Covalent Structure, Synthesis, and Structure-Function Studies of Mesentericin Y 10537, a Defensive Peptide from Gram-positive Bacteria Leuconostoc mesenteroides

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A 37-residue cationic antimicrobial peptide named mesentericin Y 10537 was purified to homogeneity from cell-free culture supernatant of the Gram-positive bacterium Leuconostoc mesenteroides. The complete amino acid sequence of the peptide, KYYNGVHTKSGCSVNG-WGEAASAGHRLANGNGFW, has been established by automated Edman degradation, mass spectrometry, and solid phase synthesis. Mesentericin Y 10537 contains a single intramolecular disulfide bond that forms a 6-membered ring within the molecule. Mesentericin Y 10537 was synthesized by the solid phase method. The synthetic replicate was shown to be indistinguishable from the natural peptide with respect to electrophoretic and chromatographic properties, mass spectrometry analysis, automated amino acid sequence determination, and antimicrobial properties. At nanomolar concentrations, synthetic mesentericin Y 10537 is active against Gram⁺ bacteria in the genera Lactobacillus and Carnobacterium. Most interestingly, the peptide is inhibitory to the growth of the food-borne pathogen Listeria. CD spectra of mesentericin Y 10537 in low polarity medium, which mimic the lipophilicity of the membrane of target organisms, indicated 30-40% α-helical conformation, and predictions of secondary structure suggested that the peptide can be configured as an amphipathic helix spanning over residues 17-31. To reveal the molecular basis of the specificity of mesentericin Y 10537 targeting and mode of action, NH₂- or COOH-terminally truncated analogs together with point-substituted analogs were synthesized and evaluated for their ability to inhibit the growth of Listeria ivanovi. In sharp contrast with broad spectrum α-helical antimicrobial peptides from vertebrate animals, which can be shortened to 14-18 residues without deleterious effect on potency, molecular elements responsible for anti-Listeria activity of mesentericin Y 10537 are to be traced once to the NH₂-terminal tripeptide KYY, the disulfide bridge, the putative α-helical domain 17-31, and the COOH-terminal tryptophan residue of the molecule. It is proposed that the amphipathic helical domain of the peptide interacts with lipid bilayers, leading subsequently to alteration of the membrane functions, whereas residues 1-14 form part of a recognition structure for a membrane-bound receptor, which may be critical for peptide targeting. Because mesentericin Y 10537 is easy to synthesize at low cost, it may represent a useful and tractable tool as a starting point for the design of more potent analogs that may be of potential applicability in foods preservation.

The production of gene-encoded antimicrobial peptides as an immune strategy is widely used in nature and has been conserved in evolution. As a first line of defense against infections, vertebrate and invertebrate animals have developed chemical defense systems based on cationic antimicrobial peptides 22-46 residues long that are synthesized and secreted by non-myeloid cells (1-4). The microbicidal effects of these broad spectrum peptide antibiotics very likely result from their capacity to interact with membranes and to permeate the target cells. Gene-encoded antibiotics are considered ancestral effectors of immunity because microbicial peptides, named bacteriocins, have also been used by a number of Gram-positive and Gram-negative bacteria for millions of years for containing the proliferation of organisms that are closely related or confined within the same ecological niche (5-12), helping the producing microbe to compete for limited resources.

Gene-encoded peptides of the chemical defense so far isolated from eukaryotic and prokaryotic organisms differ in several respects from the "classical" antibiotics or secondary metabolites and may provide a wholly new approach to fighting infectious diseases and noscomial infections (13). Whereas many antibiotics disable or kill pathogens over a period of days by inhibiting essential enzymes, most gene-encoded antimicrobial peptides kill microorganisms rapidly by destroying or permeating the microbial membrane and impairing the ability to carry out anabolic processes (1, 2). In addition, antimicrobial peptides are of relatively small size and made as pre-proteins that are processed to the mature peptide by dedicated pathways. These peptides are thus unlikely to face the same antimicrobial resistance mechanisms that limit current antibiotic use.

In this regard, the bacteriocins produced by lactic acid bacteria have gained much attention as potentially useful food additives against food-borne pathogens (9, 10, 12). Class I bacteriocins (lantibiotics) undergo extensive post-translational modifications and contain very unusual amino acids (5). Nisin, for instance, is a 34-residue peptide produced by Lactococcus
lactis that is very active against most Gram-positive bacteria, including genera Lactococcus, Lactobacillus, Bacillus, Micrococcus, and Listeria and Staphylococcus aureus and Clostridium botulinum. In contrast, class II nonantibiotic-containing bacteriocins, such as the lactococci, the pediococci, the lactacins, and leucocin A, are 36–44 amino acid peptides that are minimally modified (14–18). Most of these class II bacteriocins are potent against Listeria monocytogenes, Gram-positive pathogenic bacteria that are responsible for severe infections of the central nervous system following the absorption of contaminated dairy products (9). The approval and use of nisin as an additive in processed cheese spreads raised the interesting possibility that direct addition of bacteriocins, especially those belonging to class II that are easier and cheaper to produce either by chemical synthesis or genetic engineering, may provide a novel means of preserving foods from pathogenic bacteria.

In recent years, a wealth of information has been gained about the effectiveness of class II bacteriocins against undesirable bacteria in vitro. However, most of these data have been obtained through the use of cell-free culture supernatants of bacteriocin-producing bacterial strains or semi-purified bacteriocins, and no class II bacteriocin has been chemically synthesized and assayed for antimicrobial activity. In addition, little if anything is known with regard to structural and conformational determinants that confer stability and activity to these peptides (11). These studies would provide molecular models for the conception of more potent structural analogues and a starting point for the design of new preventive or therapeutic agents.

### MATERIALS AND METHODS

Chemicals—9-fluorenylethoxycarbonyl (Fmoc)-protected 1-aminoc acids and polyethylene glycol polylysine-graft copolymer support (substituted at 0.18 molar eq/g) were from Milligen (Bedford, MA). Chemicals for peptide synthesis (dimethylformamide, dichloromethane, disopropylcarbodiimide, hydroxybenzotriazole, piperidine, trifluoroacetic acid, and acetonitrile) were obtained from commercial sources and were of the highest purity available.

**Bacterial Strains and Media—** Leuconostoc mesenteroides Y 105 was grown aerobically to the late exponential phase at 30°C for 18–20 h in lactobacilli MRS broth (DIFCO Laboratories, Inc., Detroit, MI). The indicator strain L. ivanovi was grown at 30°C for 18 h in Tryptic Soy broth (DIFCO Laboratories). Cleavage of the peptidyl resins and side chain deprotection were carried out at a concentration of 40 mg of peptidyl resin in 1 ml of a mixture composed of trifluoroacetic acid, phenol, thioanisole, water, and ethyl methyl sulfide (82.5:5:5:5:2.5, v/v/v/v/v) for 2 h at room temperature. After filtering to remove the resin and ether precipitation at 20°C, the crude peptides were recovered by centrifugation at 5,000 rpm for 10 min, washed three times with cold ether, dried under nitrogen, cleaved, and lyophilized. Trityl groups were removed by elution with a mixture composed of trifluoroacetic acid, phenol, thioanisole, water, and ethyl methyl sulfide (82.5:5:5:5:2.5, v/v/v/v/v) for 2 h at room temperature. After filtering to remove the resin and ether precipitation at 20°C, the crude peptides were recovered by centrifugation at 5,000 rpm for 10 min, washed three times with cold ether, dried under nitrogen, dissolved in 20% acetic acid, and lyophilized. The purity of the synthetic peptides was assessed by solid phase sequence analysis, mass spectrum analysis, and analytical HPLC on a Lichrospher ODS 2 column (5 µm, 4.6 × 250 mm) eluted at a flow rate of 0.8 ml/min by a linear gradient of acetonitrile in 0.1% trifluoroacetic acid/water. Fractions were monitored for absorbance at 280 and 220 nm and for activity against the indicator strain L. ivanovi 496. The active fractions were further purified using homogeneity on HPLC using the same column and solvent system and lyophilized. Quantification of free thiois was achieved with the Ellman’s reagent as described previously (19).

**Phenylacetic Acid Sequence Analysis—** Sequence analyses were carried out on an Applied Biosystem 470 gas phase sequencer. Phenylhydantoin amino acids were detected with an on-line Applied Biosystem 120 A analyzer. Data collection and analysis were performed with an Applied Biosystem 900 A module calibrated with 25 pmol of phenylhydantoin amino acid standards. Alternatively, analysis were carried out on a Milligen 6600 solid phase sequencer after solvent precipitation (20) of the samples (250 pmol) to Sequelon aryamide membranes. Phenylhydantoin amino acids were detected with an on-line HPLC column (Waters MS HPLC; SequaTag C-18 phenylhydantoin analysis column; 350 mm x 3.9 mm) developed with ammonium acetate (pH 4.8) and acetonitrile and calibrated with 15 pmol of phenylhydantoin amino acid standards. Data collection and analysis were performed with a Maxima-phenylhydantoin chromatography analysis software package (Dynamic Solution Corp., Division of Waters Chromatography, Milford MA).

Fast Atom Bombardment and Electrospray Ionization Mass Spectrometry—Mass spectral analyses were performed using a quadrupole-coupled elecrospray mass spectrometer (VG Platform). The mass scale was calibrated using myoglobin. The accuracy was monitored using myoglobin as an internal mass calibrant.

**Solid Phase Synthesis of Leucocin A, Mesentericin Y 105, and Analogos—** Mesentericin Y 105, leucocin A, and mesentericin Y 105 analogs were synthesized using 9-Fmoc polyamide active ester chemistry on a Milligen 9050 synthesizer. All 9-Fmoc-amino acids were from Milligen. Polyethylene glycol polylysine resins were used for all peptides but mesentericin Y 105, leucocin A, and [Lys10]mesentericin Y 105 for which 4-hydroxyethylphenoxyacetic acid-linked polyamide/hexamethyleneguar hydrogel resin (pepsin K) were used. Side chain protections were tert-butyloxycarbonyl for lysine and histidine; 2,2,5,7,8-pentamethyl-6-chromansulfonyl for arginine; O-tart-butyl ester for glutamic acid; O-tet-butyl ether for serine, threonine, and tyrosine; and trityl for histidine, cystine, and histidine. Side chain deprotection was carried out by a double-coupling protocol: Nα-Fmoc-amino acids (4.4 molar excess) were coupled for 30–60 min with 0.23 m disopropylcarbodiimide in a mixture of dimethylformamide and dichloromethane (60:40, v/v). Acylation was checked after each coupling step by the Kaiser test.

**Clavage of the peptide resins and side chain deprotection were carried out at a concentration of 40 mg of peptide resin in 1 ml of a mixture composed of trifluoroacetic acid, phenol, thioanisole, water, and ethyl methyl sulfide (82.5:5:5:5:2.5, v/v/v/v/v) for 2 h at room temperature. After filtering to remove the resin and ether precipitation at 20°C, the crude peptides were recovered by centrifugation at 5,000 rpm for 10 min, washed three times with cold ether, dried under nitrogen, dissolved in 20% acetic acid, and lyophilized. The purity of the synthetic peptides was assessed by solid phase sequence analysis, mass spectrum analysis, and analytical HPLC on a Lichrospher ODS 2 column (5 µm, 4.6 × 250 mm) eluted at a flow rate of 0.8 ml/min by a linear gradient of acetonitrile in 0.1% trifluoroacetic acid/water. A summary of the production and characterization of the synthetic peptides is shown in Table I.

**Bacteriocin Assays—** Assays were performed by an antagonism well

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1. The abbreviations used are: Fmoc, 9-fluorenylethoxycarbonyl; HPLC, high performance liquid chromatography; MIC, minimal inhibitory concentration; Acm, acetamidomethyl; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; TFE, trifluoroethanol.
path length in a Jobin-Yvon Mark IV instrument linked to a Micromat digital II microprocessor. Spectra represented average values from six separate runs. The content of α-helix, β-sheet, and unordered structure were estimated as described (23).

RESULTS

Isolation and Purification of a Novel Bacteriocin Produced by L. mesenteroides—A bacteriocin was purified to homogeneity from a cell-free culture supernatant of L. mesenteroides by a four-step protocol involving ammonium sulfate precipitation, size-fractionation, Sep-pak filtration, and reverse-phase HPLC. Activity against the indicator strain L. ivanovii was used as a functional assay. The absorbance profile at a wavelength of 280 nm of a gel filtration fractionation on a Sephadex G-50 column of a 60% ammonium sulfate precipitate of a cell-free culture supernatant of L. mesenteroides is shown in Fig. 1, along with the anti-Listeria activity profile. Fractions 15–20 containing the peak of the wide zone of anti-Listeria activity were pooled and fractionated on Sep-pak C-18 cartridges. The active material eluting at 50% acetonitrile was further purified by reverse-phase HPLC. As depicted in Fig. 2, the initial anti-Listeria activity from G-50 was recovered after a series of HPLC runs as a symmetrical sharp peak eluting at 24.49 min and accounting for ~95% of the eluted material. Inspection of the near UV spectra of the peak indicated the presence of the classical tryptophan signature (Fig. 2). Analysis of the purified peptide by SDS-polyacrylamide gel electrophoresis followed by staining with Coomassie Brilliant Blue and overlay anti-Listeria assay revealed only a single active band in the 3.5-kDa size zone (Fig. 3), indicating that the bacteriocin has been purified to homogeneity. The concentration of peptide producing a 1-mm zone of growth inhibition against L. ivanovii in the well diffusion assay was estimated to be in the nanomolar range (see Table III). The purified bacteriocin (final yield, 17 mg) was further purified by reverse-phase HPLC. As depicted in Fig. 2, the initial anti-Listeria activity from G-50 was recovered after a series of HPLC runs as a symmetrical sharp peak eluting at 24.49 min and accounting for ~95% of the eluted material. Inspection of the near UV spectra of the peak indicated the presence of the classical tryptophan signature (Fig. 2). Analysis of the purified peptide by SDS-polyacrylamide gel electrophoresis followed by staining with Coomassie Brilliant Blue and overlay anti-Listeria assay revealed only a single active band in the 3.5-kDa size zone (Fig. 3), indicating that the bacteriocin has been purified to homogeneity. The concentration of peptide producing a 1-mm zone of growth inhibition against L. ivanovii in the well diffusion assay was estimated to be in the nanomolar range (see Table III). The purified bacteriocin (final yield, 17 mg/l culture supernatant) was directly subjected to amino acid sequence analysis and mass spectrometric analysis.

Amino Acid Sequence and Mass Spectrometric Analysis—The primary structure of the purified bacteriocin was successfully determined up to the 37th residue as KYGYNGVHTCKTS-GCSVNWGEAASAGIHRLANGNGNFW by automated Edman degradation of the peptide (250 pmol) using either a gas phase sequencer or a solid phase sequencer after carboxylic covalent binding of the sample to a Sequanal alyamine membrane. The purified peptide was shown to contain less than 1% free thiols as assessed by Ellman’s method (20), suggesting the presence of a single disulfide bond between Cys⁹ and Cys¹⁴. Because the sequence analysis does not yield information on additional post-translational modifications of amino acid side chains, the
purified bacteriocin was also subjected to mass spectral analysis using electrospray ionization spectrometry. As shown in Fig. 4, three unequivocal pseudo molecular ions \([M + nH]^+\) were observed at \(m/z\) corresponding to \(n = 3, 4, 5\) proteinated species whose averaged molecular mass was 3868.24 ± 0.1 atomic mass units. An almost identical value, 3868.73 atomic mass units, was obtained using fast atom bombardment spectrometry (not shown). These values corresponded to that expected theoretically for the experimentally determined amino acid sequence of 37 residues minus 2 atomic mass units. The discrepancy of 2 Da between the measured and calculated molecular weights resulted from the presence of the disulfide bridge between the 2 cysteines at positions 9 and 14.

There is almost complete agreement between the amino acid sequence of the 37-residue bacteriocin isolated in the present study and mesentericin Y 105, a 36-residue bacteriocin recently isolated from \(L.\) mesenteroides by Héchalard et al. (24). The only exception is the additional tryptophan residue at the COOH terminus of the 37-residue bacteriocin. Because this amino acid is missing from mesentericin Y 105 and has a profound influence on activity (see below), it may have been removed by proteolysis during isolation and extraction. Accordingly, the novel 37-residue bacteriocin was designated mesentericin Y 10537.

Solid Phase Synthesis of Mesentericin Y 10537—Mesentericin Y 10537 and related analogs were synthesized by the solid phase method to confirm that antimicrobial activity of the native peptide reflected intrinsic properties. Purification of the synthetic peptides was performed by reverse-phase preparative HPLC, and thiol oxidation was conducted as described previously (19). After purification, synthetic oxidized mesentericin Y 10537 was shown to be indistinguishable from the natural product by the following chemical and physical criteria: (i) the purified synthetic peptide showed by HPLC a unique sharp peak eluting exactly at the position of the corresponding natural product (Fig. 2); (ii) co-injection of the 2 peptides resulted in only one peptide peak; (iii) the sequence of synthetic mesentericin Y 10537 could be determined up to Trp37 by automated Edman degradation after covalent binding of the peptide to Sequelon arylamine membrane; (iii) mass spectrometry of a sample of oxidized synthetic mesentericin Y 10537 gave molecular ions \([M + nH]^+\) whose calculated molecular mass was 3868.22 atomic mass units, identical to that obtained with the natural peptide; moreover, unequivocal molecular ions were observed for non-oxidized synthetic mesentericin Y 10537 at \(m/z\) values whose average corresponded precisely to that of the natural product plus 2 atomic mass units; (iv) the concentration of synthetic replicate that exhibited growth inhibition against \(L.\) ivanovii is almost identical to that of natural mesentericin Y 10537 (see Table III).

Because the synthetic oxidized peptide was found to be indistinguishable from its natural counterpart, it was used in the following to evaluate its antimicrobial spectrum and to analyze structural and conformational requirements for anti-Listeria activity.

Spectrum of Antibacterial Activity of Synthetic Mesentericin Y 10537—Synthetic mesentericin Y 10537 was investigated for its ability to affect the growth of various strains of bacteria. The peptide revealed to be endowed with a narrow spectrum of activity, being inactive against Gram-negative bacteria but highly potent against a few strains of Gram-positive bacteria, including genera \(Listeria,\) Lactococcus, and Carnobacterium (Table II). However, the degree of sensitivity of these strains varies, \(L.\) ivanovii, Leucostoc paramesenteroides, and Lactobacillus sake being approximately 20 times more sensitive than the others.

To gain insight into the mechanism of action of mesentericin Y 10537, suspensions of \(L.\) ivanovii (10⁸ cells/ml) were incubated at 30°C with 3.5 \(\mu\)M of peptide. After various incubation times, the microorganisms were harvested by centrifugation, thoroughly washed, and inoculated to fresh agar medium for 48 h. After 5 min of treatment with 3.5 \(\mu\)M of mesentericin Y 10537, washed \(L.\) ivanovii did not proliferate. These results, which remained unchanged after 180 min of treatment, demonstrated that the effects of mesentericin Y 10537 are irreversible.

Analysis of Structural Requirements for Anti-Listeria Activity by Analog Design and Enzymatic Fragmentations—To evaluate the structural features that impart antibacterial activity to mesentericin Y 10537, COOH- or NH₂-terminally truncated fragments of the peptide, either obtained through solid phase synthesis or enzymatic digestion, were assayed against \(L.\) ivanovii. As shown in Table III, shortening the peptide chain to mesentericin Y 10537-[1–36] produced a dramatic 10,000-fold decrease in the anti-Listeria activity of the peptide. It is worth noting that the 36-mer peptide corresponds to that isolated previously by Héchalard et al. from \(L.\) mesenteroides cell-free culture supernatant (24). Further reduction of the chain length to residues 1–28 and 1–20 (75 and 54% of the peptide chain length, respectively) yielded peptide derivatives that were virtually devoid of activity (Table III). On the other hand, mesentericin Y 10537-[4–37] (92% of the peptide chain length) displayed only residual activity. Stepwise elimination of the NH₂-terminal 28 residues of mesentericin Y 10537 to give mesentericin Y 10537-[15–37] (62% of the peptide chain length), mesentericin Y 10537-[21–37], and mesentericin Y 10537-[29–37] yielded compounds that were inactive (Table III). To address the role the central region of mesentericin Y 10537 plays in conferring to the peptide anti-Listeria potency, deletion molecules made of the NH₂-terminal 1–8 or 1–14 segments and the 28–37 COOH-terminal segment of mesentericin Y 10537, i.e., mesentericin Y 10537-[1–8] (28–37) and mesentericin Y 10537-[1–14] (28–37), were synthesized and tested for activity. When compared with the parent compound, both analogs were found to be inactive against \(L.\) ivanovii (Table III). Hence it appears that whereas the COOH-terminal residue Trp37 is essential for full potency, the entire chain length is required for anti-Listeria activity.
To obtain evidence of the contribution of the disulfide bridge linking Cys⁹ and Cys¹⁴ to the antibacterial activity of mesentericin Y 105³⁷, the effect of chemical modifications and amino acid substitutions of residues 9 and 14 were investigated. As reported in Table III, modifications of the side chain of Cys⁹ and Cys¹⁴ by the Acm group led to an analog showing a marked loss in inhibitory potency relative to the parent compound. In addition, the ability of Ser⁹,¹⁴ substituted analog to inhibit the growth of *Listeria* was reduced by a factor of 20,000 relative to that of mesentericin Y 105³⁷. Altogether, these results indicate that the disulfide bridge is mandatory for high anti-Listeria activity.

Through amino acid substitutions, we have further investigated whether the augmentation of the positive charge of mesentericin Y 105³⁷ would enhance inhibitory potency as reported for linear cationic antimicrobial peptides isolated from vertebrate sources (25). As shown in Table III, augmentation of the positive charge of mesentericin Y 105³⁷ by one unit through substituting Lys for Thr in position 10 produced no significant change in the capability of the peptide to inhibit the growth of *Listeria*. Also consistent with this finding is the observation that augmentation of the net positive charge of mesentericin Y 105³⁷-[1–36] by one unit through substitution of Thr¹⁰ by Lys did not alter the inhibitory potency of the 36-mer peptide (not shown).

Finally, the inhibitory potency of mesentericin Y 105³⁷ was compared with that of synthetic leucocin A, i.e., [Phe²², Val²⁶], mesentericin Y 105³⁷, a bacteriocin isolated from *Leuconostoc gelidum* (16). As reported in Table III, introduction of an aromatic side chain in position 22 and reduction of the side chain length of the β-branched amino acid in position 26 afforded a peptide derivative that exhibited a potency to inhibit the
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### Table II

| Organism          | Strain   | MIC* \(\text{nmol/L}\) | Mesentericin Y 105\(^{37}\) | Leucocin A |
|-------------------|----------|------------------------|-----------------------------|------------|
| L. mesenteroides  | Y 105    | 263.50                 | ND*                         | ND*        |
| L. ivanovii       | Y 105    | 34                     | 69                          | 69         |
| L. paracasei      | 19       | 38                     | 78                          | 78         |
| L. curvatus       | DSM 20 019 | 708                   | 521                         | 521        |
| L. sake           | DSM 20 017 | 37                     | 39                          | 39         |
| Carnobacterium divergens | DSM 20 623 | 538                   | 662                         | 662        |
| Carnobacterium pisciols  | DSM 20 730 | 742                   | 623                         | 623        |

*The MIC was defined as the peptide concentration (nm) producing a diameter of inhibition zone of 1 mm in the well diffusion method. The results are the mean of at least two independent determinations with a divergence of not more than one MIC value.

**Table III**

| Peptide          | Amino acid sequencea  | Relative potencyb % |
|------------------|-----------------------|---------------------|
| Mesentericin Y 105\(^{37}\) | KYYNGVHCTKSGSCLVNGGLEAFVRLANGGNGFW | 100 |
| Mesentericin Y 105\(^{32}\) | KYYNGVHCTKSGSCLVNGGLEAFVRLANGGNGFW | 96 |
| Leucocin A       | KYYNGVHCTKSGSCLVNGGLEAFVRLANGGNGFW | 50 |
| [Lys19] mesentericin Y 105\(^{37}\) | KYYNGVHCTKSGSCLVNGGLEAFVRLANGGNGFW | 65 |
| [Asn36] mesentericin Y 105\(^{37}\) | KYYNGVHCTKSGSCLVNGGLEAFVRLANGGNGFW | 0.042 |
| Mesentericin Y 105\(^{37}\) [4-37] | GNGVHCTKSGSCNVWEEASAGIHRLANGGNGFW | 0.0022 |
| Mesentericin Y 105\(^{37}\) [15-37] | SVNVVWEEASAGIHRLANGGNGFW | <3.10 \(\times 10^{-4}\) |
| Mesentericin Y 105\(^{37}\) [21-37] | AASAGIHRLANGGNGFW | <3.10 \(\times 10^{-4}\) |
| Mesentericin Y 105\(^{37}\) [29-37] | LANGGNGFW | <3.10 \(\times 10^{-4}\) |
| Mesentericin Y 105\(^{37}\) [3-36] | KYYNGVHCTKSGSCLVNGGLEAFVRLANGGNGFW | 0.01 |
| Mesentericin Y 105\(^{37}\) [1-28] | KYYNGVHCTKSGSCLVNGGLEAFVRLANGGNGFW | 3.10 \(\times 10^{-4}\) |
| Mesentericin Y 105\(^{37}\) [1-20] | KYYNGVHCTKSGSCLVNGGLE | 3.10 \(\times 10^{-4}\) |
| Mesentericin Y 105\(^{37}\) [1-14] [28-37] | KYYNGVHCTKSCRLANGGNGFW | 3.10 \(\times 10^{-4}\) |
| Mesentericin Y 105\(^{37}\) [1-8] [28-37] | KYYNGVHRLANGGNGFW | 3.10 \(\times 10^{-4}\) |

\(a\) Amino acid residues are indicated by the single-letter code.

\(b\) The relative potency of a peptide analog to inhibit the growth of \(L.\) ivanovii is expressed as the ratio (MIC for mesentericin Y 105\(^{37}\)/MIC for the tested peptide) \(\times 100\).

**DISCUSSION**

The present study reported for the first time the complete amino acid sequence, synthesis, and structure-activity relationship of mesentericin Y 105\(^{37}\), an antimicrobial peptide secreted by Gram-positive bacteria \(L.\) mesenteroides. The sequence of mesentericin Y 105\(^{37}\) is almost identical to that of the 36-residue peptide mesentericin \(Y\) 105 isolated previously by Héchard et al. (24), except for the presence of an extra tryptophan residue at the COOH terminus of the 37-mer version. This difference is more than trivial because mesentericin \(Y\) 105\(^{37}\) is 10,000-fold more potent than the 36-mer peptide in inhibiting the growth of selected Gram-positive bacteria. The considerable disparity in biological activity of the two peptides, together with the fact that we have not been able to detect a COOH-terminally truncated form of mesentericin \(Y\) 105 isolated previously by Héchard et al. (24), for the presence of an extra tryptophan residue at the COOH terminus of the 37-mer version (33), pediocins PA-1 and AcH (34, 35), and sakacin A and 674 (36, 37). However, with a few exceptions, the antimicrobial activity of these peptides was determined through examination of growth of \(L.\) mesenteroides roughly similar to that of mesentericin \(Y\) 105\(^{37}\). Moreover, both peptides exhibited a similar spectrum of antimicrobial activity (Table II).

Circular Dichroism Spectra of Mesentericin Y 105\(^{37}\) in Low Polarity Medium—To obtain conformational information on mesentericin Y 105\(^{37}\), CD measurements were performed in either a polar or a helix-promoting media. The far-UV CD spectrum of mesentericin Y 105\(^{37}\) in water was characteristic of nonstructured conformations (Fig. 5). In the presence of 25% of trifluoroethanol, however, mesentericin Y 105\(^{37}\) showed a significant level, i.e., 33%, of helix conformation. 40% helix formation was induced in the presence of 50% TFE. Prediction of the secondary structure of mesentericin Y 105\(^{37}\) according to Chou and Fasman (26) identified 40% helical zone and 60% coil. Tentative localization of the helix indicated a domain spanning residues 17–31. When plotted as an \(\alpha\)-helical wheel, the central \(\alpha\)-helix of mesentericin exhibited clearly distinguishable hydrophobic and hydrophilic domains (Fig. 6). In this conformation, there are 6 hydrophilic or charged residues on one side of the cylindrical surface and 8 hydrophobic residues with no charged residue on the opposite side. The polar face of the helix subtends an average angle of 150° perpendicular to the long axis of the helix. Hence, both theoretical predictions and CD measurements suggest that the central portion residues 17–31 of mesentericin Y 105\(^{37}\) adopt an amphipathic helical structure in low polarity medium.
of the total inhibitory activity of the producer strain when grown on agar medium or by testing semi-purified bacteriocin preparations against only a few bacterial species. In addition, none of these bacteriocins has been chemically synthesized and assayed. It is increasingly acknowledged that crude bacteriocin preparations and highly purified bacteriocins often differ markedly in their ability to inhibit microbial proliferation. This may be due either to the presence of bacteriocin inhibitors in liquid medium or to the secretion of several bacteriocins by a single bacterium, each bacteriocin targeting specific microorganisms. In that regard, the antibacterial activity of synthetic mesentericin Y 105<sup>37</sup> was evaluated against Gram-positive and Gram-negative microorganisms. Synthetic mesentericin Y 105<sup>37</sup> was not active against Gram-negative bacteria. On the other hand, it was inhibitory to growth of a variety of related Gram-positive cocci and rods in the genera <i>Lactobacillus</i> and <i>Carnobacterium</i> (Table II). However, inspection of the tabulated values revealed complex patterns of antimicrobial potencies. For instance, although mesentericin Y 105<sup>37</sup> is very potent at inhibiting the proliferation of the bacterium <i>L. paramesenteroides</i> (MIC = 38 nM), it is 20-fold less active against <i>Lactobacillus curvatus</i> (MIC = 708 nM), a Gram-positive rod belonging to the same family. Conversely, within the genus <i>Lactobacillus</i>, mesentericin Y 105<sup>37</sup> was highly efficient at inhibiting the growth of <i>L. sake</i> (MIC = 37 nM). Most interestingly, the peptide was inhibitory to the growth of <i>L. ivanovii</i> (MIC = 34 nM).

The antimicrobial spectrum of activity of synthetic leucocin A, a 37-residue bacteriocin isolated from <i>L. gelidum</i> that differs from mesentericin Y 105<sup>37</sup> only at two sites (positions 22 and 26; Table IV), is reported in Table II. Although comparative analysis of several reports (24, 27, 38, 39) showed considerable disparity in biological activity of mesentericin Y 105<sup>37</sup> and leucocin A, synthetic replicates of these peptides were found to be indistinguishable at inhibiting the growth of selected Gram-positive bacteria. This finding adds further support to the proposal that synthetic replicates of bacteriocins should be used instead of cell-free culture supernatants or semi-purified preparations to delineate their precise biological spectrum.

The propensity of small-sized cationic peptides to form helical amphipathic structures in apolar medium has been proposed as a prerequisite for their membrane disrupting activity (1, 2, 40). Accordingly, both theoretical predictions and CD measurements suggest that residues 17–31 of mesentericin Y 105<sup>37</sup> can form a nearly perfect amphipathic helix in hydrophobic medium. At the NH<sub>2</sub>-terminal side of the putative helix, the 6-membered disulfide loop linking Cys<sup>5</sup> and Cys<sup>14</sup> should impose a compact local structure. Interestingly, breaking the
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**TABLE IV**
Amino acid sequence of class II bacteriocins produced by lactic acid bacteria

| Bacteriocin | Amino acid sequence | Similarity score (%) |
|------------|---------------------|----------------------|
| Mesentericin Y105\textsuperscript{37} | -KYYGNGVHCTKXCSVNVNGGAS-----NIRHLKAN-------GONGFW----- | 100 |
| Leucocin A | -KYYGNGVHCTKXCSVNVNGGAFS-----AGVHLRAN-------GONGFW----- | 94.5 |
| Sakacin A | ARSYAGYGVCKNFRKXSVXGQI/SIGMIGWAS-----GLAGM----- | 38 |
| Sakacin 674 | -KYYGNGVCGKXSCVTWGAFAQNSGIGNANAWATG-------GNAGNK----- | 45 |
| Pediocin PA-1 | -KYYNGVCTKXCSVNBGQATTCIINMGMAWATG-----GHQGNNK | 46 |
| Carnobacteriocin B2 | V-NSYNGV/NXSKTXCSVNVQGAPQRYRTAGINSFYGVSQASSQAGCIGGRP- | 41 |

The sequences are aligned to demonstrate the most conserved amino acids. The numbering is indexed at the bottom of the longest of the bacteriocins. Multiple sequence alignments were performed by using CLUSTAL V multiple sequence alignment software (43) with a fixed gap penalty. Identical (*) and similar (·) residues among sequences are indicated. The similarity scores for pairwise sequence alignments with mesentericin Y 105\textsuperscript{37} were calculated by the method of Wilbur and Lipman (44).

The NH\textsubscript{2} terminus Lys-Tyr-Tyr, the local constraint imposed by the disulfide bond by substituting or derivatizing the cysteiny residues induced a significant decrease of the peptide helical content (Fig. 5). This may argue in favor of a structural inter-action between the disulfide loop and the helix.

A comparison of the primary structure of mesentericin Y 105\textsuperscript{37} with that of several other class II bacteriocins that inhibit the growth of Listeria sp. is presented in Table IV. All the peptides are 30–49 residues long, are cationic, and contain a consensus sequence Tyr-Gly-Asn-Gly-Val-Xaa-Cys (residues 3–9 in mesentericin Y 105\textsuperscript{37}) at their NH\textsubscript{2} termini. This consensus sequence has been suggested to be important for the activity and specificity against Listeria of this group of bacteriocins. With the aim of checking for this hypothesis and identifying for the first time the structural and conformational determinants leading to activity of class II bacteriocins against Listeria, a series of 13 mesentericin Y 105\textsuperscript{37} analogs were tested for their potency to inhibit the proliferation of L. ivanovii (Table III). The results showed that the NH\textsubscript{2}-terminal tripeptide Lys\textsuperscript{4}-Tyr-Tyr\textsuperscript{5} is essential for activity, mesentericin Y 105\textsuperscript{37}-[4–37] derivative being virtually inactive. Similarly, the COOH-terminal nonapeptide Leu\textsuperscript{39}-Ala-Asn-Gly-Gly-Asn-Gly-Phe-Trp\textsuperscript{39}, and especially the tryptophan residue at the carboxyl end, are mandatory for activity; whereas the 36-residue version is only marginally active (MIC = 6 \(\mu\)m), the 1–28 derivative is devoid of activity. Thus, the NH\textsubscript{2}-terminal sequence 1–3 of mesentericin Y 105\textsuperscript{37} rather than the consensus sequence spanning residues 3–9 is mandatory but not sufficient for conferring activity to the peptide. Removal of both the 6-membered disulfide loop and the central helical domain of mesentericin Y 105\textsuperscript{37} yielded a compound, mesentericin Y 105\textsuperscript{37}-[1–8]-[28–37] with no activity. Excision of the central helical domain of mesentericin Y 105\textsuperscript{37} to give mesentericin Y 105\textsuperscript{37}-[1–14]-[28–37] also yielded an inactive derivative (Table III). Also, substitution of Cys\textsuperscript{9} and Cys\textsuperscript{14} by Ser abolished antimicrobial potency. Taken together, these results strongly support the view that the entire chain length of mesentericin Y 105\textsuperscript{37} is required for anti-Listeria activity. It thus appears that the COOH-terminal tryptophan residue is each requested for giving full potency to the peptide against Listeria and act in a cooperative manner.

Although the precise mechanism of the action of class II bacteriocins remains to be defined, these peptides appear to act by perturbing the barrier function of membranes (12), thereby resembling helical antimicrobial peptides that are produced by vertebrate animals (1, 2). It is, however, noteworthy that structural determinants imparting activity to the two classes of peptides differ. Whereas the molecular elements responsible for the antimicrobial potency of 23–34-residue-long vertebrate antimicrobial peptides, such as the magainins and the derma-septins, are to be traced to the NH\textsubscript{2}-terminal helical segment spanning residues 1–15 to 1–20 of these molecules (41, 42), the entire chain length of mesentericin Y 105\textsuperscript{37} is requested for activity. Moreover, vertebrate membrane-active peptides are endowed with broad spectrum antimicrobial activity, being active against Gram-positive and Gram-negative bacteria, yeast, fungi, and protozoa (1, 2), whereas mesentericin Y 105\textsuperscript{37} and related bacteriocins have narrower spectrum of activity. Our study suggested that mesentericin Y 105\textsuperscript{37} exists as a random coil in water but assumes that the central region of the peptide adopts a defined disulfide loop-hinge-amphipathic helix conformation in low polarity environment, which mimics the lipophilicity of the membrane of a target organism. This may suggest an evident role for the putative \(\alpha\)-helical domain of mesentericin Y 105\textsuperscript{37} in interacting with lipid bilayers, leading subsequently to alterations of the membrane functions, whereas the NH\textsubscript{2}-terminal domain residues 1–14 and possibly the COOH terminus may form part of a recognition structure for a membrane bound protein "receptor" or anionic cell surface polymers like teichoic acid and lipoteichoic acid, which may be critical for peptide targeting. Evidence for the existence of bacteriocin receptor proteins in the membranes of sensitive bacteria have been recently reported (12).

Mesentericin Y 105\textsuperscript{37} is a member of a small but growing family of bacterial defensive peptides that are of the utmost interest to the food fermentation industry because they inhibit the growth of food-borne pathogenic microorganisms during food processing. In addition, these peptides also exert inhibitory action against microorganisms that cause food spoilage. The present study demonstrated that mesentericin Y 105\textsuperscript{37} is easy to synthesize at low cost. Thereby, it may represent a useful and highly tractable tool for identifying key features responsible for membrane permeabilization and a starting point for the design of more potent structural analogues that may be of potential applicability in food preservation.

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