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In vitro system for high-throughput screening of random peptide libraries for antimicrobial peptides that recognize bacterial membranes

QIUHONG XIE, SHIGERU MATSUNAGA, ZHESHENG WEN, SETSUKO NIIMI, MIYUKI KUMANO, YOSHIKIYO SAKAKIBARA and SACHIKO MACHIDA*

National Food Research Institute, 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, JAPAN

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Abstract: Antibacterial peptides have been isolated from a wide range of species. Some of these peptides act on microbial membranes, disrupting their barrier function. With the increasing development of antibiotic resistance by bacteria, these antibacterial peptides, which have a new mode of action, have attracted interest as antibacterial agents. To date, however, few effective high-throughput approaches have been developed for designing and screening peptides that act selectively on microbial membranes. *In vitro* display techniques are powerful tools to select biologically functional peptides from peptide libraries. Here, we used the ribosome display system to form peptide-ribosome-mRNA complexes *in vitro* from nucleotides encoding a peptide library, as well as immobilized model membranes, to select specific sequences that recognize bacterial membranes. This combination of ribosome display and immobilized model membranes was effective as an *in vitro* high-throughput screening system and enabled us to identify motif sequences (ALR, KVL) that selectively recognized the bacterial membrane. Owing to host toxicity, it was not possible to enrich any sequence expected to show antimicrobial activity using another *in vitro* system, e.g. phage display. The synthetic peptides designed from these enriched motifs acted selectively on the bacterial model membrane and showed antibacterial activity. Moreover, the motif sequence conferred selectivity onto native peptides lacking selectivity, and decreased mammalian cell toxicity of native peptides without decreasing their antibacterial activity. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: antimicrobial peptide; *in vitro* screening; ribosome display; peptide library; model membrane; phage display

INTRODUCTION

Over the last two decades, antimicrobial peptides (AMPs) have been identified for a wide range of organisms. These peptides belong to a subset of host defense peptides and occur widely in plants, insects, invertebrates, and vertebrates, including humans [1,2]. In particular, a specific type of host defense peptide, which acts on the biomembranes of microorganisms, has been shown to kill microorganisms by destroying the barrier function of their membranes [3,4]. The membranes of microorganisms are distinguished from those of mammalian cells by their structural composition of phospholipids and cholesterols. On the basis of their mechanism of action and their specificity, these AMPs may act as antibiotics with a low potential for inducing antibiotic-resistant microbials [5]. To obtain more effective host defense peptides with stable and desired antibacterial spectra, attempts have been made to modify known sequences to produce peptides with improved activity [6,7], but current methods are time-consuming and the efficiency obtained is not as high as expected. In recent years, high-throughput screening has been performed frequently using combinatorial chemistry or similar approaches [8,9]. To date, however, there have been no reports showing that peptides capable of acting specifically on the membranes of microorganisms can be obtained successfully from random peptide libraries. In addition, it is difficult to synthesize libraries of peptides with long chain lengths and to determine the amino acid sequence of a peptide directly after screening. These drawbacks can be avoided by associating the amino acid sequence of the peptide with its genetic information; i.e. the amino acid sequence of the peptide can be determined from the DNA sequence encoding it [10,11]. All these techniques rely on living cells for protein expression and must include a step that uses the functions of an organism, such as a microorganism or a phage. Therefore, it is not possible to use these techniques for the selection of peptide sequences that have potential antimicrobial properties or that affect the growth of organisms, such as host defense peptides.

To overcome these problems, *in vitro* techniques, such as ribosome display, in which all steps in the screening procedure are carried out in a cell-free system, have been developed [12–14]. In the case of ribosome display, a protein-ribosome-mRNA complex is formed and screened for proteins with a specific function [15,16]. Such methods make it possible to select the amino acid sequence of a peptide that is

*Correspondence to: Machida S, Sachiko Machida, National Food Research Institute, 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan; e-mail: lili@affrc.go.jp

potentially lethal toward microorganisms. It is difficult, however, to generate selection pressure during peptide screening. Here, we report that a combination of ribosome display and an immobilized model membrane can enrich the sequences that selectively recognize membrane structures (Figure 1(B)).

EXPERIMENTAL PROCEDURES

Library Construction

To construct a DNA library, genes were constructed in three portions (Cassette 1, Cassette 2, and Cassette 3; Figure 1(A)) so that they could be applied to the design of any library. Cassette 1 involved the insertion of the cDNA sequence of the gene corresponding to a member of the library to be screened; this sequence lacked a termination codon so that the translated peptides and the mRNA would be coupled via the ribosome in a peptide-ribosome-mRNA complex. An *Nde*I site was incorporated at the 5′ end and an *Xba*I site was incorporated at the 3′ end of Cassette 1, for ligation to Cassettes 2 and 3, respectively.

Cassette 2 involved the introduction of a cDNA sequence encoding a FLAG epitope tag for prescreening at its 5′ end, followed by a sequence encoding a 3′ stem-loop to increase the stability of the mRNA and a linker sequence (Gly-Gly-Gly-Ser) to enable flexible movement of the translated peptide. The linker could be extended by including multiples of the unit sequence (Gly-Gly-Gly-Ser), and $(Gly\text{-}Gly\text{-}Gly\text{-}Ser)_4$ was used in this study. Furthermore, an *Xba*I site was incorporated at the 5′ end of Cassette 2 for ligation to Cassette 1.

Cassette 3 comprised all the cDNA sequences required for transcription and translation (a T7 promoter sequence, a Shine-Dalgarno sequence (SD), and a 5′ stem-loop to increase the stability of post-transcriptional mRNA). The T7 promoter and the SD sequence were separated by the optimal distance. An *Nde*I site was incorporated at the 3′ end of Cassette 3 for ligation to Cassette 1.

DNA sequences corresponding to the peptide library for screening were chemically synthesized to prepare Cassette 1. Cassettes 1 and 2 were ligated via the restriction sites described above using DNA ligase, and the ligated product was amplified using primers 3 and 4. The resultant PCR product was digested with *Nde*I, purified, and ligated to Cassette 3 using DNA ligase. The resultant ligation product was amplified

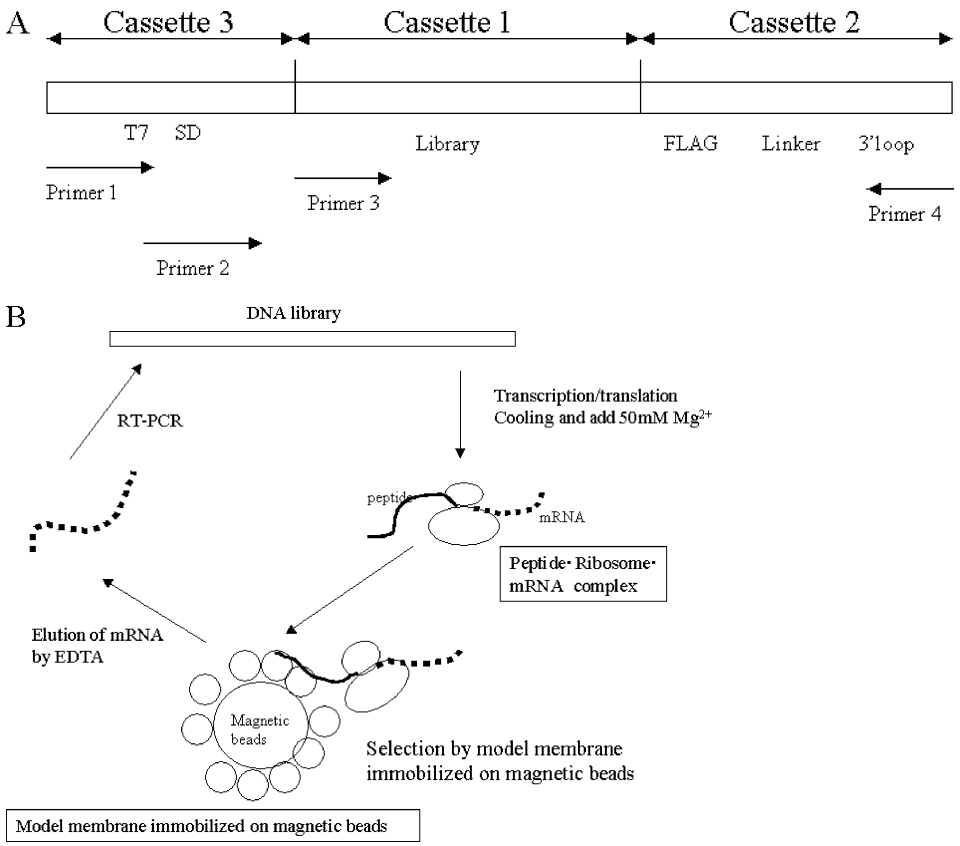

Figure 1 Screening scheme for ribosome display combined with immobilized model membrane. (A) Schematic representation of DNA constructs. The full-length DNA library consisted of three cassettes. Cassettes 2 and 3 were common to each component in the library, whereas Cassette 1 carried the unique sequence for screening. (B) *In vitro* selection of mRNA-encoded peptides. The DNA library was transcribed and translated in one tube, and the reaction was stopped by cooling on ice. Following stabilization of the peptide-ribosome-mRNA complex by addition of 50 mM Mg^{2+}, it was incubated with the model membrane immobilized on magnetic beads via streptavidin. mRNA was eluted from the bound complex in buffer containing EDTA, amplified by RT-PCR, and used for the next round of screening.

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using primers 1 and 4, and the PCR product was used as the DNA library.

Transcription and Translation of DNA in Cell-Free System

We used 0.5 μg of the constructed DNA library in a one-step transcription/translation system, which contained *Escherichia coli* S30 extract solution and 20 amino acids, in a total volume of 20 μl. Following the addition of 0.8 μl T7 RNA polymerase, the solution was incubated at 37 °C for 30 min, RNase-free DNase I was added, and the solution was incubated at 37 °C for 20 min to completely digest the template DNA. The solution was cooled on ice, and 1 M magnesium acetate was added to a final concentration of 50 mM to stabilize the peptide-ribosome-mRNA complex (PRM complex).

Prescreening

The prescreening process was used to remove complexes that did not contain a full-length peptide of interest, owing to the generation of a termination codon by an error in DNA synthesis or PCR. This reduced the probability that incomplete DNA sequences were included in the actual screening system. Biotinylated antiFLAG antibodies were immobilized on magnetic beads coated with streptavidin and incubated with the PRM complex solution for 1 h. DNAs containing a frameshift or a termination codon did not correctly express the FLAG tag and were therefore not bound by antiFLAG antibodies or subsequently recovered by the magnet, and remained in the supernatant. The beads recovered by the magnet were washed five times with cold TBS (20 mM Tris-HCl, 150 mM NaCl, pH7.6) containing 50 mM magnesium acetate. It was then incubated on ice for 10 min with 20 mM EDTA to elute the mRNAs, which were purified on a G-25 microspin column (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) and reverse transcribed and amplified using a *Campylobacter therm*. polymerase One-Step RT-PCR kit (Roche, Indianapolis, IN, USA). Each 25 μl reaction solution contained 0.3 pM of primers 2 and 4, 10 pg to 2 ng of mRNA, 0.4 mM of each dNTP, 5% DMSO, 0.5% DTT, and 0.8 units of RNase inhibitor. The amplification protocol consisted of 30 min at 60 °C and 5 min at 95 °C, followed by 25 cycles of denaturation at 95 °C for 30 sec, annealing at 57 °C for 30 sec, and extension at 72 °C for 1 min, with a final extension step of 72 °C for 6 min. After RT-PCR, the resultant amplification product was purified and subjected to additional screening.

Preparation of Immobilized Membrane Model

The bacterial membrane model employed was a liposome preparation consisting of a 1 : 1 mixture of phosphatidyl choline and phosphatidyl glycerol, plus 1.4% biotinylated phosphatidyl ethanolamine. The mammalian cell membrane model employed was a liposome preparation consisting of a 10 : 1 : 1 mixture of 'phosphatidyl choline, phosphatidyl glycerol, and cholesterol, plus 1.4% biotinylated phosphatidyl ethanolamine. Biotinylated small unilamellar vesicles (SUV), of diameter 100 nm or less, were prepared as described in the literature [7]. Biotinylated liposomes with a phospholipid content of 16.8 μg were immobilized on magnetic beads coated with streptavidin (Roche, Indianapolis, IN, USA).

Screening of DNA Library using Immobilized Membrane Model

Using a one-step transcription/translation system, we utilized 0.5 μg of the prescreened DNA library in a total volume of 20 μl to form a PRM complex. The latter was incubated with magnetic beads on which biotinylated liposomes (either the bacterial or the mammalian membrane model) had been immobilized. The beads were recovered with a magnet, washed with cold tris buffered saline (TBS) containing 50 mM magnesium acetate, and incubated on ice with 20 mM EDTA solution for 10 min to dissociate the mRNA. The eluted mRNA was purified, reverse transcribed, and amplified using primers 2 and 4. The amplified product was further purified and subjected to another cycle of screening.

Five cycles of screening were performed in order to enrich for peptides that could act specifically on the bacterial or mammalian cell membrane. TA cloning and nucleotide sequencing were performed after the final RT-PCR step, and the amino acid sequence was obtained.

Screening by a Combination of Phage Display and Immobilized Membrane Model

To compare our approach with previously developed systems, we performed a screening in which phage display (T7 phage system; Novagen, Madison, WI, USA) was combined with an immobilized membrane model system, as described above.

Evaluation of the Membrane Specificity of the Peptides using Liposomes with Entrapping Calcein

Peptides synthesized by Sawady Technology (Tokyo, Japan) were purified to at least 95% by HPLC, and the molecular weight of each purified peptide was determined by mass spectrometry. The three model membranes consisted of the following: liposome A, phosphatidylcholine : phosphatidylglycerol (PC : PG = 1 : 1), representative of a typical bacterial membrane model; liposome B, (PC : PG = 10 : 1), a model in which the negative charge of liposome A was decreased; and liposome C (PC : PG : Cholesterol = 10 : 1 : 1), representative of a healthy mammalian cell membrane. Each type of liposome contained the fluorescent dye calcein [17], and the peptide activity toward each liposome was evaluated by monitoring calcein leakage. Standard assays were done in 96-well Fluoro Nunc Plate (Nunc, Denmark). Each type of calcein-containing liposomes was diluted to 70 μM with 10 mM HEPES buffer and added to 96-well Fluoro Nunc. To each well containing 100 μl calcein-containing liposomes, aqueous peptide solution was added till final concentrations of 0.5, 1, 2, 3, 4, 5, 7.5, and 10 μM were reached. Following incubation at 25 °C for 1 min, the amount of leaked calcein was measured using a micro plate fluorometer (SPECTRAmax GEMINI; Molecular Devices Corp., Sunnyvale, CA, USA) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm; where indicated, fluorescence intensity was measured every 2 min thereafter. Calcein does not emit fluorescence within the liposomes owing to fluorescence quenching; however, in the presence of a peptide that destabilizes liposomes, calcein will leak out of the liposomes and emit fluorescence at the excitation wavelength. As a positive control, we added Triton X-100 to a final concentration of 1%; as a negative control, we added

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buffer solution. The peptide concentration that caused 50% calcein efflux (EC_{50}) was determined from the dose-response curve.

Evaluation of Hemolytic Activity

Hemolytic activity was evaluated as described previously [7]. Briefly, 80 µl aliquots of an isotonic solution of each peptide were incubated for 30 min at 37 °C with 720 µl of an erythrocyte suspension (2×10^7 cells/ml). Each suspension was centrifuged at 700 *g* for 5 min, and the hemoglobin recovered in the supernatant was measured by determining the absorbance at 540 nm. As a positive control, we added Triton X-100 to a final concentration of 1%; as a negative control, we measured the absorbance of isotonic solution alone.

Analysis of the Interaction of Peptide with Liposomes

Small unilamellar vesicles (SUV) were prepared as described in the literature [17]. The interaction of peptides with SUV was analyzed by circular dichroism (CD) spectroscopy, recorded at 25 °C on a Jasco J-820 spectropolarimeter (Jasco, Tokyo, Japan) using a quartz cell with a pathlength of 1 mm. Each peptide sample was dissolved to 50 µM in 10 mM phosphate buffer (pH7.0) and mixed with 0.7 mM SUVs. Each sample was scanned five times at 10 nm/min over the range 195–250 nm.

Evaluation of Antibacterial Activity

Antibacterial activity was evaluated in accordance with the US National Committee for Clinical Laboratory Standard (NCLL Documents M7-A3) [18]. Specifically, the minimum concentration of peptide capable of inhibiting the growth of bacteria was determined using micro titer plates. Bacteria were cultured for 16 h in sensitivity measurement broth medium, and the absorbance at 600 nm (A_{600}) was measured. According to the correlation between the previously obtained turbidity and the number of colony forming units (CFU), each bacterial strain was diluted to a prescribed CFU with sensitivity measurement broth to a final concentration of 5×10^5 CFU/ml, and 50 µl was added to each well of the 96-well microplates. A 5 mM aqueous solution of each peptide was serially diluted from 800 µM to 0.78 µM, and 50 µl of each dilution was added to each well, and the plates were agitated (the negative control contained no peptide). The plates were incubated at 37 °C for 18 h to determine the minimum inhibitory concentration (MIC).

Sequences

Primer 1: gatctcgatc ccgcgaaatt a
Primer 2: gcgaaattaa tacgactcac tatagggaga ccacaacgg
Primer 3: tgctctagag cagcctccag a
Primer 4: cggaattcc cgcacaccagt a

RESULTS

Selection of Model Antibacterial Peptides

Initially, we attempted to select two model peptides – mast21, which recognizes only bacterial membranes [7], and mastoparanX, which recognizes both bacterial and mammalian membranes [19] – to confirm the viability of our *in vitro* strategy. The DNA sequence encoding mast21 was inserted into Cassette 1, and a cycle of ribosome display coupled to the selection on an immobilized membrane was carried out. A signal was obtained at the molecular weight expected for the corresponding DNA after RT-PCR only when the model bacterial membrane was used (Figure 2, lane 2). This result was consistent with the specificity of mast 21 toward the bacterial membrane. When the DNA sequence was determined after several repeated cycles of screening, most clones encoded the full-length mast21 sequence, with only a few clones having deletions. This finding showed that our method was effective for screening and enriching peptides that act specifically on bacterial membranes. When we used a combination of phage display and immobilized model membrane, we found that its efficiency in concentrating the DNA sequence encoding the peptide was considerably poorer, and the obtained DNA sequence always had deletions (data not shown). This showed that the method allows screening even when the subject being screened is a peptide capable of affecting the growth of the host.

The DNA sequence encoding mastoparanX was inserted into Cassette 1 and the ribosome display-based screening was performed. Using either the bacterial or the mammalian model membrane, a signal corresponding to mastoparanX cDNA was obtained after RT-PCR (lanes 3 and 6 in Figure 2). This result was consistent with the lack of selectivity of mastoparanX, which acts on all types of membranes. Even when the DNA sequence was determined after repeated screening cycles, most clones encoded the full-length sequence and only a few had deletions. These observations indicated that the method is effective in screening for a peptide capable of acting on various biomembranes, including those of bacterial and mammalian origin. Replacement of the screening system with a combination of phage display and an immobilized membrane made the efficiency of cDNA concentration considerably poorer, with the cDNA sequence obtained always having deletion mutations.

Selection of Peptides from Random Peptide Libraries

Next, we examined the ability of this approach to select peptide sequences with antibacterial activity from a random DNA library. DNA sequences encoding peptides of 21 random amino acids were inserted into Cassette 1; note that perfectly random amino acid sequences may contain termination codons, preventing the generation

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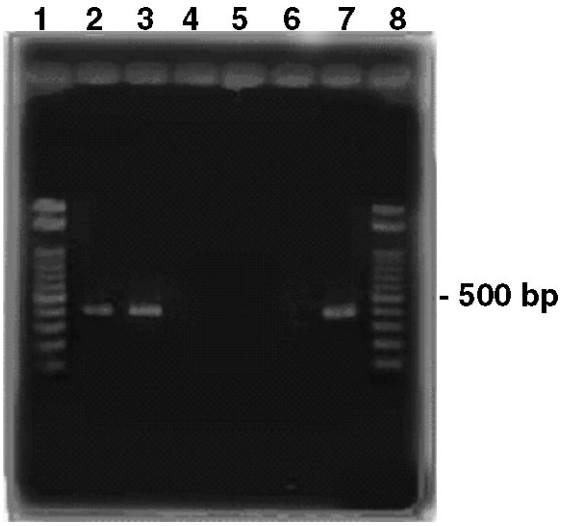

Figure 2 RT-PCR amplification of DNA encoding mast21 or mastoparanX after selection by ribosome display combined with an immobilized model membrane. cDNA encoding mast21 or mastoparanX was inserted into Cassette 1. After screening by the procedure shown in Figure 1(b), the mRNA obtained was used as a template for RT-PCR. Lanes 1 and 8: molecular weight marker; lane 2: mRNA obtained after screening the DNA construct encoding mast21 with a bacterial model membrane (PC : PG = 1 : 1); lane 3: mRNA obtained after screening the DNA construct encoding mastoparanX with the same bacterial model membrane; lane 4: control mRNA obtained after screening the DNA construct encoding mast21 with the same bacterial model membrane in the absence of *in vitro* transcription/translation; lane 5: control mRNA obtained after screening the DNA construct encoding mast21 with the same bacterial model membrane but without inclusion of amino acids in the *in vitro* transcription/translation; lane 6: mRNA obtained after screening the DNA construct encoding mast21 with a mammalian model membrane (PC : PG : Chl = 10 : 1 : 1); lane 7: mRNA obtained after screening the DNA construct encoding mastoparanX with the same mammalian model membrane, using the resultant mRNA as the template. A specific amplified signal was obtained in lane 2 for mast21, and in lanes 3 and 7 for mastoparanX.

of full-length peptides. We therefore used the DNA sequences, $(XXB)_{20}XAG$, where X represents A, T, G, or C and B represents T or C; limiting the third codon to T or C prevents the occurrence of a possible termination codon [14]. For the ligation cassettes, it was necessary for the DNA sequence encoding the 21st amino acid to be XAG, limiting the terminal amino acid to Try, His, Asn, and Asp. This random peptide library was inserted into Cassette 1 and ligated to the other cassettes, followed by prescreening using the FLAG tag to exclude any sequences that did not express the full-length peptide; following prescreening, the library diversity was 10^8 per μg DNA. Screening using the immobilized bacterial model membrane was performed, and the final RT-PCR product obtained after five screening cycles was purified and subjected to TA cloning. Of the resultant colonies, 50 clones were sequenced, 48 of which encoded 21 amino acids without a deletion (Table 1A). We were unable to obtain similar results by phage display or similar techniques.

We obtained a high frequency of motifs consisting of several specific amino acids (e.g. ALR, RVG) in the peptides obtained from the random peptide library. These motifs consisted of a basic amino acid, K or R, and several hydrophobic amino acids, and may represent the minimum units essential for interaction with the bacterial membrane. Since repeats of these motifs might confer an amphipathic helix or amphipathic sheet structure on the peptide, we designed and screened a library to obtain a peptide capable of binding more effectively to the bacterial membrane. Specifically, a DNA sequence library encoding 14 amino acids, ZXXXZXXZXXXZXX (where Z represents K or R and X represents any amino acid), in which K or R was placed at a position that would provide an amphipathic peptide, was subjected to screening with the method used for the semi-random library. Of the resultant colonies, 50 clones were subjected to sequencing, of which 47 encoded a full-length peptide without a deletion (Table 1B). Sequences such as KVZ and RVZ were present in several of the peptides, and K/R was often followed by a hydrophobic amino acid in most of these, and rarely by a hydrophilic but nonpolar amino acid, but never by a basic or acidic amino acid. These observations supported our expectation that peptides obtained by this screening method were likely to have an amphipathic helix or β-sheet structure and will be useful as lead sequences for antibacterial peptides.

Specific Activity of Enriched Peptides

Inspection of the peptide motifs suggested that ALR and KVL might be the minimum units for acting on a cell membrane structure. We also hypothesized that the length of each peptide was related to its ability to penetrate and destroy the membrane. Therefore, we chemically synthesized units of ALR and KVL sequences linked in tandem and tested their abilities to act specifically on a model membrane. We found that the activity of each peptide was enhanced as a function of its length. In addition, we found that $(KVL)_6$ and $(ALR)_6$ acted on the bacterial model membrane, liposome A, but showed significantly weaker action on the mammalian model membrane, liposome C. The ability to leak calcein increased markedly when the acidic phospholipid contents of the liposome increased (compare liposomes A and B). Moreover, these minimum motifs conferred selectivity toward microorganisms. For example, mastoparanX had potent activity toward liposomes A, B, and C, whereas, when the KVL motif was fused to mastoparanX, its activity toward liposome C was markedly reduced (Table 2A).

To determine their activity towards mammalian membranes, we evaluated the activities of these peptides

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Table 1 Amino acid sequences selected by bacterial model membrane

A

1. VWAWVFGASTRERA**RVG**WQGY
2. CFVLP**GVR**PCSSHILTLSFSY
3. E**GVR**DMFRRCLWISLRSWCVH
4. GGVYASCASYLL***ALR***S**RVG**GN
5. SDSASVS**RVG**GLWPTCCPH
6. WGRVDNSGSWG**RVG**APWRYLH
7. CAAVYLVTSLFGIVT**GVR**EDH
8. DSR**GVR**AFACDYVLFVLWVPY
9. DLAPV**RVG**FYN***ALR***ELRVFRY
10. **RVG*ALR***YFMVWYLMWFFLLFH
11. PVLDAGSVYLGYL**GVR**FLSY
12. VRNRVIA**RVG**GVPYVGGPCYN
13. ACCLVRFYSHGRGK**RVG**FLWY
14. LRYSGLLGFPLWVGRIFVCVD
15. RMRCVSLELVVYGG**GVR**MWEN
16. FMGYGRSVWVVSSSLVLCIYD
17. ARMLWGRGTTLLLIRRRVSAY
18. VDLSWYASCRVSICVFVVVY
19. WPNYQSREHM***ALR***SRMYYYFY
20. CVV***ALR***NVKAAALIPGVVSRH
21. WWCLLGYWALGGNHSA***ALR***SY
22. YGSYLE***ALR***WGTSACW***ALR***Y
23. GVAVDCAVVGW***ALR***VLGVHSY
24. RCLEAGKIWWG***ALR***SHLAVYD
25. GSGSAVGW***ALR***SYASGLAIAY
26. HAWARWMGWGHGGVLSW***ALR***Y
27. FVSW***ALR***YSRCLVWLCWFPNY
28. VKGNPVFDHRHFSLWG***ALR***EY
29. FVQHWSFTAGSRSDRAPYPGH
30. AGWVN***ALR***MWSLMPLMWLWSY
31. DRTTGRWFYIRRTAEVLGWTY
32. RFINPTSHCFGSLSLWRQLSY
33. VGCLVSVGSVWGCSSVVVRVY
34. RMESGAPLAAYGKMRLRPGTH
35. VWNRVIA**RVG**GVLYVGGPCTN
36. SGHMHSYWPTTWILVLIRRTY
37. LEVLVWYSLWSYWLDVAAASH
38. SWGGGFYDWSYVGGGAYWAY
39. MGLFRSYKYRFVHDSESSFN
40. MALYLAWYGCSDSAVVMLADD
41. MYCWRMLANSC***ALR***MVLAMRN
42. VIVNVAVLYRRCWPCAEFWPY
43. RLGSFYPLLWRLVSHEYSLWH
44. RYWFGRWRCFYGPFVSSYFLY
45. VCCCRCLPWSYMCEWGSMRLY
46. VLKIHSWHNWVYGVMLYDMEY
47. MGYAWDL***ALR***MGPYFLMDLIN
48. SDKCAPVCYVMDRLCLANWD

Nucleotide sequences $(XXB)_{20}XAG$ (where X represents A, T, G, or C, and B represents T or C) were inserted into Cassette 1 and the resultant DNA constructs were screened using the bacterial model membrane as described in the Methods section. Amino acid motifs appearing over 5 times are highlighted in bold. The most frequent motifs (ALR, RVG) are underlined and ALR is marked in italics.

B

1. RPVFRTYRSVVKSG
2. NRACLKRPRYLRKH
3. KACVRFSKSTSKRY
4. RFRPKAVRYRIKFN
5. RSHFRRVKRHSKTP
6. KDVLRNHKHSDRVG
7. KSAR**KVL**KLYRKIT
8. KKNSCRDGRFSRKC
9. KGYFRGRRSYLRAF
10. KGCA**KVL**KRITRHI
11. KGRHRHCRYILRGN
12. KCTFRRRVLIIKPS
13. KTDWF**KVL**MTFLMD
14. RGFVRLIKPYAEAS
15. RNLCRSLRSHLEA
16. KIPGRFTRAGRKTT
17. KSDH**KVL**KNLPKTI
18. RRTGRIDKVSVKAY
19. **KVL**IKLAKCCIRIS
20. RAACRDSKLCSRYY
21. KHFVRCPKCAVRSS
22. KISDRNSKHHCRSS
23. KVGLIVDKASVKTA
24. RDVCKSSRHSHKGS
25. RFVSKGTDAINRRS
26. KGNCRLYRLRCKVV
27. RLLLKAVRFCCKCF
28. KGGGKVGKHTRSR
29. RHFRKNCKFCHRHC
30. KRCT**KVL**RAYTKLT
31. KSYGKAPKFVGRIC
32. RAAIRHFRSATKRP
33. KYSARFCKYGGRSH
34. RFTARVRKSVFRSC
35. KVYSRSSKSAHKCF
36. KRAYKDARHIYLCS
37. KIFVRTIRAAHKRD
38. KGGGKVGKHTRSR
39. KSLTKCC**KVL**RLSC
40. RCDIKSVKHILRCS
41. KASVRNS**KVL**PRFC
42. KGAFRLA**KVL**IRHY
43. RHVPKANKGADRSC
44. KTSWVRAAALVVVH
45. KSVNKDVRISLRD
46. KCIARRGRLPVKRY
47. **KVL**FRHARSSCKHY

cDNAs encoding ZXXXZXXZXXXZXX (where Z represents K/R, and X represents any amino acid) were inserted into Cassette 1 and the resultant DNA constructs were screened using the bacterial model membrane as described in the Methods section. The amino acid motif (KVL), which appeared over 5 times, is highlighted in bold.

on erythrocytes. Whereas mastoparanX, which can act on mammalian cell membranes, had high hemolytic activity, all the peptides obtained from the screening exhibited a low level of hemolysis (Table 2A). These

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Table 2 Properties of selected peptides
A: Specific membrane activity of peptides synthesized according to the enriched motif sequences.

Peptide name (amino acid sequence)	EC_{50} (μM)			Hemolysis
	Liposome A	Liposome B	Liposome C	L(%)
ALR (ALR)	nd	nd	nd	nd
ALR_5 (ALRALRALRALRALR)	6.2	>10	>10	nd
ALR_6 (ALR ALRALRALRALRALR)	2.6	nd	nd	1.8
KVL (KVL)	nd	nd	nd	nd
KVL_3 (KVLKVLKVL)	>10	5.8	nd	nd
KVL_5 (KVLKVLKVLKVLKVL)	6.8	8.2	>10	nd
KVL_6 (KVLKVLKVLKVLKVLKVL)	3.2	6.9	>10	1.9
KVL-mastX (KVLINWKGIAAMAKKLL)	2.1	0.52	>10	9
MastoparanX (INWKGIAAMAKKLL)	0.31	0.81	1.2	28
No.12 (VRNRVIARVGGVPYVGGPCYN)	>10	nd	nd	nd
No.35 (VWNRVIARVGGVLYVGGPCTN)	nd	nd	nd	nd
$RVGG_5$ (RVGGRVGGRVGGRVGGRVGG)	>10	nd	nd	1.9
$WALR_5$ (WALRWALRWALRWALRWALR)	5.8	9.3	>10	11
$ALRY_5$ (ALRYALRYALRYALRYALRY)	4.6	8.3	>10	12

EC_{50} values are the peptide concentrations causing 50% calcein release after 1 min incubation with peptides at 25 °C.
The fluorescence intensity corresponding to 100% calcein release was obtained by the addition of Triton X-100 to a final concentration of 1% (v/v). Liposome A (PC : PG = 1 : 1); Liposome B (PC : PG = 10 : 1); Liposome C (PC : PG : Chl = 10 : 1 : 1). L (%) represents the percentage of lysed red blood cells at a peptide concentration of 10 μM after 1 h at 37 °C.
nd: not detectable.
No.12 and No.35 were sequences in Table I.

B: Antibacterial activities of peptides synthesized according to the enriched motif sequences.

Peptide	*B. subtilis*	*S. aureus*	*E. coli*	*E. coli*	*E. coli*	*S. enteritidis*	*S. enteritidis*
	IFO13722	IFO13276	JCM1649	CR-3	CE-273	ATCC1891	ATCC14028
	μM	μM	μM	μM	μM	μM	μM
KVL	>200	>200	>200	>200	>200	>200	>200
KVL3	200	200	200	200	200	200	200
KVL5	25	100	12.5	25	6.3	25	100
KVL6	3.1	25	25	25	25	25	25
ALR	200	>200	200	200	200	200	200
ALR5	25	>200	100	100	100	100	100
ALR6	25	50	25	12.5	12.5	12.5	12.5
MastoparanX	3.1	1.56	3.1	3.1	3.1	3.1	3.1
No.12	>200	>200	>200	>200	>200	>200	>200
No.35	—	—	—	—	—	—	—
$RVGG_5$	>200	>200	>200	>200	>200	>200	>200
$WALR_5$	>200	>200	>200	>200	>200	>200	>200
$ALRY_5$	—	—	—	—	—	—	—

Each number represents the Minimum Inhibitory Concentration (MIC) of a peptide, defined as the lowest concentration of peptide that completely inhibited the growth of tested microorganisms. *E. coli* 2R-3: O157 : H7 lacks the vero toxin gene. *E. coli* CE-273: O157 : H7, but is not pathogenic.

observations support the validity of our approach, in which we screened for peptides incapable of acting on mammalian model membranes.

Besides the three amino acid motifs, we observed several longer motifs at high frequency, including RVGG, WALR, and ALRY. When we attempted to evaluate the activity of tandem repeats of these peptides, we observed little specific activity to destabilize liposomes and no antimicrobial activity (Table 2). It is possible that tandem repeats of each individual motif

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lack activity, whereas the mixture of several motifs shows specific activity. Sequence No.12 and No.35 contained several motif sequences and these two closely resemble each other. So sequences 12 and 35 could be a common ancestor. Then we checked the ability of both sequences, however the synthesized peptides had no activity toward liposomes and microorganisms (Table 2A, B).

When we monitored calcein leakage induced by the serial addition of peptides and the time course of leakage, we found that leakage occurred rapidly within 1 min (Figure 3). While the addition of low concentrations of each peptide resulted in partial leakage, serial addition of peptides every 10 min continued to induce calcein leakage (Figure 3).

When we examined the structure of $(ALR)_6$ and $(KVL)_6$, with or without liposomes, by CD, we found that their CD spectra in 50% trifluoroethonol (TFE) showed α-helical patterns (double minima at 208 nm and 222nm, and a maximum below 200nm), indicating that both peptides could form helical structures. The CD spectra of these two peptides showed that they did not form clear α-helical patterns in buffer solution; however, following incubation with SUVs, they did form α-helical structures. Thus, the increase in acidic phospholipid content is consistent with the increase in helicity (Figure 4(A), (B) line 2,4)

Next, we evaluated the antibacterial activities of these sequences. KVL, ALR, and the tandem peptide sequences that acted specifically on the model microorganism membrane were shown to have antibacterial activity. The activities of both KVL and ALR

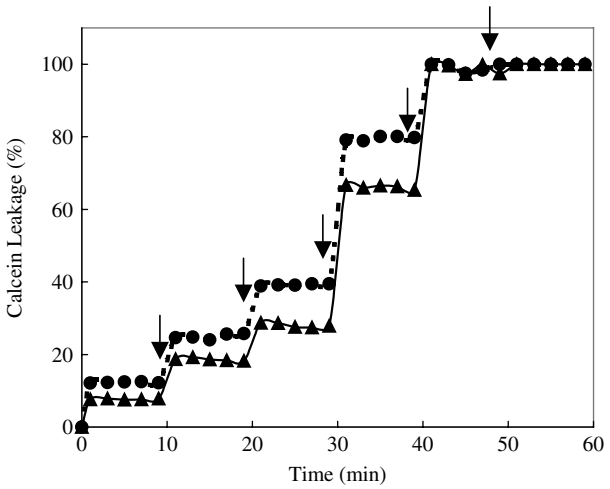

Figure 3 Time course of calcein leakage induced by $(KLV)_6$ and $(ALR)_6$. At time zero, 0.5 μM of the test peptide was incubated with 70 μM liposome A (PC : PG = 1 : 1) at 25 °C, and increasing concentrations of the peptides (1, 2, 4, 5, and 7.5 μM) were added at 10, 20, 30, 40, and 50 min, as indicated. Dye leakage was monitored fluorometrically (excitation at 490 nm and emission at 530 nm) every 2 min. ●: (ALR)6; ▲: (KVL)6.

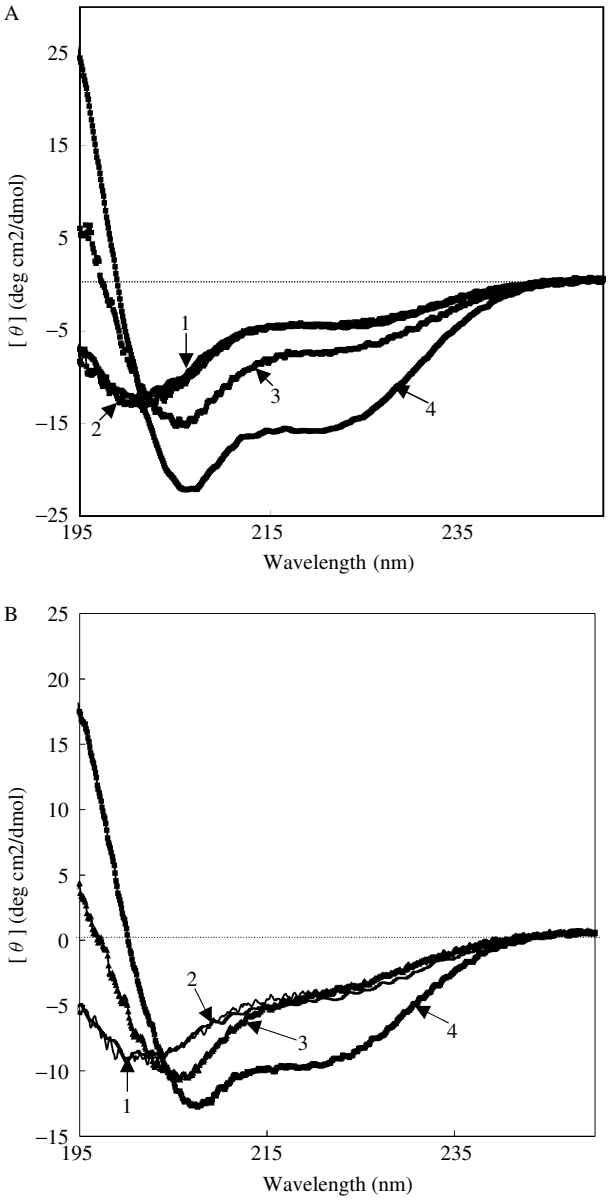

Figure 4 Circular dichroism of peptides (A) $(ALR)_6$ and (B) $(KLV)_6$. Peptides were dissolved to 50 μM in 10 mM phosphate buffer (pH7.0), 0.7 mM SUV, or 50% trifluoroethanol (TFE) and their spectra were obtained. 1, buffer; 2, PC : PG (10 : 1); 3, PC : PG (1 : 1) SUVs; 4, TFE.

increased with increasing chain length. The antibacterial spectrum of $(KVL)_6$ and $(ALR)_6$ extended from Gram-negative bacteria, such as *E. coli*, to Gram-positive bacteria, such as *Staphylococcus aureus*. These observations showed good agreement with our findings using the model bacterial membrane.

DISCUSSION

We have developed a new high-throughput screening system for obtaining AMP sequences. This method, which combined a ribosome display and immobilized

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model membranes, enriched sequences from random peptide libraries that selectively recognized bacterial membranes within a week, and identified two motif sequences, ALR and KVL. Several motifs of AMPs, such as RW-rich sequences, have been described. These motifs are characterized by at least two charged residues and a minimum of one aromatic amino acid, and have been found in both native peptide sequences and a synthetic combinatorial library [20,21]. Similar to most AMPs, our motifs contain positively charged residues, e.g. Arg or Lys, which interact with negatively charged membranes, but do not contain any aromatic residues. The motifs we obtained recognized bacterial membranes and showed antibacterial activity. Moreover, these motifs conferred selectivity toward bacterial membranes on peptides that originally lacked selectivity.

We also found that leakage of calcein was induced within 1 min and that addition of higher peptide concentrations caused additional leakage. It is not clear how these peptides destabilize the membranes, but our results indicate that these peptides do not lyse liposomes immediately. Rather, the time course of membrane destabilization by these peptides is relatively short, similar to that of magainin 2 and mastoparanX [17]. In addition, the dose-response relationship between peptide concentration and calcein leakage activity is not clear, although other peptides have been found to have a sigmoidal dependence [22]. These findings suggested that the peptides act on membranes according to a two-state model [3,23]. According to this model, there may be a concentration threshold above which these peptides have severe destabilizing activity. Unfortunately, the antimicrobial activity of these peptides was not as strong as that of mastoparanX. However, these peptides showed low activity toward mammalian type cell membranes.

There have been a number of studies of the structure/activity relationship of AMPs, and mimics and hybrids of natural AMPs, as well as novel AMPs, have been found to have enhanced activity [24–26]. Using computational tools and algorithms, these strategies have been combined, giving rise to *in silico*-based [26,27], as well as *in vivo*-based [28] and *in vitro*-based [29,30] high-throughput screening systems. In an *in vivo* study, bioactive peptides were isolated successfully from a randomized oligonucleotide library encoding up to 20 amino acids [31]. While this approach and its results are very interesting, this system has difficulties in screening peptides according to their biological activity, such as destroying bacterial cell membrane structure.

The increasing resistance of pathogenic bacteria to conventional antibiotics has become a serious problem, necessitating the development of antibiotics with novel modes of action. The host defense AMPs, which act by disrupting the bacterial cell membranes, can be considered a new class of antibiotics. The *in vitro* system described here may facilitate the isolation of novel AMPs from random libraries. Although previously described *in vitro* systems have the advantage of allowing synthesis of peptides at high speed and overcoming the problem of cell toxicity, there have been few reports of their application to AMPs, perhaps because some AMPs can bind DNA or RNA and inhibit peptide synthesis. Another reason is that there are difficulties in constituting the screening system. The new *in vitro* strategy described here has shown that a combination of ribosome display and an immobilized model membrane can identify motifs that recognize bacterial membranes. This strategy could be a powerful tool for screening lead sequences of AMPs that act selectively on specific membrane structures and to develop novel antibiotics.

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REFERENCES

1. Huttner KM, Bevins CL. Antimicrobial peptides as mediators of epithelial host defense. *Pediatr. Res.* 1999; **45**: 785–794.
2. Barra D, Simmaco M. Amphibian skin: a promising resource for antimicrobial peptides. *Trends Biotechnol.* 1995; **13**: 205–209.
3. Matsuzaki K. Why and how are peptide–lipid interactions utilized for self-defense? Magainins and tachyplesins as archetypes. *Biochim. Biophys. Acta* 1999; **1462**: 1–10.
4. Zasloff M. Antimicrobial peptides of multicellular organisms. *Nature* 2002; **415**: 389–395.
5. Binder S, Levitt AM, Sacks JJ, Hughes JM. Emerging infectious diseases: public health issues for the 21st century. *Science* 1999; **284**: 1311–1313.
6. Park Y, Lee DG, Jang SH, Woo ER, Jeong HG, Choi CH, Hahm KS. A Leu–Lys-rich antimicrobial peptide: activity and mechanism. *Biochim. Biophys. Acta* 2003; **1645**: 172–182.
7. Machida S, Niimi S, Shi X, Ando Y, Yu Y. Design of a novel membrane-destabilizing peptide selectively acting on acidic liposomes. *Biosci. Biotechnol. Biochem.* 2000; **64**: 985–994.
8. Blondelle SE, Houghten RA. Novel antimicrobial compounds identified using synthetic combinatorial library technology. *Trends Biotechnol.* 1996; **14**: 60–65.
9. Hong SY, Oh JE, Kwon MY, Choi MJ, Lee JH, Lee BL, Moon HM, Lee KH. Identification and characterization of novel antimicrobial decapeptides generated by combinatorial chemistry. *Antimicrob. Agents Chemother.* 1998; **42**: 2534–2541.
10. Whaley SR, English DS, Hu EL, Barbara PF, Belcher AM. Selection of peptides with semiconductor binding specificity for directed nanocrystal assembly. *Nature* 2000; **405**: 665–668.
11. Zou W, Ueda M, Tanaka A. Screening of a molecule endowing Saccharomyces cerevisiae with n-nonane-tolerance from a combinatorial random protein library. *Appl. Microbiol. Biotechnol.* 2002; **58**: 806–812.
12. Hanes J, Pluckthun A. *In vitro* selection and evolution of functional proteins by using ribosome display. *Proc. Natl. Acad. Sci. U.S.A.* 1997; **94**: 4937–4942.

DOI: 10.1002/psc

13. Zhou JM, Fujita S, Warashina M, Baba T, Taira K. A novel strategy by the action of ricin that connects phenotype and genotype without loss of the diversity of libraries. *J. Am. Chem. Soc.* 2002; **124**: 538–543.
14. Cho G, Keefe AD, Liu R, Wilson DS, Szostak JW. Constructing high complexity synthetic libraries of long ORFs using *in vitro* selection. *J. Mol. Biol.* 2000; **297**: 309–319.
15. Sidhu SS, Weiss GA, Wells JA. High copy display of large proteins on phage for functional selections. *J. Mol. Biol.* 2000; **296**: 487–495.
16. Keefe AD, Szostak W. Functional proteins from a random-sequence library. *Nature* 2001; **410**: 715–718.
17. Matsuzaki K, Murase O, Tokuda H, Funakoshi S, Fujii N, Miyajima K. Orientational and aggregational states of magainin 2 in phospholipid bilayers. *Biochemistry* 1994; **33**: 3342–3349.
18. National Committee for Clinical Laboratory Standards. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically* (3rd edn). Approved standard NCCLS Documents M7-A3. The National Committee for Clinical Laboratory Standards: Wayne, Pennsylvania, USA, 1993.
19. Wieprecht T, Dathe M, Epand RM, Beyermann M, Krause E, Maloy WL, MacDonald DL, Bienert M. Influence of the angle subtended by the positively charged helix face on the membrane activity of amphipathic, antibacterial Peptides. *Biochemistry* 1997; **36**: 12869–12880.
20. Wessolowski A, Bienert M, Dathe M. Antimicrobial activity of arginine- and tryptophan-rich hexapeptides: the effects of aromatic clusters, $_{D}$-amino acid substitution and cyclization. *J. Pept. Res.* 2004; **64**: 159–169.
21. Rezansoff AJ, Hunter HN, Jing W, Park IY, Kim SC, Vogel HJ. Interaction of the antimicrobial peptide Ac-FRWWHR-NH_2 with model membrane systems and bacterial cells. *J. Pept. Res.* 2005; **65**: 491–501.
22. dos Santos Cabrera MP, de Souza BM, Fontana R, Konno K, Palma MS, Azevero WF Jr, Neto JR. Conformation and lytic activity of eumenine mastoparan: a new antimicrobial peptide from wasp venom. *J. Pept. Res.* 2004; **64**: 95–103.
23. Huang HW. Action of antimicrobial peptides: two-state model. *Biochemistry* 2000; **39**: 8347–8352.
24. Park Y, Lee DG, Hahm KS. HP(2–9)-magainin 2(1–12), a synthetic hybrid peptide, exerts its antifungal effect on *Candida albicans* by damaging the plasma membrane. *J. Pept. Sci.* 2004; **10**: 204–209.
25. Del Borgo MP, Hughes RA, Wade JD. Conformationally constrained single-chain peptide mimics of relaxin B chain secondary structure. *J. Pept. Sci.* 2005; **11**: 564–571.
26. Sun X, Chen S, Li S, Yan H, Fan Y, Mi H. Deletion of two C-terminal Gln residues of 12-26-residue fragment of melittin improves its antimicrobial activity. *Peptides* 2005; **26**: 369–375.
27. Giangaspero A, Sandri L, Tossi A. Amphipathic α-helical antimicrobial peptides. A systematic study of the effects of structural and physical properties on biological activity. *Eur. J. Biochem.* 2001; **268**: 5589–5600.
28. Raventos D, Taboureau O, Mygind PH, Nielsen JD, Sonksen CP, Kristensen H-H. Improving on nature's defenses: optimization & high throughput screening of antimicrobial peptides. *Comb. Chem. High Throughput Screen.* 2005; **8**: 219–233.
29. Taguchi S, Nakagawa K, Maeno M, Momose H. *In vivo* monitoring system for structure-function relationship analysis of the antimicrobial peptide apidaecin. *Appl. Environ. Microbiol.* 1994; **60**: 3566–3572.
30. Rungpragayphan S, Nakano H, Yamane T. PCR-linked *in vitro* expression: a novel system for high throughput construction and screening of protein libraries. *FEBS Lett.* 2003; **540**: 147–150.
31. Walker JR, Roth JR, Altman E. An *in vivo* study of novel bioactive peptides that inhibit the growth of *Escherichia coli*. *J. Pept. Res.* 2001; **58**: 380–388.

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