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Developmental and Comparative Immunology 29 (2005) 83–90
Developmental & Comparative Immunology
www.elsevier.com/locate/devcompimm

Host-defense peptides isolated from the skin secretions of the Northern red-legged frog *Rana aurora aurora*

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Received 2 March 2004; revised 25 April 2004; accepted 4 May 2004

Available online 15 June 2004

Abstract

Antimicrobial peptides in the skin secretions of anurans constitute a component of the innate immunity that protects the organism against invading pathogens. Four peptides with antimicrobial activity were isolated in high yield from norepinephrine-stimulated skin secretions of the Northern red-legged frog *Rana aurora aurora* and their primary structures determined. Ranatuerin-2AUa (GILSSFKGVAKGVAKNLAGKLLDELKCKITGC) showed potent growth-inhibitory activity against a range of Gram-positive and Gram-negative bacteria (minimum inhibitory concentrations <20 μM) but low hemolytic activity against human erythrocytes (50% hemolysis at 290 μM). Brevinin-1AUa (FLPILAGLAAKLVPKVFCSITKKC) and brevinin-1AUb (FLPILAGLAANILPKVFCSITKKC) also showed potent antimicrobial activity but were strongly hemolytic ($HC_{50} < 10$ μM). Temporin-1AUa (FLPIIGQLLSGLL.NH_2) atypically lacked a basic amino acid residue and showed very weak antimicrobial and hemolytic activity. Its biological function remains to be established. The primary structures of the antimicrobial peptides are consistent with a close phylogenetic relationship between *R. aurora*, *Rana boylii* and *Rana luteiventris*.

Keywords: Antimicrobial peptide; Frog skin; Ranatuerin-2; Brevinin-1; Temporin; Phylogeny

1. Introduction

Peptides with antimicrobial activity play an important role in the innate immunity that constitutes the first-line defense against invading pathogens for a wide range of vertebrate and invertebrate species [1]. The synthesis of such peptides in granular glands

Abbreviations: MIC, minimum inhibitory concentration; HC_{50}, concentration producing 50% hemolysis.

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0145-305X/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.dci.2004.05.003

located in the skin is a feature of several anuran (frog and toad) species, particularly those belonging to the families Bombinatoridae, Hylidae, Hyperoliidae, Myobatrachidae, Pipidae, and Ranidae (reviewed in Refs. [2–4]). Frogs of the genus *Rana*, comprising at least 250 species worldwide, produce a remarkably diverse array of antimicrobial peptides with virtually no single peptide from one species being found with an identical amino acid sequence in another [4]. This molecular heterogeneity is considered important in protecting the organism against invasion by a wide range of pathogenic microorganisms [5]. The peptides from ranid frogs comprise between 12 and 48 amino acid residues and can be grouped on the basis of limited structural similarity into families, many of which are believed to be evolutionarily related having arisen as a result of multiple gene duplication events [6]. Although, lacking any consensus amino acid sequences that are associated with biological activity, the *Rana* skin peptides, with few exceptions, are cationic, relatively hydrophobic and have the propensity to form an amphipathic α-helix in a membrane-mimetic solvent such as trifluoroethanol [7].

The red-legged frog *Rana aurora* is the largest native ranid frog in California and is divided into two subspecies based on morphological and behavioral characteristics but the precise systematic relationship between them is unclear [8]. The smaller Northern red-legged frog *Rana aurora aurora* occupies the northwestern region of California, north to southern British Columbia. The California red-legged frog *Rana aurora draytonii* traditionally occupied central and southern portions of the state but the subspecies has disappeared from 75% of its historic range and has now been declared a threatened species by the fish and wildlife services [9]. The habitat of *R. aurora aurora* is generally cool, well-vegetated areas near deep pools and intermittent streams, although the frogs may leave riparian zones for upland forests during the non-breeding season.

In the laboratory (or in the field), skin secretions may be collected from ranid frogs under non-invasive conditions that do not appear to cause major discomfort or long-term harm to the animal. Mild electrical stimulation [10] or injection of norepinephrine into the dorsal lymph sac [11] is generally effective. The aim of the present study is the purification and structural characterization of the antimicrobial peptides present in norepinephrine-stimulated skin secretions from *R. aurora aurora* and an investigation of their growth-inhibitory properties against a range of pathogenic microorganisms and their hemolytic activities against human erythrocytes.

2. Methods

2.1. Collection of skin secretions

Three adult male specimens of *R. aurora aurora* were collected in the neighbourhood of Korbel, Humboldt County, Northern California (California Department of Fish and Game permit number 801134-5). Skin secretions were collected as previously described [12]. In brief, the animals were injected bilaterally with norepinephrine (2 nmol/g body wt) and placed in a solution (100 ml) of composition 25 mM sodium chloride/25 mM ammonium acetate, pH 7.0 for 15 min. The combined secretions and washings were acidified by addition of trifluoroacetic acid (1 ml) and passed at a flow rate of 2 ml min^{-1} through 4 Sep-Pak C-18 cartridges (Waters Associates, Milford, MA) connected in series. Bound material was eluted with acetonitrile/water/trifluoroacetic acid (70.0:29.9:0.1, v/v/v) and freeze-dried. All animals were released unharmed at the exact sites of collection within 24 h.

2.2. Antimicrobial assays

Standard strains were purchased from the American Type Culture Collection (Rockville, MD) and from the Hungarian National Type Culture Collection (Budapest, Hungary). Purification of the peptides was monitored by incubating lyophilized aliquots of chromatographic effluent in Mueller–Hinton broth (50 μl) with an inoculum (50 μl of 10^6 colony forming units ml^{-1}) from a log-phase culture of *Staphylococcus aureus* (NCTC 8325) and *Escherichia coli*(ATCC 25922) in 96-well microtiter cell-culture plates for 18 h at 37 °C in a humidified atmosphere of air. After incubation, the absorbance at 630 nm of each well was determined using a microtiter plate reader. Minimum inhibitory concentrations (MIC)

for *E. coli*, *S. aureus*, *Staphylococcus epidermidis* (RP62A), *Enterococcus faecalis* (ATCC 29212), *Streptococcus* Group B (HNTCC 80130), *Enterobacter cloacae* (NHTCC 53001), *Klebsiella pneumoniae* (KK3 9904), *Proteus mirabilis* (ATCC 25933), *Pseudomonas aeruginosa* (ATCC 27853) were measured by a standard microdilution method [13] and were taken as the lowest concentration of peptide where no visible growth was observed. Incubations with the yeast *Candida albicans* (ATCC 90028) were carried out in RPMI 1640 medium for 48 h at 35 °C. In order to monitor the validity of the assays, incubations with bacteria were carried out in parallel with increasing concentrations of the broad-spectrum antibiotic, bacitracin and incubations with *C. albicans* in parallel with amphotericin B.

2.3. Hemolytic assay

Peptides in the concentration range 1–300 μM were incubated with washed human erythrocytes (2×10^7 cells) from a healthy donor in Dulbecco's phosphate-buffered saline, pH 7.4 (100 μl) for 1 h at 37 °C. After centrifugation (12,000 × *g* for 15 s), the absorbance at 450 nm of the supernatant was measured. A parallel incubation in the presence of 1% v/v Tween-20 was carried out to determine the absorbance associated with 100% hemolysis. The HC_{50} value was taken as the mean concentration of peptide producing 50% hemolysis.

2.4. Peptide purification

The skin secretions, after partial purification on Sep-Pak cartridges, were redissolved in 0.1% (v/v) trifluoroacetic acid/water (2 ml) and injected onto a (1 × 25-cm) Vydac 218TP510 (C-18) reverse-phase HPLC column (Separations Group, Hesperia, CA) equilibrated with 0.1% (v/v) trifluoroacetic acid/water at a flow rate of 2.0 ml min^{-1}. The concentration of acetonitrile in the eluting solvent was raised to 21% (v/v) over 10 min and to 63% (v/v) over 60 min using linear gradients. Absorbance was monitored at 214 and 280 nm and fractions (1 min) were collected. The ability of freeze-dried aliquots (50 μl) of the fractions to inhibit the growth of *S. aureus* and *E. coli* was determined as described in Section 2.3. Fractions with antimicrobial activity were successively chromatographed on a (1 × 25-cm) Vydac 214TP510 (C-4) column and a (1 × 25-cm) Vydac 219TP510 (phenyl) column. The concentration of acetonitrile in the eluting solvent was raised from 21 to 56% over 50 min and the flow rate was 2.0 ml min^{-1}.

2.5. Structural characterization

The primary structures of the peptides were determined by automated Edman degradation using an Applied Biosystems model 494 Procise sequenator. Mass spectrometric analysis was performed on a Voyager RP MALDI-TOF instrument (Perspective Biosystems Inc.) equipped with a nitrogen laser (337 nm). The instrument was operated in reflector mode with delayed extraction and the accelerating voltage in the ion source was 25 kV. The accuracy of mass determinations was within 0.1%.

3. Results

3.1. Peptide purification

The elution profile on a semipreparative Vydac C-18 column of the skin secretions, after partial purification on Sep-Pak C-18 cartridges, is shown in Fig. 1. The prominent peaks designated 1 (subsequently shown to contain ranatuerin-2AUa), 2 (brevinin-1AUa), and 3 (brevinin-1AUb) contained components that inhibited the growth of *E. coli* and *S. aureus*. The peak designated 4 (temporin-1AUa) contained material that inhibited the growth of *S. aureus* only. The peptides were purified to near homogeneity, as assessed by a symmetrical peak shape and by mass spectrometry, by further chromatography on Vydac C-4 and Vydac phenyl columns. The purification of ranatuerin-2AUa is illustrated in Fig. 2. The final yields of purified peptides were ranatuerin-2AUa 810 nmol, brevinin-1AUa 495 nmol, brevinin-1AUb 1420 nmol, and temporin-1AUa 450 nmol.

3.2. Structural characterization

The primary structures of the antimicrobial peptides were established without ambiguity by automated Edman degradation and are shown in

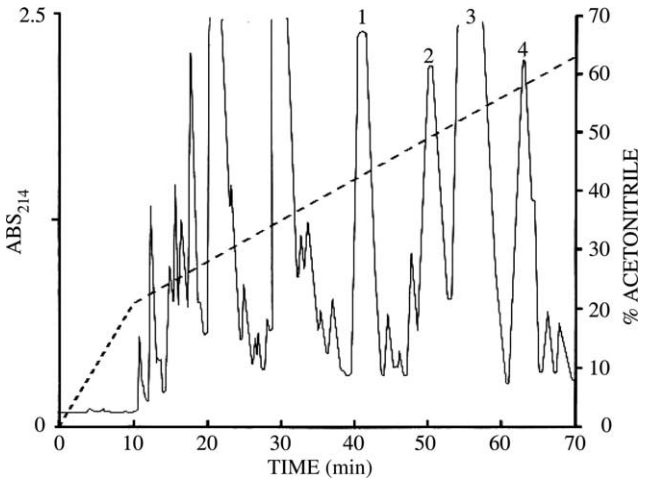

Fig. 1. Reverse-phase HPLC on a semipreparative Vydac C-18 column of skin secretions of *R. aurora aurora* after partial purification on Sep-Pak cartridges. Peak 1 contained ranatuerin-2AUa; peak 2, brevinin-1AUa; peak 3, brevinin-1AUb; and peak 4, temporin-1AUa. The dashed line shows the concentration of acetonitrile in the eluting solvent.

Fig. 3. The presence of an intramolecular disulfide bridge in ranatuerin-2AUa, brevinin-1AUa and brevinin-1AUb and the presence of a C-terminally α-amidated leucine residue in temporin-1AUa were demonstrated by mass spectrometry.

3.3. Antimicrobial and hemolytic properties

The MIC of ranatuerin-2AUa, brevinin-1AUa, and brevinin-1AUb against a range of reference strains of Gram-positive and Gram-negative bacteria and the HC_{50} values against human erythrocytes are shown in Table 1. Temporin-1AUa was tested only against the Gram-positive bacteria and MIC values were >80 μM. The HC_{50} value of temporin-1AUa was >300 μM.

4. Discussion

This study has identified in the skin secretions of *R. aurora aurora* a peptide (ranatuerin-2AUa)

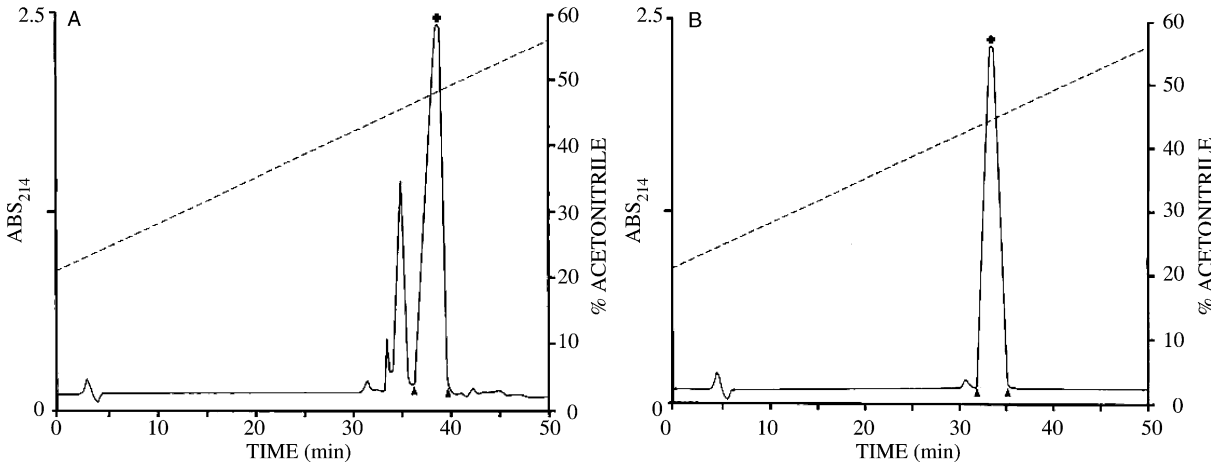

Fig. 2. Purification of ranatuerin-2AUa from *R. aurora aurora* skin secretions on semipreparative (A) Vydac C-4 and (B) Vydac phenyl columns. The peak containing the active peptide is denoted by (+) and the arrowheads show where peak collection began and ended.

		$M_{r(obs)}$	$M_{r(calc)}$
Ranatuerin-2AUa	GILSSFKGVAKGVAKNLAGKLLDELKCKITGC	3261.2	3260.8
Brevinin-1AUa	FLPILAGLAAKLVPKVFCSITKKC	2559.5	2559.5
Brevinin-1AUb	FLPILAGLAANILPKVFCSITKKC	2559.4	2559.5
Temporin-1AUa	FLPIIGQLLSGLL.NH_2	1382.7	1382.7

Fig. 3. Primary structures, observed molecular masses M_r (obs) and calculated molecular masses M_r (calc) of the antimicrobial peptides isolated from *R. aurora aurora* skin secretions.

belonging to the ranatuerin-2 family of antimicrobial peptides that shows relatively high potency against several species of Gram-positive (*S. aureus*, *S. epidermidis*, *Streptococcus* Group B) and Gram-negative (*E. coli*, *E. cloacae*, *P. aeruginosa*, *K. pneumoniae*) bacteria but has very low hemolytic activity (Table 1). In common with all other frog skin peptides that have been studied in the investigator's laboratory [14–16], the peptide was not active against *P. mirabilis* at the maximum concentration tested (40 μM). The microorganisms selected for study are those that are most commonly found in infected external lesions, such as foot ulcers in diabetic patients [17]. The ranatuerin-2 family, first identified in the skin of the bullfrog *Rana catesbeiana* [18], is widely distributed in ranid frogs of North American origin and members of the family have been isolated from skin secretions and/or skin extracts of *Rana grylio* [19], *Rana clamitans* [20], *Rana boylii* [21], *Rana berlandieri* [22], *Rana pipiens* [22], *Rana luteiventris* [22], *Rana tarahumarae* [12], *Rana palustris* [23], and *Rana aerolata* [24]. At this time, ranatuerin-2 has been identified in only one Eurasian species, *Rana pirica* [15]. The primary structure of the peptide has been poorly conserved across species with several residue deletions and only five amino acids (Gly^1, Ala^{15}, Lys^{22}, Cys^{23}, and Cys^{28} in the *R. catesbeiana* sequence) invariant. This variation in amino acid sequence is matched by a correspondingly wide range of antimicrobial potencies but, in general, the previously studied ranatuerin-2 peptides are only weakly active against Gram-positive bacteria and activity against *C. albicans* is generally very low or absent [4].

The present study has also led to the isolation of two structurally closely related peptides belonging to the brevinin-1 family that showed particularly high potency against Gram-positive bacteria

Table 1
Antimicrobial and hemolytic activities of peptides isolated from the skin secretions of *Rana aurora aurora*

	Ranatuerin-2AUa	Brevinin-1AUa	Brevinin-1AUb	Temporin-1AUa
E. coli	5	13	25	ND
P. aeruginosa	5	25	25	ND
E. cloacae	5	13	25	ND
K. pneumoniae	10	50	>50	ND
P. mirabilis	>40	>50	>50	ND
S. aureus	20	3	3	>80
S. epidermidis	20	6	6	>80
E. faecalis	>40	13	6	>80
Streptococcus Group B	20	13	13	>80
C. albicans	>40	3	3	ND
HC_{50}	290	5	7	>300

Data for microorganisms show minimum inhibitory concentrations (μM); HC_{50}, concentration (μM) producing 50% hemolysis of human erythrocytes; ND, not determined.

and against *C. albicans* (Table 1). However, the peptides were strongly hemolytic towards human erythrocyes, which severely limits their therapeutic potential. Brevinin-1 peptides, first identified in the skin of the Asian frog *Rana brevipoda porsa* [25], are the most widely distributed family of antimicrobial peptides among the ranid frogs, occurring in most Eurasian and N. American species [4]. Brevinin-1AUa showed greater potency than brevinin-1AUb against Gram-negative bacteria. As shown in Fig. 3, Lys^{11}-Leu^{12}-Val^{13} in brevinin-1AUa is replaced by Asn-Ile-Leu in brevinin-1AUb resulting in a decrease in cationicity (a net charge of +2.73 compared with 3.73 at pH 7.5). The cationic residues in an antimicrobial peptide are considered to be important in the initial binding to the negatively charged phospholipids in the cell membranes of Gram-negative bacteria, and up to a certain limit, there is a good correlation between peptide cationicity and antimicrobial potency for several peptides that have been investigated [26].

A comparison of the amino acid sequences of the brevinin-1 and ranatuerin-2 peptides from *R. aurora* with orthologs from other ranid frogs demonstrates closest structural similarity with peptides from the foothill yellow-legged frog *R. boylii* [21] and the Columbia spotted frog, *R. luteiventris* (formerly *Rana pretiosa*) [22] (Fig. 4). By way of comparison, this figure depicts the sequences of the orthologous peptides from the bullfrog *R. catesbeiana* [18]. This observation is consistent with the conclusion based upon the nucleotide sequences of mitochondrial DNA that *R. aurora*, *R. boylii*, *Rana cascadae*, *Rana muscosa* and *R. pretiosa* form a well-defined monophyletic group that is approximately 8 million years old [27].

The antimicrobial properties of ranatuerin-2AUa and ranatuerin-2BYa (from *R. boylii*) were measured

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Ranatuerin-2AUa      G*ILSSFKGVAKGVAKNLAGKLLDELKCKITGC
Ranatuerin-2Lb       -*----I----------V-AQ---T--------
Ranatuerin-2BYa      -*---T---L------D---N---KF-------
Ranatuerin-2Ca       -LF-DTL--A--D--****GKL*EG--------KLP

Brevinin-1AUa        FLPILAGLAAKLVPKVFCSITKKC
Brevinin-1AUb        ----------NIL-----------
Brevinin-1La         ---M------SM---LV-L-----
Brevinin-1Lb         ---M------SM---FV-L-----
Brevinin-1BYa        ------S----FG--L--LV----
Brevinin-1BYb        ------S-----G--L--LV----
Brevinin-1BYc        ------S---T-G--LL-L-----
Brevinin-1Ca         ---FI-R----VF-SII--V----

Temporin-1AUa        FLPIIGQLLSGLL.NH2
Temporin-1La         V--L-SMA-GK--.NH2
Temporin-1BYa        -----AKV-----.NH2
Temporin-1Ca         -ISA-ASM-GKF-.NH2
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Fig. 4. A comparison of the primary structure of antimicrobial peptides of the brevinin-1, ranatuerin-2 and temporin families from *R. aurora* (AU), *R. luteiventris* (L), *R. boylii* (BY) and *R. catesbeiana* (C). [–] denotes residue identity. In order to maximize structural similarity, gaps denoted by [*] have been introduced into the ranatuerin-2 sequences.

under identical conditions and potencies against *E. coli* and *S. aureus* are comparable. Neither peptide was active against the yeast opportunistic pathogen, *C. albicans*. Ranatuerin-2AUa, however, was appreciably less hemolytic than ranatuerin-2BYa ($HC_{50} = 290$ μM compared with 120 μM). Ranatuerin-2AUa contains six amino acid substitutions compared with ranatuerin-2BYa ($Thr^5 \rightarrow Ser$, $Leu^9 \rightarrow Val$, $Asp^{16} \rightarrow Asn$, $Asn^{20} \rightarrow Lys$, $Lys^{24} \rightarrow Glu$, and $Phe^{25} \rightarrow Leu$) (Fig. 4). These substitutions do not affect the cationicity of the peptides, which have a predicted net charge of +3.73 at pH 7.5 in both cases (EMBL WWW gateway to isoelectric point service; http://www.embl-heidelberg.de/cgi/pi-wrapper.pl). A good correlation between increasing hydrophobicity and mammalian cell toxicity has been demonstrated for several antimicrobial peptides [7,28] and so it is suggested that the decreased hemolytic activity of ranatuerin-2AUa is a consequence of replacement of Thr, Leu, Asn and Phe by less hydrophobic amino acids.

The biological role of the peptides present in the skin secretions of *R. aurora* is a matter for speculation. Peptides of the ranatuerin-2 [12,29] and brevinin-1 [12] families are active at relatively low concentrations against *Batrachochytrium dendrobatidis*, the chytrid fungus associated with global amphibian declines and ranatuerin-2P (from *R. pipiens*) will inactivate frog virus 3, a potentially pathogenic iridovirus infecting anurans [30]. Thus, these peptides may be important in protecting the frog against the kind of pathogen to which it might be exposed in the wild. The biological role of temporin-1AUa, which was present in the skin secretions in high concentration, is unclear. Peptides of the temporin family were first isolated from the skin of the Eurasian frog, *Rana temporaria* [31] and are widely distributed among ranid species [4]. Temporin-1AUa lacks measurable activity against *E. coli* and has only very weak activity against the Gram-positive bacteria tested (MIC >80 μM). Unlike the active orthologs from *R. boylii* [21] and *R. luteiventris* [22] (Fig. 4), temporin-1AUa lacks a basic residue (isoelectric point 7.85), which is probably the factor responsible for the low antimicrobial activity. Peptides of the temporin family have been shown to be active against *B. dendrobatidis* but all peptides tested were strongly cationic [32]. Consequently, it seems unlikely that the temporin isolated in this study has a direct role in defense against invading microorganisms and an alternative biological function should be sought.

Acknowledgements

This work was supported by an Interdisciplinary Grant (03/12-8-03-01) and a Faculty Support Grant (NP/04/02) from the United Arab Emirates University. The authors thank Lowell Diller and Laura Burkholder, Department of Wildlife, Humboldt State University for help in collection of specimens.

References

[1] Hoffmann JA, Kafatos FC, Janeway CA, Ezekowitz RA. Phylogenetic perspectives in innate immunity. Science 1999; 284:13–18.
[2] Simmaco M, Mignogna G, Barra D. Antimicrobial peptides from amphibian skin: what do they tell us ? Biopolymers 1998; 47:435–50.
[3] Kawasaki H, Isaacson T, Iwamuro S, Conlon JM. A protein with antimicrobial activity in the skin of Schlegel's green tree frog *Rhacophorus schlegelii* (Rhacophoridae) identified as histone H2B. Biochem Biophys Res Commun 2003;312: 1082–6.
[4] Conlon JM, Kolodziejek J, Nowotny N. Antimicrobial peptides from ranid frogs: taxonomic and phylogenetic markers and a potential source of new therapeutic agents. Biochim Biophys Acta 2004;1696:1–14.
[5] Mor A, Hani K, Nicolas P. The vertebrate peptide antibiotics dermaseptins have overlapping structural features but target specific microorganisms. J Biol Chem 1994;269:31635–41.
[6] Duda Jr TF, Vanhoye D, Nicolas P. Roles of diversifying selection and coordinated evolution in the evolution of amphibian antimicrobial peptides. Mol Biol Evol 2002;19: 858–64.
[7] Giangaspero A, Sandri L, Tossi A. Amphipathic α-helical peptides. A systematic study of the effects of structural and physical properties on biological activity. Eur J Biochem 2001;268:5589–600.
[8] Hayes MP, Miyamoto MM. Biochemical, behavioral, and body size differences between *Rana aurora aurora* and *Rana aurora draytoni*. Copeia 1984;1018–22.
[9] Davidson C, Shaffer HB, Jennings MR. Declines of the California red-legged frog: climate, UV-B, habitat and pesticides hypotheses. Ecol Appl 2001;11:464–79.
[10] Tyler MJ, Stone DJ, Bowie JH. A novel method for the release and collection of dermal, glandular secretions from the skin of frogs. J Pharmacol Toxicol Methods 1992;28:199–200.

[11] Nutkins JC, Williams DH. Identification of highly acidic peptides from processing of the skin prepropeptides of *Xenopus laevis*. Eur J Biochem 1989;181:97–102.
[12] Rollins-Smith LA, Reinart LK, Miera V, Conlon JM. Antimicrobial peptide defenses of the Tarahumara frog, *Rana tarahumarae*. Biochem Biophys Res Commun 2002; 297:361–7.
[13] National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 4th ed. Approved standard M7-A4, Wayne, PA: NCCLS; 1997.
[14] Ali MF, Soto A, Knoop FC, Conlon JM. Antimicrobial peptides isolated from skin secretions of the diploid frog, *Xenopus tropicalis* (Pipidae). Biochim Biophys Acta 2001; 1500:81–9.
[15] Conlon JM, Sonnevend A, Patel M, Al-Dhaheri K, Nielsen PF, Kolodziejek J, Nowotny N, Iwamuro S, Pál T. A family of brevinin-2 peptides with potent activity against *Pseudomonas aeruginosa* from the skin of the Hokkaido frog, *Rana pirica*. Regul Pept 2004;118:135–41.
[16] Sonnevend A, Knoop FC, Patel M, Pál T, Soto AM, Conlon JM. Antimicrobial properties of the frog skin peptide, ranatuerin-1and its [Lys-8]-substituted analog. Peptides 2004;25:29–36.
[17] Ge Y, MacDonald D, Hait H, Lipsky B, Zasloff M, Holroyd K. Microbiological profile of infected diabetic foot ulcers. Diabetic Med 2002;19:1032–5.
[18] Goraya J, Knoop FC, Conlon JM. Ranatuerins: antimicrobial peptides isolated from the skin of the American bullfrog *Rana catesbeiana*. Biochem Biophys Res Commun 1998;250: 589–92.
[19] Kim JB, Halverson T, Basir YJ, Dulka J, Knoop FC, Conlon JM. Purification and characterization of antimicrobial peptides from skin extracts and skin secretions of the North American pig frog *Rana grylio*. Regul Pept 2000;90:53–60.
[20] Halverson T, Basir YJ, Knoop FC, Conlon JM. Purification and characterization of antimicrobial peptides from the skin of the North American green frog *Rana clamitans*. Peptides 2000;21:469–76.
[21] Conlon JM, Sonnevend A, Patel M, Davidson C, Nielsen PF, Pál T, Rollins-Smith LA. Isolation of peptides of the brevinin-1 family with potent candidacidal activity from the skin secretions of the frog *Rana boylii*. J Peptide Res 1999;62: 207–13.
[22] Goraya J, Wang Y, Li Z, O'Flaherty M, Knoop FC, Platz JE, Conlon JM. Peptides with antimicrobial activity from four different families isolated from the skins of the North American frogs, *Rana luteiventris*, *Rana berlandieri* and *Rana pipiens*. Eur J Biochem 2000;267:894–900.
[23] Basir YJ, Knoop FC, Dulka J, Conlon JM. Multiple antimicrobial peptides and peptides related to bradykinin and neuromedin N isolated from the skin secretions of the North American pickerel frog, *Rana palustris*. Biochim Biophys Acta 2000;1543:95–105.
[24] Ali MF, Lips KR, Knoop FC, Fritzsch B, Miller C, Conlon JM. Antimicrobial peptides and protease inhibitors in the skin secretions of the crawfish frog, Rana areolata. Biochim Biophys Acta 2002;1601:55–63.
[25] Morikawa N, Hagiwara K, Nakajima T. Brevinin-1 and -2, unique antimicrobial peptides from the skin of the frog, *Rana brevipoda porsa*. Biochem Biophys Res Commun 1992;189: 184–90.
[26] Yeaman MR, Yount NY. Mechanisms of antimicrobial peptide action and resistance. Pharmacol Rev 2003;55:27–55.
[27] Macey JR, Strasburg JL, Brisson JA, Vredenburg VT, Jennings M, Larson A. Molecular phylogenies of western North American frogs of the *Rana boylii* species group. Mol Phylogenet Evol 2001;19:131–43.
[28] Wieprecht T, Dathe M, Krause E, Beyermann M, Maloy WL, MacDonald DL, Bienert M. Modulation of membrane activity of amphipathic, antibacterial peptides by slight modifications of the hydrophobic moment. FEBS Lett 1997;417:135–40.
[29] Rollins-Smith LA, Carey C, Longcore J, Doersam JK, Boutte A, Bruzgal JE, Conlon JM. Activity of antimicrobial skin peptides from Ranid frogs against *Batrachochytrium dendrobatidis*, the chytrid fungus associated with global amphibian declines. Dev Comp Immunol 2002;26:471–9.
[30] Chinchar VG, Wang J, Murti G, Carey C, Rollins-Smith L. Inactivation of frog virus 3 and channel catfish virus by esculentin-2P and ranatuerin-2P, two antimicrobial peptides isolated from frog skin. Virology 2001;288:351–7.
[31] Simmaco M, Mignogna G, Canofeni S, Miele R, Magoni ML, Barra D. Temporins, antimicrobial peptides from the European red frog *Rana temporaria*. Eur J Biochem 1996;242: 788–92.
[32] Rollins-Smith LA, Carey C, Conlon JM, Reinert LK, Doersam JK, Bergman T, Silberring J, Lankinen H, Wade D. Antimicrobial activity of temporin family peptides against the chytrid fungus (*Batrachochytrium dendrobatidis*) associated with global amphibian declines. Antimicrob Agents Chemother 2003;47:1157–60.