]: class I, the lantibiotics; class II, the small heat-stable non-lantibiotics which are divided into the subgroups IIa (pediocin-like bacteriocins, *Listeria*-active peptides with a YGNGVxC consensus sequence); IIb (bacteriocins whose activity depends on the complementary activities of two peptides) and IIc (which is uncertain and has been successively suggested to include thiol-activated and sec-dependent secreted bacteriocins); class III, large heat-labile bacteriocins and class IV claimed to consist in an undefined structure constituted of proteins, lipids and carbohydrates. Most of the so far described bacteriocins produced by lactococcal strains belong to the lantibiotics and the IIb bacteriocin groups. To date, no class IIa bacteriocin arising from such bacteria has ever been identified. At least 16 members of the class IIa have been well characterized and their structure described, primarily due to development of efficient and standardized purification protocols for these hydrophobic and cationic peptides.

The principle aim of this work was to purify and characterize a new bacteriocin produced by *Lactococcus lactis* MMFII strain isolated from a Tunisian dairy product.
2. Materials and methods

2.1. Strains, culture medium and chemicals

*L. lactis* MMFII was isolated from Rigouta, a traditional Tunisian cheese. The other class IIa bacteriocin producer strains used in this study were previously described [11]. All indicator strains from our laboratories collection are listed in Table 1. Lactococcal strains were grown in M17 (Difco, USA) medium with lactose (5 g l⁻¹) at 30°C. *Listeria* and enterococcal strains were grown in BHI medium (Difco, USA) at 37°C. Other LABs were grown in MRS (Difco, USA) medium at 37°C. Acetonitrile, high-performance liquid chromatography (HPLC) grade, was supplied by Chromanorm. Others chemicals were provided by Sigma (St. Louis, MO, USA).

2.2. Effects of temperature, pH and proteases on lactococcin MMFII activity

For thermosensitivity assays, culture supernatants were treated at different temperatures. The effect of pH on bacteriocin activity was determined by adjusting the pH of the supernatant with diluted HCl or NaOH. Samples were incubated for 2 h at 30°C and the pH was adjusted to 6.8. For protease-susceptibility assays, culture supernatants were incubated for 2 h with hydrolases (proteinase K, trypsin, papaine, glucoamylase, lipase, α-amylase and lysozyme) at a final concentration of 1 mg ml⁻¹ [12]. For testing residual inhibitory activities, each sample was assayed in the agar well diffusion test (ADT) [13].

2.3. Bacteriocin purification procedure

*L. lactis* MMFII strain was grown in 4 l of M17 at 30°C to stationary phase. The cells were removed by centrifugation at 10 000 g for 15 min, and the cell-free supernatant fluid was used as the starting material for bacteriocin purification (fraction I). Ammonium sulfate concentration was adjusted to 60% saturation, stirred at 4°C overnight and centrifuged at 14 000 g for 45 min. The precipitate was collected and dissolved in 100 ml of 10 mM phosphate buffer, pH 6.8 (fraction II). Fraction II was desalted by dialysis (dialysis membrane Spectra/Por; USA, MW cut-off: 1000) against 5 l of 10 mM phosphate buffer, pH 6.8, for 18 h. The final volume of dialysate (95 ml) was loaded on a phosphocellulose cation-exchange column that had been equilibrated with 10 mM phosphate buffer, pH 6.8. After washing the column with the same buffer, the inhibition activity was eluted with a continuous gradient of 0.8 M NaCl in the same buffer. Fractions were collected, and the protein concentrations were quantified by the method of Bradford [14], then the bacteriocin activity was estimated. The resulting active fractions were pooled, dialyzed and concentrated by acetone precipitation. The dried pellet was dissolved in 3 ml of 10 mM phosphate buffer, pH 6.8 (fraction III). Fraction III was applied on *sep-pack* column (Waters), eluted at 50% acetonitrile, lyophilized and suspended in 400 µl 10 mM phosphate buffer, pH 6.8 (fraction IV). This last fraction was then purified by two HPLC steps (Applied Biosystems; with a 5-µm C18 VYDAC column). The column was maintained at 30°C with a column heater. After equilibration of the C18 column with water/trifluoroacetic acid 0.1% (v/v), at a flow rate of 0.8 ml min⁻¹, peptides were eluted by increasing the concentration gradient of this solvent (0.1% trifluoroacetic acid in acetonitrile) as follows: 0–5 min: 0% (v/v) acetonitrile; 5–45 min: 0–60% (v/v) acetonitrile. Peptides were detected spectrophotometrically by measuring the optical density at 220 and 280 nm. Fractions corresponding to all peaks were collected independently, lyophilized, and assayed for bacteriocin activity.

2.4. Mode of action of lactococcin MMFII

Bacteriocin arbitrary activity unit (AU) was defined as the reciprocal of the highest dilution of culture supernatant giving an inhibition zone in the ADT against indicator strains. Different amounts of crude bacteriocin were added to cultures of the indicator strain *L. lactis* subsp. *cremonis* ATCC11603 in early exponential phase and then incubated at 30°C. Bacterial growth was monitored by measuring the optical density at 600 nm and by counting viable cells on M17 agar plates.

2.5. Mass spectrometry

The lyophilized fractions were analyzed with the mass spectrometer model Voyager-DERP (Perkin-Elmer, USA). The MALDI-TOF system (matrix-assisted laser desorption ionization time-of-flight) was used with a matrix type α-cyano-hydroxycinnamic acid.

2.6. Determination of the amino acid composition

The bacteriocin was hydrolyzed in 6 N HCl under vacuum at 110°C for 72 h and amino acids were identified with an amino acid analyzer (Applied Biosystems, USA).

2.7. Amino acid sequencing

Amino acid sequencing was performed by Edman degradation using a 477 X automatic sequence analyzer (Applied Biosystems).

2.8. Minimal inhibitory concentration determination

Lactococcin MMFII and class IIa bacteriocins were purified from 200 ml of overnight cultures by the method of Guyonnet et al. [11]. Purified bacteriocins were analyzed by mass spectrometry on a Perkin-Elmer Siex MPI 165 mass spectrometer equipped with an ion spray source.
Minimum inhibitory concentrations (MICs) were determined by the ADT method as being the lower concentration of bacteriocin solution inducing a measurable inhibition zone using *Listeria ivanovi* BUG 496 as indicator strain.

### 3. Results

#### 3.1. Screening for bacteriocinogenic LABs

About 60 LAB strains (*Lactococcus, Lactobacillus*), isolated from Tunisian dairy products were examined for their bacteriocin-like activity by the ADT. Subsequently, three strains were selected for the production, purification and characterization of bacteriocins. Among them, the strain MMFII which showed the strongest bacteriocin activity against *L. cremoris* ATCC11603 was retained for further experiments.

#### 3.2. Identification of the selected lactococcal strain

On the basis of biochemical and morphological characteristics, the strain MMFII was identified as a *L. lactis* according to Schleifer and Kilpper-Balz [15]. This identification was confirmed by 16S RNA sequence analysis performed by the Laboratoire d’Identification Moléculaire des Bactéries de l’Institut Pasteur de Paris (France). A phylogenetic study was provided by this Laboratory. Comparison of the *rrs* gene sequence of the MMFII strain with that of lactococcal reference strains strongly suggests that the MMFII strain is a *L. lactis* subsp. *lactis* (data not shown). Nevertheless, PCR analysis of the histidine biosynthesis operon or RAPD analysis [16] should confirm subspecies identification.

#### 3.3. Sensitivity to hydrolases, pH and heat treatments of *L. lactis* MMFII culture supernatants

Inhibitory activity levels of culture supernatants were not modified after a treatment for 30 min at 70°C. However, the activity decreased at higher temperatures, since supernatants retained only 58.3 and 25% of the initial activity.

### Table 1

<table>
<thead>
<tr>
<th>Indicator strain</th>
<th>Bacteriocin</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. cremoris</em> ATCC11603</td>
<td>mesentercin Y105</td>
<td>ATCC</td>
</tr>
<tr>
<td><em>L. cremoris</em> INA15</td>
<td>sakacin P</td>
<td>INA</td>
</tr>
<tr>
<td><em>L. lactis</em> INA45</td>
<td>pediocin PA-1</td>
<td>INA</td>
</tr>
<tr>
<td><em>Lactococcus diacetylactis</em> INA18</td>
<td>lactococcin MMFII</td>
<td>INA</td>
</tr>
<tr>
<td><em>L. diacetylactis</em> CNRZ125</td>
<td>--</td>
<td>CNRZ</td>
</tr>
<tr>
<td><em>L. lactis</em> ATCC11454</td>
<td>--</td>
<td>ATCC</td>
</tr>
<tr>
<td><em>L. delbrueckii</em> DSM 20081</td>
<td>+/-</td>
<td>DSM</td>
</tr>
<tr>
<td><em>Lactobacillus casei</em> DSM 20011</td>
<td>+/+</td>
<td>DSM</td>
</tr>
<tr>
<td><em>Enterococcus faecium</em> ENSAIA 631</td>
<td>+/-</td>
<td>ENSAIA</td>
</tr>
<tr>
<td><em>E. faecalis</em> JH22</td>
<td>+</td>
<td>D. Clewell, University of Michigan</td>
</tr>
<tr>
<td><em>E. faecalis</em> V583</td>
<td>+</td>
<td>M. Gilmore, University of Oklahoma</td>
</tr>
<tr>
<td><em>L. ivanovi</em> BUG 496</td>
<td>+</td>
<td>Institut Pasteur Paris</td>
</tr>
</tbody>
</table>

All strains were a collection from the Laboratoire de Microbiologie Fondamentale et Appliquée de l’Université de Poitiers, France. ATCC, American Type Culture Collection; INA, Institut National d’Agronomie, Paris, France; CNRZ, Centre National de Recherche Zootechnique, Jouy en Josas, France; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany; ENSAIA, Ecole Nationale d’Agronomie et des Industries Alimentaires, Nancy, France.

*a* *L. lactis* subsp. *cremoris*.

*b* *L. lactis* subsp. *lactis*.

*c* *L. lactis* subsp. *lactis* biovar *diacetylactis*.

*d+: inhibition; +/-: inhibition for 100 ng ml⁻¹, not for 50 ng ml⁻¹; --: no inhibition.*

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**Fig. 1.** Cation-exchange chromatogram of the antimicrobial peptide produced by *L. lactis* MMFII. Aliquot of each fraction was analyzed by the Bradford method and optical density at 595 nm was determined (---). Inhibitory activity against *L. cremoris* ATCC11603 (histogram) and NaCl gradient (---) are shown.
activity after treatment for 30 min at 80 and 100°C respectively. Only 8.3% of this activity was conserved after a 30-min treatment at 110°C. Inhibitory activity was totally lost by proteinase K, trypsine and papaine treatments, whereas glucoamylase, lipase, α-amylase and lysozyme had no effect. This result strongly suggests that a heat-stable proteinaceous compound was responsible for the inhibitory activity of the culture supernatant of *L. lactis* MMFII. Thus, we designed purification steps to isolate this biomolecule that was termed lactococcin MMFII.

### 3.4. Purification of lactococcin MMFII

The proteinaceous compound secreted by *L. lactis* MMFII was purified to homogeneity by ammonium sulfate precipitation and cation-exchange chromatography (Fig. 1). Active fractions were pooled and applied to a C18 *sep-pack* chromatography column, followed by two steps of reverse-phase chromatography. The final step of purification by silica C18 reverse-phase chromatography gave a single symmetrical peak with antimicrobial activity (Fig. 2). The 29.58-min peak was collected and the proteinaceous compound was subjected to sequence analysis and mass determination.

### 3.5. Sequence analysis and mass determination

The primary sequence of the lactococcin MMFII was determined by Edman degradation. The amino acid composition deduced after HCl hydrolysis of the purified peptide revealed the presence of two cysteine residues. Thus, the two blank cycles obtained, when sequencing the lactococcin MMFII, likely corresponded to cysteine residues. To verify this hypothesis, reduction and alkylation of the biomolecule was performed, and this led to the identification of two alkylated cysteines in positions 9 and 14. Consequently, the sequence of lactococcin MMFII corresponded to a 37-amino acid peptide with a calculated mass of 4144.6 Da. Otherwise, lactococcin MMFII MALDI-TOF analysis gave a mass of 4142.6 Da (Fig. 3), suggesting the presence of one disulide bond within the purified bacteriocin. By comparison of the calculated mass with the observed mass obtained by MALDI-TOF analysis, it appeared that lactococcin MMFII does not contain any modified amino acid. Deduced amino acid sequence of lactococcin MMFII is shown in Fig. 4 (Swiss-Prot accession number P83002). The N-terminal part of the molecule contains the class IIa bacteriocin YGNGVxC(x)4C motif. Sequence comparison between class IIa bacteriocins indicated that lactococcin MMFII constitutes a new member of the anti-*Listeria* bacteriocin group (Fig. 4).

### 3.6. Mode of action of lactococcin MMFII against *L. lactis* subsp. cremoris ATCC11603

Addition of MMFII strain culture supernatant contain-
ing 20–60 AU ml\(^{-1}\) of lactococcin MMFII to a growing \textit{L. cremoris} ATCC11603 culture led to a total growth inhibition of this indicator strain. Viable cells were counted after washing the challenged cells with fresh medium. It has been noted that viable-cell counts had decreased from 37.5\% to 20\% after addition of lactococcin MMFII with activity ranging from 20 to 60 AU ml\(^{-1}\), respectively (Fig. 5A). The optical density at 600 nm of test culture remained unchanged after exposure to lactococcin MMFII at various concentrations (Fig. 5B). Conclusively, under our experimental conditions, the effect of lactococcin MMFII appears bactericidal rather than bacteriostatical, although this bacteriocin has no any lytic activity on \textit{L. cremoris} ATCC11603.

3.7. Inhibitory spectrum and MIC determination

The antagonism spectrum of lactococcin MMFII was compared to that of three class IIa bacteriocins. Antagonism spectra presented in Table 1 were assayed using 50 and 100 ng ml\(^{-1}\) bacteriocin concentrations on each tested strain according to the ADT with 50 \(\mu\)l of bacteriocin solution per well. \textit{Enterococcus faecalis}– and \textit{Listeria}–tested strains were all sensitive to these class IIa bacteriocins. Only two of the six-tested lactococcal strains were sensitive to lactococcin MMFII, whereas all these strains were insensitive to the three other bacteriocins. This indicates that the inhibitory spectrum of lactococcin MMFII encompasses a limited number of strains in the \textit{Lactococcus} genus. \textit{Lactobacillus delbrueckii} DSM20081 sensitivity to mesentericin Y105, sakacin P and pediocin PA-1 was not previously reported, most probably due to lower concentrations of tested bacteriocin in the study of Guyonnet et al. [11]. Lactococcin MMFII MIC on \textit{L. ivanovi} BUG 496 was estimated to be 12 ng ml\(^{-1}\). MICs of sakacin P, mesentericin Y105 and pediocin PA-1 were identical to those previously published, i.e. 65 ng ml\(^{-1}\), 33 ng ml\(^{-1}\) and 4 ng ml\(^{-1}\) [11].

4. Discussion

A bacteriocin-like inhibitor substance was detected in the culture supernatant of \textit{L. lactis} MMFII isolated from a Tunisian dairy product. To identify this compound, several purification approaches were applied to the culture supernatant of the producer strain. One of them, designed for the isolation of hydrophobic peptides,
led us to purify the inhibitory substance, allowing its identification. The purified product is sensitive to proteases, but insensitive to other hydrolases, confirming its proteinaceous nature. The compound secreted by L. lactis MMFII was named lactococcin MMFII. Edman degradation and acid hydrolysis analysis of the purified peptide identified 37 residues with a calculated mass of 4144.6 Da. Sequence analysis indicated that lactococcin MMFII contains two cysteine residues, in positions 9 and 14. Mass spectrometry analysis of lactococcin MMFII identified a 4142.66-Da peptide. These results, taken together, suggested the presence of a disulfide bond and the absence of unusual amino acids in the natural peptide.

Lactococcin MMFII contains in its N-terminal region the YNGGV consensus amino acid motif, i.e. the signature of the anti-Listeria, class IIa bacteriocins [8]. Nevertheless, primary sequences comparison between class IIa bacteriocins described so far indicates that lactococcin MMFII constitutes a new member of this group, and the first described class IIa bacteriocin produced by a lactococcal strain.

The role of disulfide bonds for the antimicrobial activity of the class IIa bacteriocins has not been fully defined [10]. We have obtained the reduced form of lactococcin MMFII by chemical synthesis and its activity was tested against indicator strains. The reduced form inhibited the growth of L. cremoris ATCC11603, but no inhibition of L. ivanovi BUG 496 was detected. This result suggests that the disulfide bond was necessary for antimicrobial activity against Listeria. Nevertheless, the use of the reduced form of the bacteriocin did not ensure which form was delivered to the target bacteria. Indeed, oxidation can occur in the environment of the target cells, therefore precluding from differentiation between the activity of both forms. We observed quick and spontaneous formation of the disulfide bridge in water, by oxidation in contact with the ambient air (data not shown). Specific modifications blocking the side chain of the cysteine residues or chemical synthesis of the bacteriocin with substitution of cysteine residues should be performed first, as done for activity studies on mesentericin Y105, pediocin AcH and carnobacteriocin B2 [17–19]. Such modifications have abolished or dramatically reduced the activity of the corresponding class IIa bacteriocins. Thus, oxidation of the reduced form of lactococcin MMFII could explain the apparent activity of the reduced form of lactococcin MMFII on L. cremoris ATCC11603.

Lactococcin MMFII MIC on L. ivanovi BUG 496 was estimated to be 12 ng ml\(^{-1}\). This MIC is lower than that of mesentericin Y105, sakacin A and P, higher than that of pediocin PA-1 and dierivcin V41, and close to that of enterocin A (10 ng ml\(^{-1}\)) [11]. Thus, lactococcin MMFII is one of the most active bacteriocins within this class IIa bacteriocins, rendering this bacteriocin attractive as an anti-Listeria compound to protect food.

Lactococcin MMFII is the first anti-Listeria bacteriocin produced by a lactococcal strain described so far. The producer strain is able to coagulate milk, indicating that L. lactis MMFII is Lac\(^+\), Prt\(^+\). These characteristics strongly suggest that this strain is a good candidate to be incorporated in starters to protect fermented dairy products against listerial contamination. Lactococcal indicator strains retained for this study are not sensitive to the tested class IIa bacteriocins, except for two strains inhibited by lactococcin MMFII. Despite extension of the activity spectrum of class IIa bacteriocins to the Lactococcus genus, sensitivity to the latter vary from species to species and from strain to strain [6,10]. Finally, only a few lactococcal strains are sensitive to these bacteriocins. Nevertheless, lactococcal starter composition designed for incorporation of L. lactis subsp. lactis MMFII should be carefully chosen since lactococcin MMFII is active against some lactococcal strains.

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References


