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Purification of the Bacteriocin Bavaricin MN and Characterization of Its Mode of Action against *Listeria monocytogenes* Scott A Cells and Lipid Vesicles†

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Bavaricin MN was purified from *Lactobacillus sake* culture supernatant 135-fold with a final yield of 11%. Sequence analysis revealed bavaricin MN to be a 42-amino-acid peptide having a molecular weight of 4,769 and a calculated pI of 10.0. Computer analysis indicated that the C-terminal region may form an α-helical structure with an amphipathic nature deemed important in the interaction of bacteriocins with biological membranes. Bavaricin MN rapidly depleted the membrane potential (Δp) of energized *Listeria monocytogenes* cells in a concentration-dependent fashion. At a bavaricin MN concentration of 9.0 μg/ml, the Δp decreased by 85%. Both the electrical potential (ΔΨ) and *Z*ΔpH components of the Δp were depleted, and this depletion was not dependent on a threshold level of proton motive force. In addition to studying the effect of bavaricin MN on the Δp of vegetative cells, bavaricin MN-induced carboxyfluorescein (CF) efflux from *L. monocytogenes*-derived lipid vesicles was also characterized. Bavaricin MN-induced CF leakage was also concentration dependent with an optimum of pH 6.0. The rate of CF efflux was 63% greater in lipid vesicles in which a ΔΨ was generated compared with that in lipid vesicles in the absence of a ΔΨ.

Many antimicrobial peptides that interact with and permeabilize biological membranes have been identified in a great variety of organisms, including mammals (30), plants (42, 45), insects (28), amphibians (8), and prokaryotes (39). A great deal of interest has recently been focused on antimicrobial peptides isolated from lactic acid bacteria because of their well-documented inhibitory activity against many important food pathogens (24, 27, 33). These antimicrobial peptides or bacteriocins are defined as ribosomally synthesized proteins, which may be posttranslationally modified, that have inhibitory properties against some bacteria but are not active against the cells which produce them. They vary greatly in size and sequence homology but tend to be small cationic peptides of between 20 and 60 amino acids with amphipathic characteristics and high isoelectric points.

The bacteriocins of the lactic acid bacteria have most recently been classified by Klaenhammer (27), and they fall into four basic groups. Class I, the lantibiotics, includes lanthionine-containing bacteriocins such as nisin. Class II, small bacteriocins that do not contain lantionine residues, includes bacteriocins that contain a Gly-Gly protease-processing site between the mature peptide and the prepeptide. This class is further subdivided into bacteriocins containing the N-terminal consensus sequence Tyr-Gly-Asn-Gly-Val (IIa), bacteriocins requiring two different peptides for activity (IIb), and peptides requiring reduced cysteine residues for activity (IIc). Class III bacteriocins are large antimicrobial proteins (>30 kDa), and class IV includes complex bacteriocins containing carbohydrate or lipid components that are required for their activity. Although extensive biochemical and genetic studies have been conducted on many class I and II bacteriocins, the interaction of these bacteriocins with biological membranes and the sequence of cytotoxic effects that follow are poorly understood.

Previously we described the production kinetics in chemostat fermentations of bavaricin MN, a class IIa bacteriocin produced by *Lactobacillus sake* MN (26). Here we describe the purification, amino acid sequence analysis, and characterization of bavaricin MN. Data obtained both in vivo and in vitro indicate that the mode of action of bavaricin MN may follow a mechanism whereby the presence of a proton motive force (PMF) enhances the action of this bacteriocin. A general model for this mode of action is presented.

MATERIALS AND METHODS

Bacterial cultures and growth media. *L. sake* MN was isolated and originally identified as *Lactobacillus bavaricus* MN (31). Recent taxonomic studies have reclassified *L. bavaricus* as *L. sake* (25). Stock cultures were maintained as previously described (26). All cultures were grown at 30°C.

A strain of *Listeria monocytogenes* Scott A having plasmid-mediated resistance to chloramphenicol and erythromycin (10) was used as the indicator organism for bacteriocin quantification in a differed-antagonism assay as previously described (26), except that stock cultures were stored in brain heart infusion (Difco Laboratories, Detroit, Mich.) supplemented with 0.6% yeast extract (Difco), 20% glycerol, and 3.5-μg/ml final concentrations of chloramphenicol and erythromycin (Sigma Chemical Co., St. Louis, Mo.). All cultures were grown at 30°C in brain heart infusion broth supplemented with yeast extract and antibiotics as indicated above.

Bacteriocin assay. Samples were centrifuged at 13,000 × *g* for 10 min in a microcentrifuge to obtain cell-free supernatant. Bavaricin MN activity was determined by determining titers of twofold dilutions for activity against *L. monocytogenes* in a spot-on-the-lawn assay as previously described (26). The units of activity were defined as the recipricol of the highest dilution having a detectable zone of inhibition and were expressed as arbitrary units (AU) per milliliter.

Protein concentration determination. Protein concentrations were obtained by a modified Bradford method (3) (Coomassie Plus assay; Pierce, Rockford, Ill.) as described in the manufacturer's instructions, using bovine serum albumin as the standard.

Purification of bavaricin MN. (i) Ammonium sulfate precipitation. Cell-free supernatant (900 ml) obtained from a continuous-culture fermentation, described more fully elsewhere (26), was gradually brought to 60% saturation with ammonium sulfate and held overnight at 4°C. The precipitate was collected by centrifugation (20 min at 14,000 × *g*) and resuspended in 50 mM sodium *N*-morpholinoethanesulfonic acid (MES), pH 6.0. The resuspended precipitate

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TABLE 1. Purification of bavaricin MN

Sample	Total activity (10^3 AU)	Total protein (mg)	Protein concn (mg/ml)	Sp act (AU/mg)	Fold purification	Yield (%)
Culture supernatant	720	2,790	3.1	258	1	100
$(NH_4)_2SO_4$ precipitate (60% saturation)	870	68	2.0	12,800	50	121
Q Sepharose (anion exchange)	441.6	10.4	0.15	42,462	165	61
SP Sepharose (cation exchange)	76.8	2.2	0.18	34,909	135	10.7

was dialyzed in dialysis tubing (Spectrum; molecular weight [MW] cutoff, 2,500) at 4°C versus 5.0 M urea (enzyme grade; Fisher Scientific Co., Pittsburgh, Pa.) in 50 mM Na-MES, pH 6.5.

(ii) Anion-exchange chromatography. The dialyzed, ammonium sulfate-precipitated bavaricin MN sample was applied to a column (5.0-cm diameter) containing Q Sepharose fast-flow resin (143-ml bed volume; Pharmacia Biotechnology, Piscataway, N. J.) at 4°C. The column was equilibrated and run in 50 mM Na-MES, pH 6.5, plus 5.0 M urea at a linear flow rate of 5.0 cm^2/h. The fractions containing protein that was not retained on the column (as monitored by A_{280}) were assayed for bavaricin MN activity and pooled.

(iii) Cation-exchange chromatography. The pooled anion-exchange sample was applied to a column containing SP Sepharose fast-flow resin (2.6-cm diameter, 40-ml bed volume; Pharmacia Biotechnology) at 4°C. The column was equilibrated and run in 50 mM Na-MES, pH 6.5, containing 5.0 M urea at a flow rate of 5.0 cm^2/h. The activity was eluted stepwise from the column with one column volume of 50 mM Na-MES, pH 6.5, containing 0.5 M NaCl. The activity of each fraction was determined, and active fractions were pooled.

The pooled fractions were dialyzed against 20 mM Na-MES, pH 6.0, at 4°C and concentrated by repeatedly coating the dialysis tubing with polyethylene glycol (Sigma; average MW, 10,000) between changes of the dialysis buffer. The resulting purified bavaricin MN sample was stored at 4°C. This sample was further concentrated, as needed, in a vacuum centrifuge to obtain the desired level of activity.

(iv) SDS-PAGE. Discontinuous sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a 16.5% acryamide gel as described by Schägger and von Jagow (41). Samples were run at a constant current of 10 mA per 0.75-mm gel until the dye front entered the spacer region of the gel. The current was then increased to 15 mA per gel and run for 16 h.

Gels were silver stained (Bio-Rad Laboratories, Hercules, Calif.) by using a slightly modified version of the supplier's instructions. Each gel was fixed with 40% methanol–10% acetic acid–10% glutaraldehyde for 30 min followed by 15 min in the presence of 7% acetic acid and 5% methanol. Finally, the gel was washed for 15 min in the presence of deionized water prior to the addition of the silver stain oxidizing solution. The rest of the staining procedure was followed exactly as described by the manufacturer.

To verify the antimicrobial nature of the single silver-stained band, a direct detection method similar to that described by Bhunia et al. (2) was used. After the initial fixation step (40% methanol, 10% acetic acid, 10% glutaraldehyde) the gel was cut in half, leaving identical marker and sample lanes on each half. The unstained half was washed in deionized water, with frequent changes, for 30 min and overlaid with agar that had been seeded with *L. monocytogenes* Scott A. The plate was incubated overnight at 4°C and then overnight at 30°C.

Edman degradation N-terminal sequencing. A sample of purified bavaricin MN was submitted to the sequencing facilities at Harvard Microchemistry (Harvard University, Cambridge, Mass.). N-terminal sequence analysis was performed by Edman degradation on a G1000A Hewlett Packard Sequencer with an on-line 1090 high-pressure liquid chromatograph. The sample was prepared in a 5% trifluoroacetic acid–H_2O solution, and an appropriate amount was injected on to the sequencer.

The MacDNASIS computer program (version 3.0; Hitachi) was used to predict the secondary structure of the resulting amino acid sequence and was also used to calculate the pI of bavaricin MN.

Determination of the PMF, pH gradient, and $\Delta\Psi$. *L. monocytogenes* Scott A was grown to mid-log phase (A_{660}, 0.6 to 0.8) at 30°C with shaking (150 rpm) in brain heart infusion broth containing 0.6% yeast extract. The cells were collected by centrifugation (15 min at 20,845 × *g*), washed once with phosphate-buffered saline (0.8% NaCl, 5.0 mM KCl, 20 mM K_2HPO_4-KH_2PO_4 buffer, pH 7.0), and resuspended to an A_{660} of 1.0 in 50 mM Na-MES buffer containing 10 mM $MgSO_4$ and 10 mM KCl at the indicated pH. In experiments in which valinomycin was used, the KCl concentration was changed to 100 mM.

The pH potential and electrical potential ($\Delta\Psi$) which compose the proton motive force (PMF) were determined as previously described (5) except as indicated otherwise. Bavaricin MN, in 50 mM MES, pH 6.0, was added to appropriate tubes at the desired concentrations after the addition of the radiolabeled probes and was incubated for 30 min at room temperature. In experiments in which ionophores were used, glucose-energized cells were incubated for 30 min at room temperature in the presence of the ionophore prior to the addition of the radiolabeled probes. Valinomycin was added to a final concentration of 5.0 μM (in 95% ethanol). Nigericin was added to a final concentration of 2.0 μM (in 95% ethanol). Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was used at a final concentration of 200 μM (in 95% ethanol). Appropriate controls excluded any effect of ethanol. An internal cell volume of 1.8 μl/mg of cells (dry weight) for *L. monocytogenes* Scott A was used in the calculation of the pH potential and $\Delta\Psi$ (4).

Phospholipid vesicle formation and CF entrapment. The isolation of lipids from *L. monocytogenes* Scott A cell membranes and preparation of large unilamellar vesicles were as previously described (46, 47) with modifications. The isolated lipids were resuspended in appropriate-pH 50 mM Na-MES buffer containing 50 mM 5(6)-carboxyfluorescein (CF; Sigma) and a 14:1 molar ratio of *n*-octyl β-D-glucopyranoside (Sigma) to phospholipid (final lipid concentration, 6 to 10 mg/ml). An average molecular weight of 1,000 g per mol of phospholipid was assumed for the calculation of the molar ratio of detergent to phospholipid. This average is based on the phospholipid composition of the *L. monocytogenes* Scott A membrane (47). The lipids were thoroughly resuspended, and the solution was run on a Sephadex G-50 superfine gel filtration column (0.7 by 10 cm). The column had been previously equilibrated with a buffer of an appropriate pH. Fifty millimolar CF in the same buffer was added to the top of the column prior to loading the lipid mixture in order to enhance CF entrapment. Lipid vesicles eluted with the void volume and were collected in 1.0-ml fractions. The eluted lipid vesicles were stored on ice until used.

CF release assay. The release of CF from lipid vesicles was monitored by observing the increase in fluorescence of the lipid vesicle solution with a spectrofluorometer (model F1T11; Spex Industries; Metuchen, N. J.). The procedures were modified from those described by Winkowski (46). The CF-loaded lipid vesicle solution was monitored at an emission wavelength of 516 nm and an excitation wavelength of 490 nm. Excitation and emission slit widths were set to 0.8 nm. The effect of bavaricin MN on CF-loaded lipid vesicles was monitored by adding various concentrations of bavaricin MN in 50 to 100 mM Na-MES, pH 6.0. The assay was performed in a stirred cuvette at room temperature with 2.0 ml of assay buffer at an appropriate pH and 20 μl of the lipid vesicle solution. The assay was monitored for 600 s. Maximal CF leakage was determined by the addition of Triton X-100 (final concentration, 0.1% by volume). The intrinsic leakage of CF in the absence of other compounds increases at low pH because CF becomes protonated and is therefore more lipophilic (36). To correct for this pH-dependent leakage in the pH-dependent studies, efflux curves in the presence of 50 mM MES at the appropriate pH were subtracted from curves obtained in the presence of bavaricin MN at a given pH. Rates of efflux (percent per minute) were calculated from the slope of a tangent to the efflux curve at 100 s after the addition of bavaricin MN.

Effect of Δp on MN-induced CF release. A membrane potential (Δp) was generated in CF-loaded lipid vesicles by a valinomycin-mediated outwardly directed potassium diffusion gradient. Lipid vesicles were prepared in Na-MES buffer (pH 6.0) containing 5.0 mM KH_2PO_4. After elution from the gel filtration column, the vesicles were centrifuged at 30,000 × *g* and washed once in either 5.0 mM KH_2PO_4 or 50 μM KH_2PO_4 and resuspended to a final volume of 1.0 ml in the buffer in which they were washed. This created a theoretical $\Delta\Psi$ across the lipid vesicle membrane of either 0 or 120 mV (inside negative).

The lipid vesicles were stored on ice for up to 1.5 h. The CF release assay was performed as described above except that 5 μM valinomycin (in 95% ethanol; Sigma) was added to the buffer-lipid vesicle mixture 10 s prior to the addition of controls, Trition X-100, or bavaricin MN. The assay was monitored for 600 s. Rates of efflux were calculated as described above.

Nucleotide sequence accession number. The amino acid sequence resulting from Edman degradation N-terminal sequencing was submitted to the European Bioinformatics Institute for inclusion in the SWISS-PROT protein sequence database and was given the accession number P80493.

RESULTS AND DISCUSSION

Bavaricin MN purification. Bavaricin MN was purified 135-fold with a final recovery of about 11% of the initial activity (Table 1). Ammonium sulfate precipitation resulted in a 50-fold increase in the specific activity following dialysis in the presence of urea. After the amino acid sequence was obtained,

TABLE 2. Amino acid sequence comparison of nonlantibiotic bacteriocins of gram-positive bacteria

Bacteriocin	Sequence[a]				
	10	20	30	40	50
Bavaricin MN	TKYYGNGVYx	NSKKxWVDWG	QAAGGIGQTV	VxGWLGGAIP	GK
Bavaricin A[b]	KYYGNGVHx	GKHSxTVDWG	TAIGNIGNNA	AANxATGxNA	GG
Leucocin A[b]	KYYGNGVHC	TKSGCSVNWG	EAFSAGVHRL	ANGGNGFW	
Mesentericin Y105[b]	KYYGNGVHC	TKSGCSVNWG	EAASAGIHRL	ANGGNGF	
Pediocin PA-1[b]	KYYGNGVTC	GKHSCSVDWG	KATTCIINNG	AMAWATGGHQ	GNHKC
Sakacin P[b]	KYYGNGVHC	GKHSCTVDWG	TAIGNIGNNA	AANWATGGNA	GWNK
Sakacin A	ARSYGNGVYC	NNKKCWVNRG	EATQSIIGGM	ISGWASGLAG	M
Carnobacteriocin B2[b]	VNYGNGVSC	SKTKCSVNWG	QAFQERYTAG	INSFVSGVAS	GAGSIGRRP
Lactacin F	RNNWQTNVGG	AVGSAMIGAT	VGGTICGPAC	AVAGAHYLPI	LWTGVTAATG GFGKIRK
Carnobacteriocin A	DQMSDGVNYG	KGSSLSKGGA	KCGLGIVGGL	ATIPSGPLGW	LAGAAGVINS CMK
Lactococcin A	KLTFIQSTAA	GDLYYNNTNT	HKYVYQQTQN	AFGAAANTIV	NGWMGGAAGG FGLHH
Lactococcin M	IRGTGKGLAA	AMVSGAAMGG	AIGAFGGPVG	AIMGAWGGAV	GGAMKYSI

[a] Significant regions of homology are underlined.
[b] Starts at position 2 to maximize homology matching.

a purification protocol was designed to take into account the fact that this bacteriocin does not contain histidine (Table 2). Application of the ammonium sulfate-precipitated sample to a anion-exchange column and pooling of the fractions containing protein that did not bind to the column resulted in an about 3-fold increase in the purification over that in the ammonium sulfate precipitation step and a 165-fold increase over that in the original culture supernatant sample. The pooled fractions from the anion-exchange column were directly applied to the cation-exchange column. Despite a slight reduction in the specific activity in this final purification step, other contaminating proteins were present prior to this final column, and only after the cation-exchange column step was a single band observed on silver-stained SDS-PAGE gels.

Following ammonium sulfate precipitation, the sample was applied to an anion-exchange resin equilibrated at pH 6.5. This pH results in more histidine-containing proteins binding to the positively charged resin. Given the highly cationic nature of bavaricin MN, it passed through this column with minimal interaction with the resin. On the cation-exchange column, bavaricin MN binds to the resin while other contaminating proteins do not interact with the column. In practice, small amounts of bavaricin MN activity bound to the anion-exchange resin and eluted off with buffer containing NaCl. This is most likely due to the highly hydrophobic nature of bacteriocins, which is a general characteristic of bacteriocins (22, 35).

Dialysis of the ammonium sulfate-precipitated bavaricin MN in the presence of 5.0 M urea greatly increased the amount of activity recovered from the fractions containing bacteriocin activity following application of the sample to the Q Sepharose fast-flow anion-exchange column. In the absence of urea, bavaricin MN activity was observed in most fractions from either the cation- or anion-exchange column. The observation that a majority of bavaricin MN activity eluted from a gel filtration column (Sephacryl S-200 HR; 5,000- to 250,000-MW exclusion limit) in the void volume indicates that this peptide aggregates extensively with other proteins and/or itself. The addition of 5.0 M urea to all of the chromatography buffers used in the bavaricin MN purification greatly decreased this aggregation. Similar evidence has led other investigators to erroneously conclude that their bacteriocins were of high MW or existed as multimers (34). Garver and Muriana (14) suggested that the large antimicrobial complexes observed during the purification of curvaticin FS47 are also due to Tween 80-protein micelles. Ultrafiltration experiments have also supplied evidence that aggregation in bacteriocin purifications is very common (35).

Analysis of purified bavaricin MN by silver-stained SDS-PAGE revealed a single band corresponding to an apparent MW of 2,692 (Fig. 1). An identical gel overlaid with agar containing *L. monocytogenes* Scott A revealed a zone of inhibition corresponding to the appropriate lane and centered at the expected R_f value (as determined from the silver-stained gel).

However, this MW does not correspond to the calculated MW based on the amino acid sequence. Discrepancies in bacteriocin molecular masses are also frequently seen with MW determination by SDS-PAGE. Factors such as variable binding of SDS, a high degree of basic amino acid residues, and peptide structure may all contribute to the variable migration of small peptides in SDS-PAGE (20, 35).

N-terminal sequencing and sequence analysis. The amino acid sequence of bavaricin MN was obtained by Edman deg-

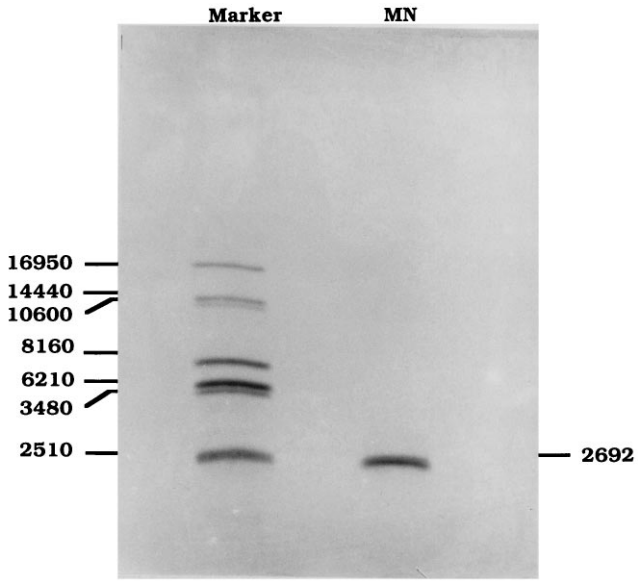

FIG. 1. SDS-PAGE of purified bavaricin MN. Marker lane, myoglobin-derived low-MW standards of the indicated MWs. MN lane, pure bavaricin MN sample.

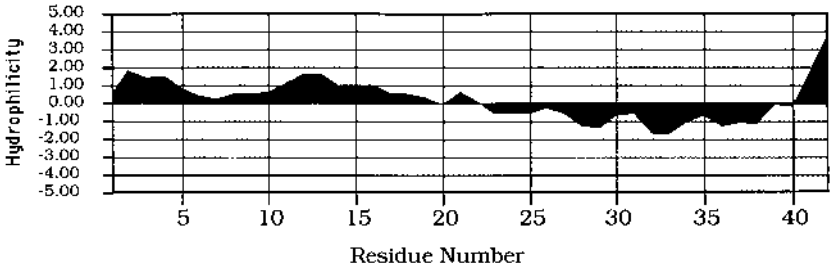

FIG. 2. Hydrophobicity plot of amino acid residues in bavaricin MN by using the Kyte-Doolittle algorithm.

radation (Table 2). Bavaricin MN is 42 amino acids in length with a calculated MW of 4,769. No unusual amino acid residues, such as didehydroalanine or didehydrobutyrine, were found. This indicates that bavaricin MN does not belong to the lanthionine-containing group of bacteriocins.

The N-terminal region of bavaricin MN contained the Lys-Tyr-Tyr-Gly-Asn-Gly-Val consensus region common to several other class IIa bacteriocins (27), including bavaricin A (29), leucocin A (17), pediocin PA-1 (20), sakacin A (21), sakacin P (43), mesentericin Y105 (19), and carnobacteriocin B2 (40) (Table 2). Other regions with some homology include valine at position 17, tryptophan at position 19, glycine at position 20, and alanine at position 22 (Table 2). The cysteine residues have not been positively identified at positions 10 and 15 in both bavaricin MN and bavaricin A (Table 2). Nonetheless, these amino acids may be cysteine, given the high degree of homology to cysteines at the same position in other class IIa bacteriocins and the fact that cysteine residues are not normally identified by N-terminal Edman degradation.

Unlike the other seven class IIa bacteriocins cited above, the first amino acids in bavaricin MN are a threonine followed by a lysine. Bavaricin MN is the only one of these eight bacteriocins that contains the entire N-terminal consensus sequence and does not start with lysine. Sakacin A and carnobacteriocin B2 are interesting in that they have a slightly truncated N-terminal consensus region having neither the initial lysine nor the first of the two tyrosines. The N-terminal Lys-Tyr-Tyr-Gly-Asn-Gly-Val consensus sequence has been coined the listeria-active region by Klaenhammer (27), since most bacteriocins with this sequence are inhibitory to listerias. However, curvaticin FS47 does not contain this sequence and is active against *L. monocytogenes* (13). Hence, the function of the N-terminal consensus sequence remains to be elucidated.

Bacteriocins also have very high isoelectric points. Based on the amino acid sequence of bavaricin MN, the calculated isoelectric point is 10.0. This is consistent with other bacteriocins such as pediocin PA-1 (9.6), leucocin A (9.5), sakacin A (10.0), lactacin F (11.3) and lactococcin M (10.2).

Bavaricin MN has a significant region of hydrophobicity between amino acids 22 and 39 (Fig. 2). By analogy to predictions made for lactococcin A (21), lactococcin G (37), and sakacin A (22), this region may form an α-helical structure. These structures have been used to support the hypothesized formation of barrel-and-stave poration complexes after the model of Ojcious and Young (38). However, bavaricin MN has a high (33%) glycine content in this region, and lactococcin A, lactococcin G, and sakacin A (22) have similarly high (25 to 33%) glycine contents in the predicted helical region. Such a high glycine content would give these small molecules a high degree of conformational freedom not found in the gamma toxin and alamethicin (which contain a single glycine) used to develop the barrel-and-stave model (38). Thus, the use of the barrel-and-stave model to describe bacteriocin poration complexes is still quite speculative. The predicted α-helical region of sakacin A is 16 residues in length. Assuming 1.5 Å (0.15 nm) per residue, the length of the α-helical region of sakacin A would be 24 Å (2.4 nm). This would be too short to span the phospholipid bilayer, which has a thickness of about 30 Å (3.0 nm) (15). Lactococcin A has a proposed 20-amino-acid α-helical region of 30 Å, allowing it to span the phospholipid bilayer. The length of the putative α-helical region in bavaricin MN is more than 30 Å and could also span the phospholipid bilayer. Chen et al. (6) have demonstrated that synthetic peptides with enhanced helical contents have increased antimicrobial activity, suggesting the importance of α-helical structures to the antimicrobial nature of these peptides.

Mode of action of bavaricin MN. Nisin, pediocin PA-1, leuconocin S, and lactacin F deplete the Δp of energized *L. monocytogenes* Scott A cells in a concentration-dependent manner, and the depletion of the PMF is a common mechanism of action of all bacteriocins (5). Bavaricin MN depleted the PMF of energized cells of *L. monocytogenes* Scott A (Fig. 3). At pH 6.0, a bavaricin MN concentration of 3.0 μg/ml caused total depletion of the pH gradient ($Z\Delta$pH) component of the total PMF and a 62% decrease in the total Δp from the control value of 144 mV (Fig. 3). A further increase in the bavaricin MN concentration to 9.0 μg/ml caused an additional 23% reduction in the total PMF from 50 to 20 mV.

The influence of various ionophores on the bioenergetics of energized *L. monocytogenes* Scott A in the presence and absence of bavaricin MN, at pH 6.0, was investigated. The addition of valinomycin, which equilibrates the potassium ion con-

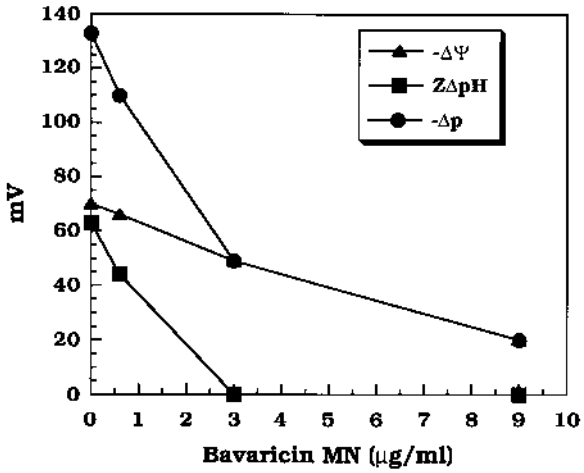

FIG. 3. Concentration dependence of bavaricin MN action on energetic parameters of glucose-energized *L. monocytogenes* Scott A cells at pH 6.0.

TABLE 3. Effect of bavaricin MN and ionophores on energized *L. monocytogenes* Scott A cells at pH 6.0

Treatment[a]	$-\Delta\Psi$ (mV)	$Z\Delta$pH (mV)	$-\Delta p$ (mV)
Control	68	76	144
Bavaricin MN	26	0	26
Valinomycin	0	90	90
Nigericin	123	0	123
Valinomycin + bavaricin MN	3	0	3
Nigericin + bavaricin MN	26	0	26
CCCP[b]	67	0	67
CCCP + bavaricin MN[b]	40	0	40

[a] Final concentrations: bavaricin MN, 9.0 μg/ml; valinomycin, 5.0 μM; nigericin, 2.0 μM; CCCP, 200 μM.
[b] Values are the averages of duplicate determinations.

centration across the membrane, results in the depletion of the $\Delta\Psi$. The addition of 6.0 μg of bavaricin MN per ml to valinomycin-treated, energized cells resulted in the total depletion of the remaining $Z\Delta$pH (Table 3). The addition of nigericin, which causes the electroneutral exchange of a proton for a potassium ion, eliminated the pH gradient across the *L. monocytogenes* membrane but not the electrical gradient. Bavaricin MN, at a concentration of 6.0 μg/ml, induced a 79% decrease in the $\Delta\Psi$ in the nigericin-treated cells, from 123 to 26 mV (Table 3).

The lipophilic protonophore CCCP was used to determine if bavaricin MN required a threshold level of Δp. CCCP equilibrates the proton concentration across the membrane by moving across the lipid bilayer in its protonated form, thereby depleting the $Z\Delta$pH. CCCP caused a 53% reduction in the total PMF in energized cells of *L. monocytogenes* Scott A (Table 3). The addition of 6.0 μg of bavaricin MN per ml to CCCP-treated cells reduced the total PMF only 27 mV, from 67 to 40 mV. Therefore, bavaricin MN acts in response to membrane potentials well below 100 mV, but the effect is not as dramatic as in the presence of a large Δp. These results are consistent with the effect of other bacteriocins, including nisin, pediocin PA-1, and leuconosin S, on energized *L. monocytogenes* Scott A cells (5).

Bavaricin MN functioned at Δp values ranging from 144 mV (control value) to 67 mV, depleted both the $\Delta\Psi$ and the $Z\Delta$pH, and allowed the passage of protons as well as other charged ions such as potassium. According to Chen and Montville (7), pediocin PA-1 also induces the release of ions such as potassium and inorganic phosphate. Both pediocin PA-1 and leuconocin S function only in the presence of a pH or electrical gradient and can deplete a Δp as low as 42 mV (5). Bavaricin MN, like other class II bacteriocins, depleted low residual PMF values, indicating that it does not require a high threshold Δp to exert its action on sensitive cells. Interestingly, bavaricin MN was unable to totally deplete the in the presence of CCCP. The reduction in the Δp was only 40% of the level in the presence of CCCP alone. Therefore, bavaricin MN appears to occupy a position somewhere between the energy-dependent action of lantibiotics and the energy-independent action of nonlanthionine bacteriocins such as pediocin PA-1. Bavaricin MN acts in a manner that could be described as energy enhanced. However, it remains unclear whether other energy-independent bacteriocins also function in an energy-enhanced fashion.

The release of CF from the lipid vesicles was both time (Fig. 4) and bavaricin MN concentration (35,500 AU/mg) dependent (Fig. 4 and 5). The rate of CF leakage increased with increasing bavaricin MN concentrations (Fig. 5). At a bavaricin

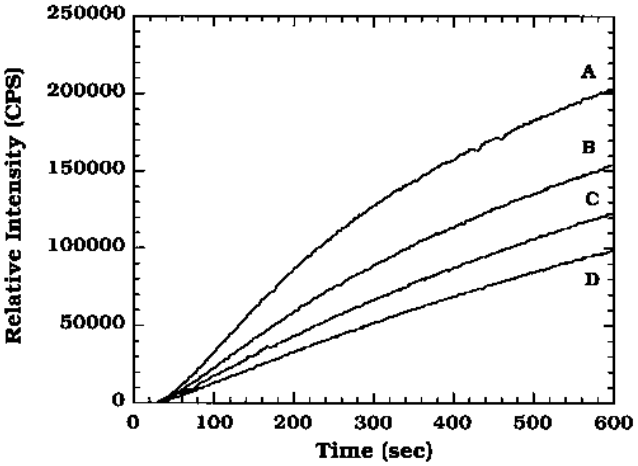

FIG. 4. CF efflux from *L. monocytogenes* Scott A-derived lipid vesicles at pH 6.0 as a function of time and bavaricin MN concentrations of 7.2 (A), 3.6 (B), 1.8 (C), and 0.9 (D) μg/ml.

MN concentration of 7.2 μg/ml, the efflux rate was 6.9%/min and the overall efflux at 600 s was 41%, while at a concentration of 0.9 μg/ml the efflux rate was 2.5%/min and the efflux seen at 600 s was only 20% (Fig. 5). Total relative intensity also increased with time, although the rate of efflux decreased after 300 s (Fig. 5).

The rate of CF efflux was also pH dependent. The rate of MN-dependent efflux of CF from *L. monocytogenes* Scott A-derived lipid vesicles was maximal at pH 6.0 (6.2%/min) compared with that at pH 6.5 (4.2%/min) and pH 5.5 (2.3%/min) (Fig. 6).

The observation that bavaricin MN caused significant CF efflux in lipid vesicles implies that bavaricin MN does not require a receptor protein, as has been suggested for lactococcin A (44) and lactostrepcin 5 (48).

The proposed energy-enhanced mode of action is supported by the in vitro CF efflux data. When a theoretical Δp of 120 mV was generated across the membrane bilayer of CF-loaded vesicles, the efflux rate was 8.8%/min at a bavaricin MN concentration of 7.2 μg/ml at pH 6.0, while in the absence of a Δp, the rate of efflux decreased 63% to 3.3%/min. Garcia-Garcera et al. (12) proposed a similar energy-enhanced mechanism for nisin. They observed nisin-induced CF efflux in the absence of

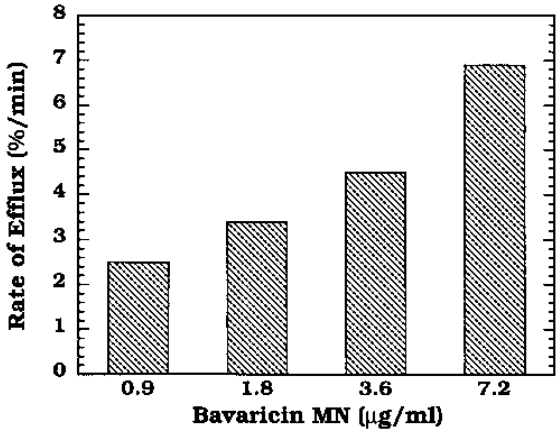

FIG. 5. CF efflux rate as a function of bavaricin MN concentration, at pH 6.0, in lipid vesicles derived from *L. monocytogenes* Scott A.

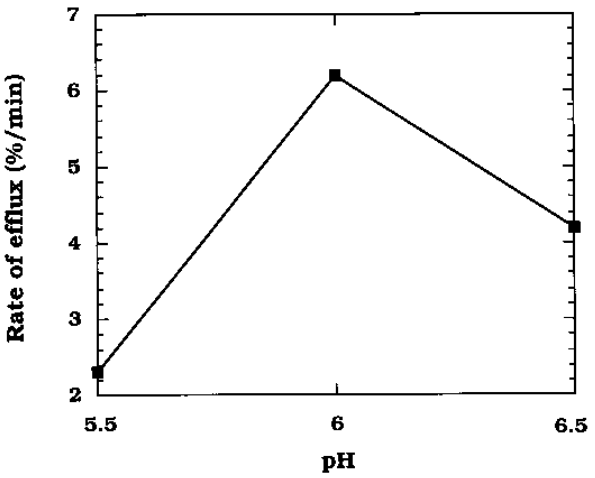

FIG. 6. The effect of pH on bavaricin MN-induced CF efflux in lipid vesicles derived from isolated *L. monocytogenes* Scott A membranes. All values were corrected for pH-induced changes in the diffusion of CF across the lipid bilayer. The final bavaricin MN concentration was 7.2 μg/ml.

a $\Delta\Psi$ or $Z\Delta$pH, while the affinity of nisin for the membrane was not effected. They concluded that a $\Delta\Psi$ was not essential for the action of nisin but that a Δp stimulated the action of nisin. Gallidermin and epidermin pore diameters increase with the application of a Δp, suggesting that the number of inserted bacteriocin molecules increases with increasing Δp (1). This result could also be interpreted as an energy-enhanced mode of action. Gao et al. (11) concluded that the rate of $\Delta\Psi$ dissipation in phosphatidylethanolamine-phosphatidylcholine liposomes increased strongly with the magnitude of the $\Delta\Psi$.

However, Driessen et al. (9) observed that the concentrations of nisin used to dissipate a low $\Delta\Psi$ were 15- to 50-fold higher than the concentrations required to dissipate an imposed $\Delta\Psi$ in phosphatidylcholine-phosphatidylglycerol liposomes. They concluded that the ability of nisin to interact electrostatically with acidic phospholipids, in the absence of a $\Delta\Psi$, may lead to a nonspecific destabilization of the membrane. This result may be different from that observed at lower concentrations of nisin. These results support the conclusion of Huang and Wu (23) and Ludtke et al. (32) that different concentrations of amphipathic peptides may interact with biological membranes in a different fashion.

Amphipathic peptides share three characteristics crucial for their cytotoxic action. First, they must interact with biological membranes. This interaction is affected by membrane phospholipid concentration (9, 12, 15), the pH of the surrounding environment, and possibly the amphipathic nature of the peptide itself (the extent and/or content of the amphipathic regions within an individual molecule). The second factor involved may be the peptide concentration, and the final factor appears to be the presence and magnitude of a Δp. These three factors appear to play a key role in the interaction of all antimicrobial peptides with biological membranes.

We suggest a general model for the mode of action of antimicrobial peptides. (i) Individual peptides bind to the membrane of sensitive cells. This appears to involve an electrostatic interaction between the cationic peptides and the negatively charged phospholipid head groups in most cases. However, evidence does exist for several bacteriocins supporting the presence of specific receptors on the surface of sensitive cells. Membrane composition may be responsible for the specificity of some peptides. (ii) The amphipathic α-helical peptides lay on the membrane with their polar surface interacting with the membrane surface and the apolar surface in contact with the surrounding aqueous environment. (iii) Membrane destabilization then occurs. In conjunction with an electrical and/or chemical gradient, this may serve to reorient the peptide into a more energetically favorable orientation. This orientation could be insertion into the membrane followed by aggregation leading to the formation of a barrel-and-stave poration complex (18, 38). This may also cause a localized aggregation of peptide, leading to a localized disruption of the membrane (15, 16). Either of these situations could be transient. Peptide concentration and the magnitude or presence of a Δp may affect the transient nature of the interaction. Peptide concentration can also effect insertion into lipid bilayers (23, 32). The findings of Garcia-Garcera et al. (12) concerning the requirement of a Δp for nisin action indicate that these ion gradients may influence how the peptides interact with lipid bilayers. The results presented for bavaricin MN are consistent with this model. The finding that the magnitude of action of bavaricin MN on lipid vesicles and whole cells changes with changes in the Δp is particularly interesting. These results could be caused by an increase in poration complex size, an increase in the number of pores, or an increase in localized surface aggregation leading to a greater general disruption of the membrane.

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