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Molecular Cloning of Two Novel Temporins From *Lithobates catesbeianus* and Studying of Their Antimicrobial Mechanisms*

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Abstract Temporins are a kind of small, hydrophobic and C-terminus amidated antimicrobial peptides from *Rana* species. They are effective against bacteria, fungi, yeast, protozoa and viruses. Two novel temporins named as temporin-La(LLRHVVKILEKYL$_{amide}$) and temporin-Lb(LFRHVVKIFEKYL$_{amide}$) were cloned from *Lithobates catesbeianus*. Synthetic peptides of temporin-La and temporin-Lb showed strong antimicrobial activities against bacteria tested, especially Gram-positive bacteria. Besides, temporin-La showed no haemolytic activity to rabbit erythrocytes at the concentration of 250 mg/L while temporin-Lb showed weak haemolytic activity($LC_{50} \approx$ 230 μmol/L). Transmission electron microscopy showed that temporin-La and temporin-Lb induced different effects on bacterial structure of *Staphylococcus aureus*.

Key words antimicrobial peptides, *Lithobates catesbeianus*, temporins, transmission electron microscopy
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Living in various environments, multicellular organisms are challenged to be infected by miscellaneous pathogenic microorganisms. Production of broad-spectrum antimicrobial peptides is a common innate immunity defense mechanism for multicellular organisms against infection[1~3]. Antimicrobial peptides are a kind of small, cationic and amphipathic molecules. They are active not only towards Gram-positive and Gram-negative bacteria, but also fungi, yeasts, protozoa and viruses[1~4]. Most of antimicrobial peptides act by compromising the structural and functional integrity of microbial membranes. In addition, there are additional or alternative modes of action, such as flocculation of intracellular contents, altering cytoplasmic membrane septum formation, inhibiting cell-wall synthesis, binding nucleic acids, inhibiting nucleic acids synthesis, inhibiting protein synthesis and inhibiting enzymatic activity[5, 6]. Skins of *Rana* species have already been known as abundant resources of antimicrobial peptides. Antimicrobial peptides are synthesized by dorsal granular glands and secreted to the surface of skins upon microbial infection[7~9]. To date, more than 200 antimicrobial peptides have been isolated and characterized from *Rana* species. Based on broad structural characteristics, they can be classified into 14 families including gaegurins, brevinins-1 and brevinins-2, ranalexin, ranatuerins-1 and ranatuerins-2, esculentins-1 and esculentins-2, palustrin, japonicin-1 and japonicin-2, nigrocin-2, rugosins and temprin[10~12].

Temporins are one of important families of antimicrobial peptides and first been isolated and characterized from *Rana erythraea*[8, 9]. After that, more members of temporins were discovered from other kinds of *Rana* species[8, 9, 13]. All temporins previously reported are small (between 10 and 18 amino acid residues), hydrophobic and C-terminus amidated[8, 9, 13].

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Compared with Gram-negative bacteria, temporins are more effective against Gram-positive bacteria. Besides, it is reported that temporins also can kill fungi, yeasts, protozoa and viruses[8, 9, 14~17]. The antimicrobial mechanism of temporins is still not very clear now. However, it is reported that it partly depends on their degree of positive charges. As a result, it is thought that temporins adopt an amphipathic α-helical structure in hydrophobic environment and act on the negatively charged bacterial membrane[18, 19]. This implies the occurrence of a barrel-stave mechanism for temporin.

In this study, we identified and cloned two novel temporins named as temporin-La and temporin-Lb from *Lithobates catesbeianus*. The peptides were synthesized by solid-phase synthesis and the antimicrobial and haemolytic activities were determined. Furthermore, transmission electron microscopy was performed to investigate clues to possible mechanisms of action of the peptides on tested bacteria. The results showed that temporin-La and temporin-Lb induced different effects on bacterial structure of *Staphylococcus aureus*.

1 Materials and methods

1.1 Materials

Adult specimens of *Lithobates catesbeianus* were captured from Xiangtan City, Hunan Province of China. SMART™ cDNA construction kit (Clontech, Palo Alto, CA, USA) was purchased from Clontech. TRIZOL Reagent was purchased from Invitrogen. mRNA isolate kit was purchased from QIAGEN. pMD18-T vector was purchased from TaKaRa Biotechnology Co. Ltd. (Dalian, China). Microorganisms used in the antimicrobial assays were as follows: *Salmonella* ATCC 20020, *Klebsislla pneumoniae* ATCC 700603, *Listeria* ATCC 54004, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 were obtained from Institute of Microbiology, The Chinese Academy of Sciences. *Streptococcus suis* 2 CVCC 606 was obtained from China Institute of Veterinary Drug Control.

1.2 SMART cDNA synthesis

Total RNA was extracted from the skin of a single sample of *Lithobates catesbeianus* with TRIzol reagent according to the instructions of the manufacturer. cDNA was synthesized by SMART™ technique using a SMART™ cDNA synthesis kit (Clontech Palo Alto, CA, USA). The first strand was synthesized using cDNA 3′ SMART CDS primer Ⅲ: 5′ATTCTAGAGGCCGAGGCGGCCGACATG-d(T)$_{30}$ N-1N 3′ (N =A, C, G or T; N-1 =A, G or C) and SMART Ⅳ oligonucleotide primer: 5′ AAGCAGTG-GTATCAACGCAGAGTGGCCATTACGGCCGGG 3′. The second strand was amplified by Advantage polymerase using 5′ PCR primer: 5′ AAGCAGTGG TATCAACGCAGAGT 3′ and cDNA 3′ SMART CDS primer Ⅲ.

1.3 Screening of the cDNAs encoding temporin-La and temporin-Lb

The cDNA synthesized was used as template for PCR screening of the cDNAs encoding temporin-La and temporin-Lb. Two oligonucleotide primers were used in PCR reactions. FP: 5′ ATGTTCACC (T/A) TGAAGAAATC 3′ based on conserved signal peptide sequences of reported antimicrobial peptides from *Rana* species in the sense direction and the antisense primer RP: 5′ ATTCTACAGGCCGAGGCGGCCGA-CATG 3′ supplied by the SMART™ cDNA synthesis kit. Advantage polymerase from Clontech (Palo Alto, CA, USA) was used and the PCR conditions were: 2 min at 94℃, followed by 30 cycles of 30 s at 94℃, 30 s at 50℃, and 1 min at 72℃, at last an extension step at 72℃ for 10 min. The PCR product was cloned into PMD18-T vector and transformed into *E. coli* DH5α. Several clones were randomly selected and sequenced. DNA sequencing was performed on an Applied Biosystems DNA sequencer, model ABI PRISM 3730.

1.4 Peptide synthesis

According to the deduced amino acid sequences of mature peptides, temporin-La and temporin-Lb were synthesized by GL Biochem (Shanghai, China) Ltd. HPLC and ESI-MS mass spectrometry were used to confirm the purity of the synthetic peptides which was higher than 95%. The synthetic peptides were dissolved in sterile water and used to evaluate biological activities.

1.5 Antimicrobial assays

Microorganisms were incubated in LB broth at 37℃ to log-phase and diluted by fresh LB broth to approximately 2×10^6 CFU/ml. Serial 1∶1 dilution of the synthetic peptides were made in a 96-well microtiter plate (50 μl/well) by fresh LB broth. 50 μl of diluted microorganisms were added to each well and the plate was incubated at 37℃ in a moist atmosphere for 16~18 h. After incubation, the absorbance of the plate at 600 nm was measured and

recorded. Minimal inhibitory concentrations (MIC) of the peptides were determined as the minimal concentrations at which no visible growth of the microorganisms occurred.

1.6 Haemolytic assays

Haemolytic assay was tested using rabbit erythrocytes in liquid medium according to the method previously reported [20]. Briefly, serial diluted peptides were incubated with washed rabbit erythrocytes at 37℃ for 30 min. After that, the cells were centrifuged and the absorbance at 595 nm of the supernatant was measured. 1% Triton X-100 was used to determine the maximum haemolysis.

1.7 Transmission electron microscopy

Transmission electron microscopy was performed to study the potential antimicrobial mechanisms of temporin-La and temporin-Lb on Gram-positive bacteria *Staphylococcus aureus* ATCC 25923 according to the method described previously [20]. Briefly, the bacteria were incubated to exponential-phase and treated with the peptides (at 10 times the MIC) for 30 min at 37℃. After treatment, the bacteria were centrifuged and the bacterial pellets were embedded, stained, dehydrated and embedded in white resin. The embedded bacteria were then sliced and stained. Finally, microscopy was performed with a JEM-1200EX Ⅱ microscope under standard operating conditions.

2 Results

2.1 cDNA cloning of temporin-La and temporin-Lb

Several clones containing an insert of approximately 320 bp were selected and sequenced. By sequencing, two groups of clones which encoding two novel antimicrobial peptides separately were identified. The first group encoded a mature peptide named as temporin-La and the second group encoded temporin-Lb (Figure 1) (GenBank accession FJ430082 and FJ430083). The deduced precursors of temporin-La and temporin-Lb contain 62 amino acid residues and can be divided into 3 segments: a signal peptide composed of 22 amino acid residues at the N-terminus, a 25-residue acidic propiece in the middle with two basic residues Lys and Arg at the C-terminus and a 13-residue antimicrobial progenitor at the C-terminus. A NCBI-BLAST search revealed that the nucleotide sequences of the precursors of temporin-La and temporin-Lb exhibit between 54% and 68% sequence identity with other members of temporins precursors from *Rana* species.

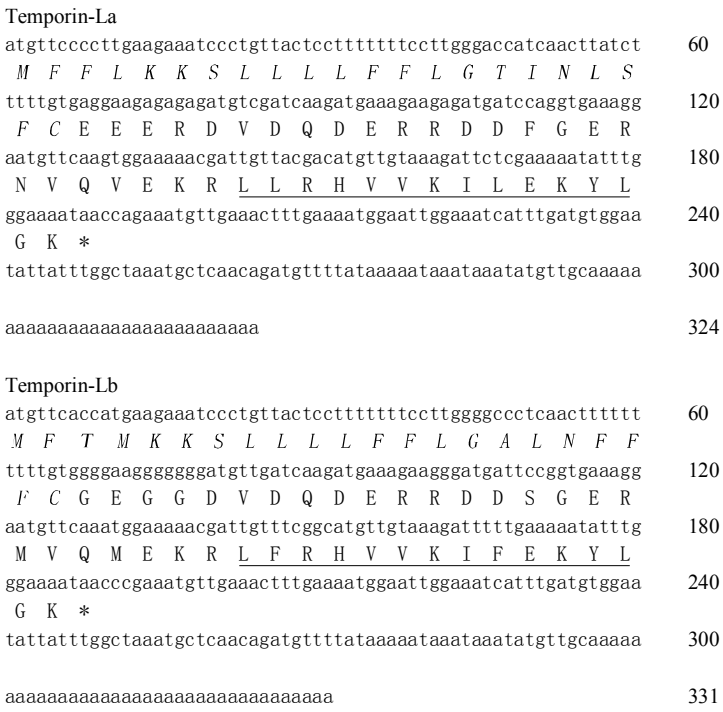

Fig. 1 Nucleotide and deduced amino acid sequences of temporin-La and temporin-Lb precursors

Sequences of the mature peptides are underlined. Signal peptides are indicated in italics. The asterisks represent the termination codon.

The deduced amino acid sequences, net charges, molecular masses, isoelectric points of temporin-La and temporin-Lb are shown in Table 1. Both of the deduced amino acid sequences of temporin-La and temporin-Lb compose of 13 amino acid residues with two residues differences at positions 2 and 9. The molecular masses are 1 623.08 u and 1 691.11 u separately. Temporin-La and temporin-Lb have net charges of +3 at pH 7.0, while most of the other temporins are less than +3. The deduced isoelectric points of temporin-La and temporin-Lb are 9.70. Like other temporins, the C-terminus of temporin-La and temporin-Lb are amidated.

Table 1 Primary structure, net charge, molecular masses and isoelectric points of temporin-La and temporin-Lb

		Net charge (pH7.0)	*M*/u	p*I*
Temporin-La	LLRHVVKILEKYLamide	+3	1 623.08	9.70
Temporin-Lb	LFRHVVKIFEKYLamide	+3	1 691.11	9.70

The isoelectric point values were calculated using the ExPASy MW/p*I* tool (http://www.expasy.ch/tools/pi_tool.html). The molecular masses were determined by ESI-MS mass spectrometry.

2.2 Antimicrobial and haemolytic activities of temporin-La and temporin-Lb

The synthetic temporin-La and temporin-Lb were used to determine the antimicrobial activity. The results were shown in Table 2. Compared with Gram-negative bacteria, temporin-La and temporin-Lb were more effective against Gram-positive bacteria which was consistent with the other temporins previously reported [8, 9, 14, 15, 17, 18]. Furthermore, it was reported that temporins not only effective against bacteria, but also fungi[8, 9, 14, 15]. However, the antifungal activities of temporin-La and temporin-Lb were not determined. Temporin-La showed no haemolytic activity against rabbit erythrocytes at the concentration of 250 mg/L, while temporin-Lb induced rabbit erythrocytes haemolysis ($LC_{50} \approx 230$ μmol/L).

Table 2 Antimicrobial activity of temporin-La and temporin-Lb

Bacterial	MIC/(mg•L^{-1})	
	Temporin-La	Temporin-Lb
Gram-positive bacteria		
Listeria ATCC 54004	ND	62.5
S. aureus ATCC 25923	7.8	7.8
S. suis 2 CVCC 606	15.6	7.8
Gram-negative bacteria		
Salmonella ATCC 20020	ND	62.5
E. coli ATCC 25922	125	250
K. pneumoniae ATCC 700603	250	125

The data represent mean values of three independent experiments performed in triplicates. ND: not determined.

2.3 Electron microscopy

Transmission electron microscopy was performed to evaluate the possible mechanisms of action of temporin-La and temporin-Lb on Gram-positive bacteria. Temporin-La and temporin-Lb showed different effects on treated cells of *Staphylococcus aureus* ATCC 25923 (Figure 2). The cytoplasmic membrane could be seen separating from the cell walls and large pores formed in temporin-La-treated cells. Meanwhile, fibers extending from the cell surface were seen in temporin-Lb-treated cells.

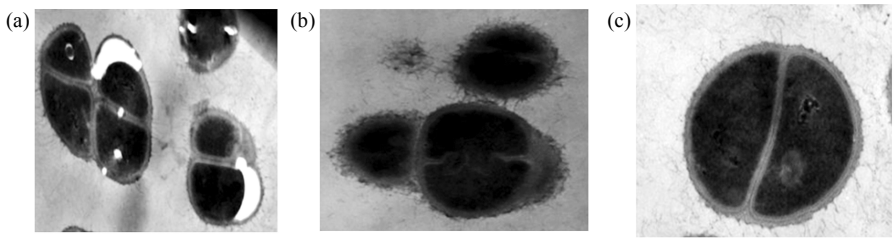

Fig. 2 Electron micrographs of temporin-La treated(a), temporin-Lb treated(b), and untreated(c) *S. Aureus*

Peptides are at the concentrations 10 times of the MIC.

3 Discussion

Being the first group of multicellucer organisms connecting water and land, amphibians are forced to adapt to various environments filled with diverse pathogenic microbes. As a result, they evolve an excellent chemical defense system composed of pharmacological and antimicrobial peptides [21 ~23]. A large number of antimicrobial peptides have been identified from *Rana* species and they can be divided into 14 different families according to broad structural characteristics[10~12, 24].

Temporin-La and temporin-Lb originate from *Lithobates catesbeianus*, a kind of *Rana* speices originally lives in North Africa, but now distributes all around the world. The amino acid sequences of temporin-La and temporin-Lb are compared with some of the other temporins with 13 or less residues in Figure 3. In contrast with other temporins compared, which containing two or less basic residues, temporin-La and temporin-Lb contain three basic residues at position 3, 7 and 11. The existence of the three basic residues results in temporin-La and temporin-Lb more positively charged and perhaps can explain the reason of their strong antimicrobial activities. Interestingly, similar to temporin-La and temporin-Lb, most of 13-residue temporins with strong antimicrobial activities contain one or two basic residues at position 7 and 11. Their homologous structures imply that the basic residues at position 7 and/or 11 may play an important role in their antimicrobial activity. To our knowledge, most of temporins with 13 residues contain no acid residue, one exception is temporin-1Ja from *Rana japonica* with an Asp residue at position 11[25]. However, there is a Glu residue at position 10 of temporin-La and temporin-Lb. Existence of the Glu residue would make sense and needed to be further investigated. Consistent with other temporins, the two amino acid residues at the C-terminus of deduced amino acid sequences of temporin-La and temporin-Lb are Lys and Gly. During processing, removal of the Lys residue by carboxypeptidase exposes the Gly residue, which serves as an amide donor for the C-terminus

Temporin-G	FFPVIG**R**ILNG-ILGK	[*Rana temporaria*]
Temporin-A	FLPLIG**R**VLSG-ILGK	[*Rana temporaria*]
Temporin-F	FLPLIG**K**VLSG-ILGK	[*Rana temporaria*]
Temporin-H	---LSPNLLKS-LLGK	[*Rana temporaria*]
Temporin-K	---LLPNLLKS-LLGK	[*Rana temporaria*]
Temporin-B	LLPIVGNLLKS-LLGK	[*Rana temporaria*]
Temporin-D	LLPIVGNLLNS-LLGK	[*Rana temporaria*]
Temporin-E	VLPIIGNLLNS-LLGK	[*Rana temporaria*]
Temporin-C	LLPILGNLLNG-LLGK	[*Rana temporaria*]
Temporin-L	FVQWFS**K**FLG**R**-ILGK	[*Rana temporaria*]
Temporin-1Ca	FLPFLA**K**ILTG-VLGK	[*Rana clamitans*]
Temporin-1Cb	FLPLFASLIG**K**-LLGK	[*Rana clamitans*]
Temporin-1Cc	FLPFLASLLT**K**-VLGK	[*Rana clamitans*]
Temporin-1Cd	FLPFLASLLS**K**-VLGK	[*Rana clamitans*]
Temporin-1Ce	FLPFLATLLS**K**-VLGK	[*Rana clamitans*]
Temporin-1La	VLPLISMALG**K**-LLGK	[*Rana luteiventris*]
Temporin-1Lc	FLPILINLIH**K**GLLGK	[*Rana luteiventris*]
Temporin-1Sa	FLSGIVGMLG**K**-IFGK	[*Pelophylar saharica*]
Temporin-1Sb	FLPIVTNLLSG-LLGK	[*Pelophylar saharica*]
Temporin-1Sc	FLSHIAGFLSN-LFGK	[*Pelophylar saharica*]
Temporin-La	LL**R**HVV**K**IL**E**K-YLGK	[*Lithobates catesbeianus*]
Temporin-Lb	LF**R**HVV**K**IF**E**K-YLGK	[*Lithobates catesbeianus*]
Temporin-1Ja	ILPLVGNLLN**D**-LLGK	[*Rana japonica*]
Temporin-GH	FLPLLFGAISH-LLGK	[*Rana guentheri*]
Temporin-1DYa	FIGPIISALASLFGGK	[*Rana dybowskii*]
Temporin-1V	FLPLVG**K**ILSG-LIGK	[*Odorrana versabilis*]
Temporin-1P	FLPIVG**K**LLSG-LLGK	[*Rana pipiens*]
Temporin-1BYa	FLPIIA**K**VLSG-LLGK	[*Rana boylii*]
Consensus	FLPLLG LL LLGK	

Fig. 3 Comparison of the primary structures of temporins with 13 or less amino acid residues from various *Rana* species

Temporin-La and temporin-Lb are underlined. The shaded residues indicate conserved amino acid residues in the peptides. The residues to be emphasized and discussed are shown in bold.

amidation [15].

Antimicrobial assays revealed that temporin-La and temporin-Lb were effective against the bacteria tested. However, consistent with other temporins, temporin-La and temporin-Lb were more effective against Gram-positive bacteria. It is assumed that the cationic peptides attract and attach to the negatively charged cytoplasmic membrane first. With the number of peptides attaching to the cytoplasmic membrane increases, peptides begin to orientate perpendicularly and insert into the membrane, forming transmembrane pores [5, 18, 19]. Several models have been proposed to explain membrane permeabilization [5]. However, the exact antimicrobial mechanisms of temporins are still unknown. For this reason, transmission electron microscopy was performed to investigate clues to possible mechanisms of action of temporin-La and temporin-Lb on *Staphylococcus aureus*. The results showed that temporin-La caused separation of cytoplasmic membrane from cell walls and formation of several large pores, while temporin-Lb induced fibers extending from the treated bacterial surfaces. The results are unexpected and interesting. They verify that formation of transmembrane pores is not the unique mechanism for cationic antimicrobial peptides. There are many other possible targets of cationic antimicrobial peptides action against bacteria, including cell wall, DNA, RNA, cell matrix and various enzymes. Different antimicrobial peptides kill bacteria in different ways, even peptides of the same family and similar amino acid sequences [5, 24, 26]. Our work reported here are meaningful for us to understand the complexity of action of antimicrobial peptides and will aid in the exploitation of antimicrobial peptides as new therapeutics.

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牛蛙两个新 Temporins 基因的克隆及其抗菌机制的研究 *

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摘要 Temporins 是从蛙属中得到的一类羧基端酰胺化的疏水性抗菌肽，具有抗细菌、霉菌、酵母菌、原虫及病毒活性．为了研究牛蛙皮肤抗菌肽的多样性及其结构特点，根据 GenBank 数据库中蛙属抗菌肽基因信号肽序列设计简并引物，从牛蛙皮肤 cDNA 文库中克隆到两个新的 temporins 家族抗菌肽，命名为 temporin-La (LLRHVVKILEKYL$_{amide}$) 和 temporin-Lb (LFRHVVKIFEKYL$_{amide}$)．合成的 temporin-La 和 temporin-Lb 肽具有很强的抗菌活性，尤其是对革兰氏阳性细菌．溶血性测定结果表明，temporin-La 浓度高至 250 mg/L 时对兔红细胞仍无溶血活性，而 temporin-Lb 具有较弱的溶血活性(半数致死浓度 $LC_{50} \approx 230$ μmol/L)．通过透射电镜观察 temporin-La 和 temporin-Lb 处理过的金黄色葡萄球菌的细胞结构，发现它们都能直接地杀死细菌，但作用机制不一样．

关键词 抗菌肽，牛蛙，temporins，透射电镜
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