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Chemical Synthesis, Molecular Modeling, and Antimicrobial Activity of a Novel Bacteriocin, MMFII

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A new antimicrobial peptide, referred to as MMFII, was purified to homogeneity from lactic acid bacteria *Lactococcus lactis*, which were isolated from Tunisian dairy product. The complete amino acid sequence of the peptide has been established by amino acid analysis, Edman sequencing, and mass spectrometry and verified by solid-phase chemical synthesis. MMFII is a single-chain 37-residue polypeptide containing a single intramolecular disulfide bond, i.e., TSYGNGVHC-NKSKCWIDVSELETYKAGTVSNPKDILW. It shares ca. 35% sequence identity with Leucocin A, a class IIa bacteriocin. Modeling based on the 3-D of Leucocin A shows three beta strands located in the N-terminal region (Thr1-Tyr3, Val7-Asn10, Lys13-Ile16) and an alpha helical domain from Asp17 to Asn31. When plotted as an α-helical wheel, the central α-helix of MMFII does not exhibit an amphipathic helical structure. The synthetic MMFII (sMMFII), obtained by the solid-phase method, was shown to be indistinguishable from the natural peptide. sMMFII is active against *Lactococcus cremoris* and *Listeria ivanovii* bacteria, whereas no activity was detected for any of the synthetic N-terminal truncated MMFII analogs Cys9-Trp37, Trp15-Trp37, and Val18-Trp37. © 2001 Academic Press

Key Words: **bacteriocin; MMFII; lactic acid bacteria; antimicrobial activity; synthetic peptide; molecular modeling.**

Bacteriocins are polypeptides produced by lactic acid bacteria (LAB). They display antimicrobial properties with a narrower spectrum of activity than antibiotics (1). They can potentially be used as biopreservatives to extend storage life or to enhance the safety of food (2). For commercial use of bacteriocins in food systems, optimization of their production is required (3). These biomolecules are classified in four classes based on their structures, sizes and modes of action: (i) the membrane-active and heat-stable peptides, including lantibiotics (class I); (ii) the small heat-stable cationic peptides which contain only natural amino acid residues (class II); (iii) the high mass heat-sensitive peptides (class III); and (iv) the complex peptides, including lipo- and glycoproteins (class IV). One subgroup, referred to as class IIa, contains bacteriocins with a particular structure motif in their N-termini and are active against the food pathogen *Listeria monocytogens* (4). This class of bacteriocin is characterized by a conserved N-terminal domain, -Tyr-Gly-Asn-Gly-Val-Xaa-Cys- (YGNGVXaaC), and a non-conserved hydrophobic C-terminus of variable length. The YGNGVXaaC motif of type IIa bacteriocins usually has the cysteine forming a disulfide bridge (5). These bacteriocins may require an undefined membrane-bound protein receptor and both the N-terminal motif as well as the C-terminal domain have been suggested to behave as recognition sequences (6).

Thus far, the only type II bacteriocin whose three-dimensional structure has been solved is leucocin A (Leu A), a 37-residue polypeptide isolated from *Leuconostoc geldium* UAL 187 (7). NMR studies have shown that Leu A adopts a well-defined 3-D structure that is composed of a three-stranded antiparallel β-sheet (residues 2–16) followed by an amphiphilic α-helix (residues 17–31) in both TFE and dodecyl phosphocholine (DPC) (8).

A novel class IIa bacteriocin produced by the lactic acid bacteria *Lactococcus lactis* from the Tunisian dairy products, named MMFII, was purified to homogeneity. Here we report for the first time the complete amino acid sequence, chemical synthesis, molec-

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ular modeling, and structure–activity relationship of MMFII.

MATERIALS AND METHODS

N-α-Fluorenylmethyloxycarbonyl (Fmoc) amino acid derivatives and 4-hydroxymethyl-phenoxymethyl-copolystyrene–1% divinylbenzene resin (HMP resin) were purchased from Perkin–Elmer. All solvents were analytical-grade commercial products from Perkin–Elmer or SDS (Peypin, France).

Purification procedure of MMFII bacteriocin. Bacteria culture and purification procedures were performed according to Ferchichi *et al.* (10). The methods used to assess the antimicrobial activity of the culture broths of *L. lactis* are the agar well diffusion test (ADT) (11) and the determination of the inhibitory titer. Before testing, cells were removed from the growth broth medium by centrifugation and the supernatant was treated with 1 mol · liter^{-1} NaOH and filter-sterilized (0.22 μm, Millipore).For determining the inhibitory titer of bacteriocin, samples to be tested were serially diluted by twofold and aliquots of 200 μl from each dilution were placed in wells of plates seeded with the bioassay strain. These plates were incubated at 30°C for 18–24 h and examined for the presence of 2 mm or larger clear zones of inhibition. The highest dilution of samples producing a defined zone of inhibition was reported as the antimicrobial titer and was expressed as arbitrary units (UA · ml^{-1}). The MIC is defined as the concentration of bacteriocin that inhibited growth of the indicator strain by 50%. *Lactococcus cremoris* was routinely used as the bioassay strain. *Listeria ivanovii* 496 was grown at 30°C for 18 h (from the Laboratoire de Microbiologie Fondamentale IBMIG Poitiers, France).

L. lactis ssbq. *Lactis* strain was grown in 4 liters of M17 at 30°C to stationary phase with agitation. The cells were removed by centrifugation at 10,000*g* for 15 min, and the cell-free supernatant was used as the starting material for bacteriocin purification. The native MMFII bacteriocin was purified to homogeneity, as follow: (i) ammonium sulfate precipitation (60%), (ii) ion-exchange chromatography, (iii) filtration with Sephadex G-50, and (iv) two steps of reversed-phase liquid chromatography (Merck, C18 Lichrospher 5 μm, 4 × 200 mm).

Amino acid sequence determination. Amino acid sequence analysis of natural MMFII was achieved by performing 40 cycles of automated Edman degradation using an applied Biosystems Model 476 A protein sequencer.

Mass spectrometry. Natural bacteriocin and synthetic peptides were analyzed on a Voyager DE RP matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer (Perseptive Biosystems, Inc., Framingham, MA). The mass spectra were recorded in the linear mode.

Chemical synthesis and physicochemical characterization of synthetic MMFII. Stepwise elongation of sMMFII (Thr1-Trp37) was carried out on 0.25 mmol of HMP resin (0.96 mmol of hydroxyl sites per gram of resin) using an automatic peptide synthesizer (Model 433A, Applied Biosystems, Inc.). To obtain sMMFII analogs (Cys9-Trp37), (Trp15-Trp37), and (Val18-Trp37), aliquots of peptide resins were removed at adequate cycles of peptide chain assembly. Trifunctional amino acids were protected on their side-chain as follows: trityl (Trt) for Cys, His and Asn; t-butyl (t-Bu) for Ser, Thr, Tyr, Glu, and Asp and Boc for Lys. *N*-α-Amino groups were deprotected by treatment with 18 and 20% piperidine/*N*-methylpyrrolidone for 3 and 8 min, respectively. The Fmoc amino acid derivatives were coupled (20 min) as their hydroxybenzotriazole active esters in *N*-methylpyrrolidone (fourfold excess). After peptide chain assembly, the peptide-resin [0.87 g for sMMFII (Thr1-Trp37), 0.45 g for (Cys9-Trp37), 0.25 g for (Trp15-Trp37), and 0.19 g for (Val18-Trp37)] was cleaved and deprotected by a 2-h treatment at 25°C with TFA containing 5% thioanisole, 5% ethanedithiol, in a final volume of 10 ml/g of peptide-resin (12). The reduced peptides, sMMFII (Thr1-Trp37) and (Cys9-Trp37), were dissolved at a concentration of 10 mg/ml in 0.2 M Tris–HCl buffer, pH 8, and stirred under air to allow folding (84 h, 25°C). The peptide solutions were filtered and the oxidized peptides (50-mg batches) were purified by preparative reversed-phase HPLC (Perkin–Elmer, ODS 20 μm, 100 × 10 mm) using a 2-h linear gradient of 0 to 50% acetonitrile in 0.1% TFA/H_2O (A) and 70% acetonitrile in water containing 0.1% TFA (B) at a flow rate of 6 ml/min, with UV detection at a wavelength of 230 nm. The identity of sMMFII (Thr1-Trp37) was verified by coelution assay with natural MMFII after injection of a mixture of both products in analytical reversed-phase HPLC (ii) amino acid analysis after acid hydrolysis (6 N HCl/1% phenol (mass/vol), 20 h, 120°C, N_2 atmosphere), and (iii) mass determination by MALDI-TOF mass spectrometry.

Molecular modeling. Search for sequence identity of MMFII protein in SWISSPROT (13) and Protein Data Bank (14) databases was carried out using both FASTA (15) and BLAST (16), respectively. Multiple amino acid sequence alignments were carried out using the HOMOLOGY module of Insight II-98 package (MSI, San Diego, CA). All steps of homology building and further energy calculations were carried out on an O2 R10000 Silicon Graphics workstation.

To choose between three PDB entries (2LEU, 3LEU, 1CW6) in which the corresponding structures can be used as templates to construct our model, PROCHECK (17) program was used to select the more appropriate starting structure according to the Ramachandran plots.

A molecular model of MMFII was obtained using the NMR structure of LeuA, an antimicrobial peptide isolated from the lactic acid bacteria *Leuconostoc geldium* UAL187 (PDB id = 2LEU). Briefly, energies of the 18 NMR structures were minimized by the DISCOVER program using the CVFF force field parameters and the steepest descent algorithm. One thousand iterations were performed for energy calculation of each structure, and the most favored conformation was selected to start building of the MMFII model. Using the BIOPOLYMER module, the mutations were done by residue replacement, and the model was refined by 1000 cycles of energy minimization, as described. The final model was obtained using the gradient conjugated method and the full energy minimization until the root mean square derivative of the energy function was less than 0.001 kcal · mol^{-1} · å$^{-1}$.

Finally, the 3-D structure coordinates were submitted to PROCHECK to validate the model. All the MMF2 residues were found to be in the favored and allowed regions of the Ramachandran plot.

RESULTS

A bacteriocin was purified to homogeneity (>99%) from a cell-free culture supernatant of *L. lactis* by a four step protocol. Antimicrobial activity against the indicator strain *L. cremoris* was used as a functional assay. The purified material was used for chemical characterization. Analysis of the near UV of the peptide solution spectrum indicated the presence of some tryptophane residues. By using a solid phase sequencer, the amino acid sequence of the purified bacteriocin (400 pmol) was established up to the 37th residue as being TSYGNGVHCNKSKCWIDVSELETYKAGTVSNPKDILW. A MALDI-TOF mass spectrometry analysis of the native peptide gave an experimental $(M + H)^+$ of 4143.66 Da, which is in agreement with the calculated M_r 4142.66. The Ellman assay did not reveal any free thiol groups in the bacteriocin, thus indicating, the presence of the disulfide bridge between the two half cystines at positions 9 and 14. A computer-

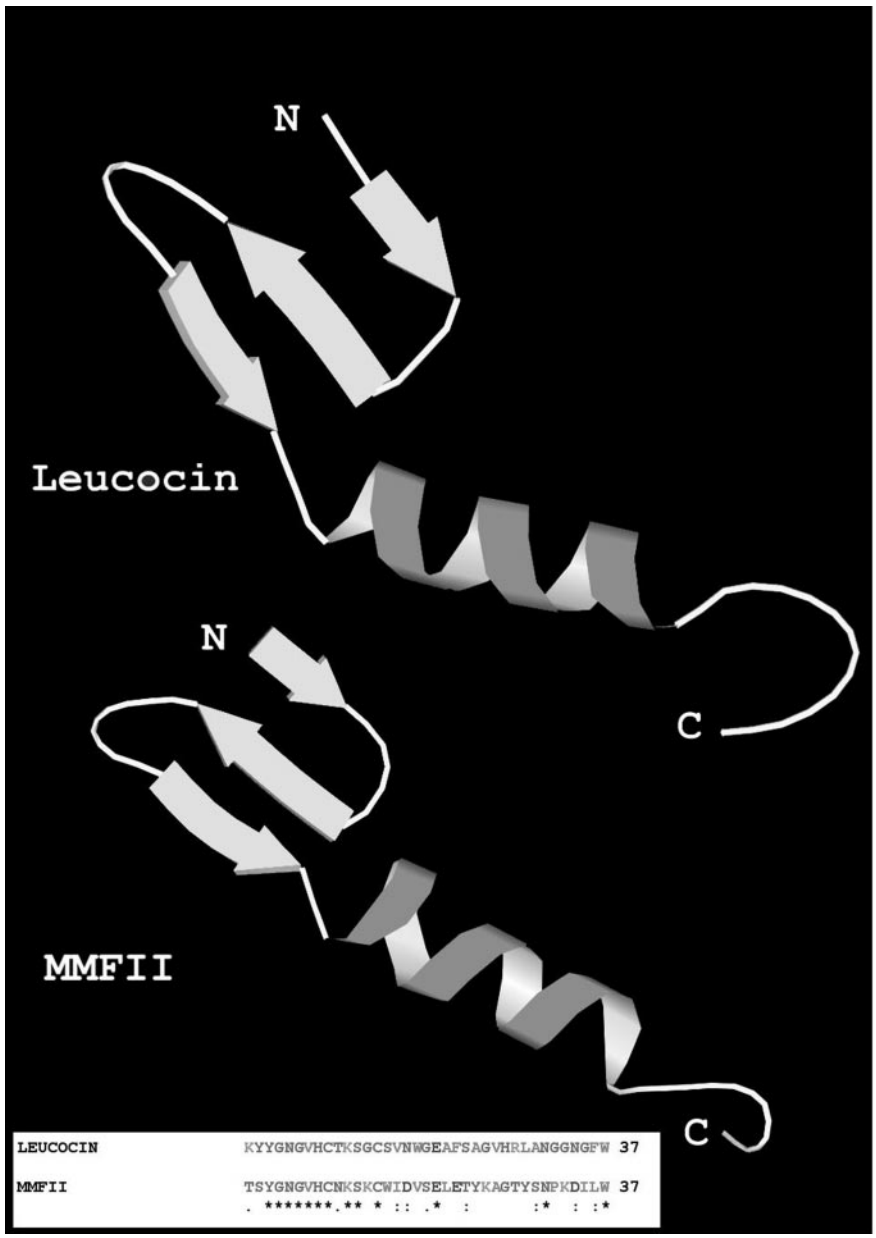

FIG. 1. Molscript 3-D representations of Leucocin (used as template) and MMFII peptides. The Leucocin model was generated according to the 1-NMR known structure (8) (PDB identification code 2LEU). The polypeptide backbone is colored in gray scale according to secondary structures: α-helix, β-sheet, and others (top). Amino acid sequence (one-letter code) of the MMFII and comparison with Leucocin (bottom). The asterisks or dots denote that the amino acid residues are either identical or similar in their properties.

assisted search in the PDB database, that compares the MMFII primary structure to other sequences reveals the existence of 35% sequence identity with peptides belonging to class IIa bacteriocins. This novel peptide was referred to as MMFII bacteriocin (Fig. 1).

sMMFII was synthesized by means of an optimized Fmoc/*tert*-Butyl chemistry to confirm that the antimicrobial activity observed with the native peptide reflects intrinsic MMFII properties. To evaluate the antimicrobial activity of the MMFII C-terminal domain, the Cys9-Trp37, Trp15-Trp37, and Val18-Trp37 analogs were also obtained by chemical synthesis. To improve yields of couplings for "difficult" sequences, a double coupling cycle was used with hydrophobic or bulky amino acid derivatives. As an example, the analytical reversed-phase HPLC profile of crude reduced MMFII after final acidolytic cleavage is shown in Fig. 2. The crude sMMFII and Cys9-Trp37 peptides were oxidized by exposure to air and purified to 99% homogeneity by preparative HPLC. The oxidation/folding process appeared to be independent of the peptide concentration and was rapid, as the oxidized peptide could

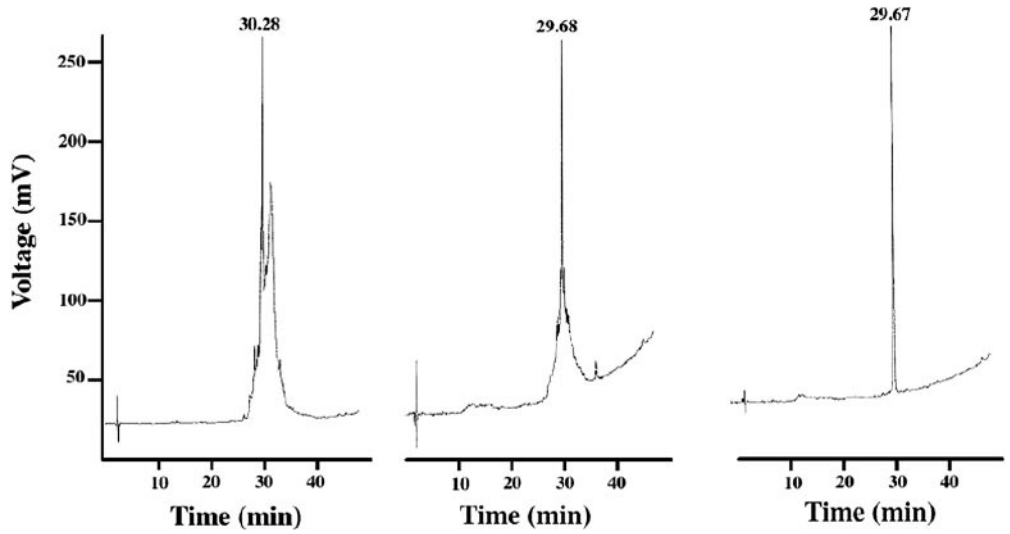

FIG. 2. Analytical C_{18} reversed-phase HPLC profiles of sMMFII at various stages of peptide synthesis. The crude peptide after trifluoroacetic acid cleavage (left), crude peptide after folding/oxidation (middle), and purified folded peptide (right). Experimental conditions are described under Materials and Methods.

be detected within 1 h whereas the reduced peptide has fully disappeared in less than 2 h (data not shown). The synthetic oxidized product was indistinguishable from its native counterpart as (i) sMMFII peptide eluted at a retention time similar to the natural bacteriocin; (ii) coinjection of the 2 peptides resulted in a single peak; (iii) amino acid content of the purified oxidized sMMFII was identical to that of natural MMFII; and (vi) the experimental molecular mass of sMMFII (4143.53 Da) agrees with the deduced mass of MMFII.

The bioactivity was assessed by the agar well diffusion test. The concentrations of peptide producing a 2-mm zone of growth inhibition against *Listeria ivanovii* BUG 469 and *L. lactis cremoris* 11603 in the well diffusion assay were found to be 2500 nM and 1.42 nM, respectively. No activity was detected for any of the N-terminal truncated peptides (i.e., Cys9-Trp37, Trp15-Trp37, and Val18-Trp37).

The calculated molecular model of MMF2 protein shows some well-defined secondary structures. The model contains three beta strands in the N-terminal region (Thr1-Tyr3, Val7-Asn10, Lys13-Ile16), and an alpha helical domain from Asp17 to Asn31. The model was compared to the 2LEU structure and the superimposed traces of the two proteins gave an RMSD value of 2.5 A (Fig. 1). When plotted as an α-helical wheel, the central α-helix of MMFII does not exhibit any amphipathic helical properties (Fig. 3).

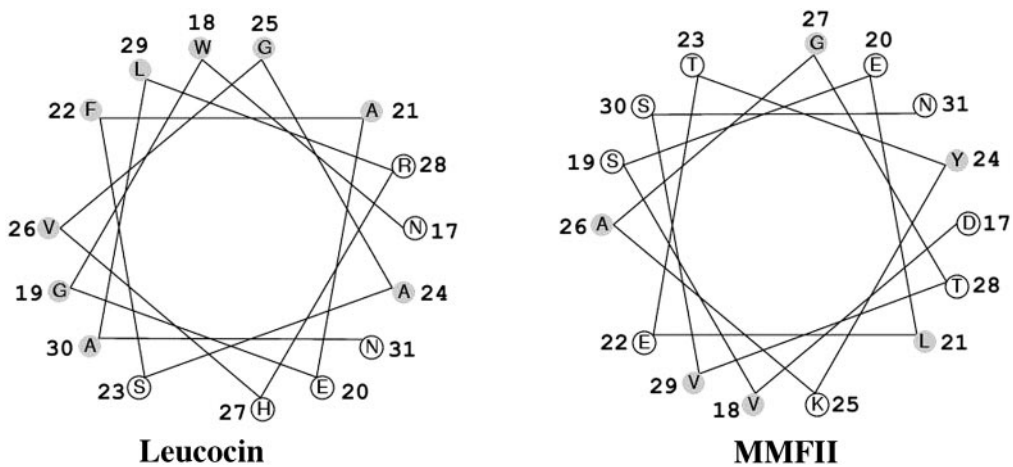

FIG. 3. Helical wheel projections for Leucocin and MMFII. A polar and hydrophilic domain (in open circles) and a hydrophobic domain (shaded circles) are clearly distinguishable on each side of the Leucocine cylindrical surface.

DISCUSSION

The range of application of antimicrobial products of LAB will certainly grow intremendeously near future because of their large spectrum of activity. Lactic acid bacteria can be exploited in feed and in pharmacological applications (18).

A novel class IIa bacteriocin, MMFII produced by the lactic acid bacteria *L. lactis,* was isolated from Tunisian dairy products and purified to homogeneity.

The present study reports the complete amino acid sequence, chemical synthesis, three-dimensional structure prediction and structure–activity relationship of MMFII. The MMFII bacteriocin does not contains any unnatural amino acid. Mass spectrometry analysis indicates a molecular mass of 4142.66 kDa, which corresponds to the 37 unmodified residues that were revealed by N-terminal Edman sequencing. The amino acid sequence of MMFII was clearly different from other published bacteriocin primary structures. It contains the -Tyr-Gly-Asn-Gly-Val-Xaa-Cys- consensus motif that is typical of the antilisterial peptides, which would therefore classify MMFII as a member of the class IIa bacteriocins, according to the nomenclature proposed by Kleanhammer (19). MMFII shares ca. 35% sequence identity with Leu A and contains a unique disulfide bridge (Cys9-Cys14) located in the highly conserved N-terminal extremity. However, comparative analysis of MMFII and known class IIa bacteriocin primary structures shows a low degree of sequence identity with regard to the central and C-terminal domains. MMFII has a net electrical charge of 0 at neutral pH value, whereas Leu A possesses 3 positive charges. Indeed MMFII contains Asp, Glu, Glu, and Asp residues at positions 17, 20, 22, and 34, instead of Asn, Glu, Phe, and Asn for Leu A at the indicated positions (Fig. 1). In addition the changes in the C-terminus are particularly striking, since this region has been proposed to be crucial for target-cell specificity (6, 8).

The MMFII bacteriocin, as well as number of N-terminal truncated MMFII analogs (i.e., Cys9-Trp37, Trp15-Trp37, and Val18-Trp37) were chemically synthesized by the solid phase method. Synthetic MMFII behavior is indistinguishable from that of its natural counterpart. It inhibits the growth of both *L. cremoris* (MIC = 1.42 nM) and *Listeria ivanovii* (MIC = 2500 nM) bacteria.

Of note, each MMFII analog did not exert any bacteriocidal activity. Therefore, it is likely that deletion of the YGNGV motif affects the β-turn structure and, consequently, the N-terminal β-sheet of MMFII, thereby altering bioactivity. Previous studies based on the use of synthetic analogs of mesentericin Y105 support the hypothesis that the highly conserved N-terminal extremity, i.e., 1–14 of this 37-mer bacteriocin forms part of a recognition domain for a membrane-bound receptor; it also indicates that the entire structure, including the N- and C-termini, plays a crucial role in antimicrobial activity (20).

Comparison of the MMFII model with the solution structure of LeuA show that both peptides share a well-defined α-helix located in their C-termini (residues 18–30 for MMFII; residues 17–31 for LeuA) despite a much greater variability in amino-acid sequence. Examination of the LeuA helical portions indicates that hydrophobic residues appear on one side, whereas the hydrophilic residues are located on the opposite side, that is typical of amphiphilic helices (8). However, analysis of the helical region shows that MMFII does not form a similar amphipathic α-helix (Fig. 3).

Apart from its antimicrobial activity, the MMFII bacteriocin shows other properties that makes it a promising food preservative. These properties include thermal stability, activity over a wide range of pH values, and maintenance of antimicrobial activity after freeze thawing, lyophilization, and prolonged storage (21).

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