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Antimicrobial properties of two purified skin peptides from the Mink Frog (*Rana septentrionalis*) against bacteria isolated from the natural habitat

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Abstract

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Numerous peptides exhibiting antimicrobial properties have been isolated from the skins of many amphibian species. These peptides offer an innate chemical defense system against various microbial agents that exist in the amphibian's environment. Amphibian skin peptides are typically tested for antimicrobial activity against microbial strains that are pathogenic to humans, but not on potential pathogenic or opportunistic bacteria that exist in the organism's habitat. Two peptides, a brevinin-2 related peptide and temporin-1SPb previously isolated from secretions of the Mink Frog, *Rana septentrionalis*, were tested for antimicrobial activity on bacterial isolates endemic to the frog's habitat. Ten isolates were identified, using 16S rRNA sequencing techniques, in the genera *Pseudomonas*, *Serratia*, *Bacillus*, *Aeromonas*, *Burkholderia*, *Microbacterium*, and *Delftia*. Bacterial isolates were tested with peptides at concentrations ranging from 0.8 μ M to 1000 μ M to determine the minimum inhibitory concentration (MIC) to inhibit growth. Growth of four of the isolates was inhibited by temporin-1SPb at the concentrations used, but all of the isolates were inhibited by the brevinin-2 related within the range of peptide concentrations used. This demonstrates the efficacy of both peptides as a component of the frog's innate chemical defense system.

Keywords

Antimicrobial peptides; Brevinin-2 related; *Rana septentrionalis*; Temporin-1SPb

Introduction

The innate immunity of vertebrates to microbial invasion is mediated by a network of host-defense mechanisms involving in part a nonspecific chemical defense system that includes broad-spectrum antimicrobial peptides (Bals, 2000). Amphibian antimicrobial peptides are

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secreted by non-lymphoid cells on the mucosal surfaces of the respiratory and gastrointestinal tracts, and by the granular glands of the skin (Nicolas and Mor, 1995; Woodhams et al., 2005). These peptides inhibit growth of numerous pathogenic microorganisms, including enveloped viruses, Gram-positive and Gram-negative bacteria, protozoa, and fungi (Nicolas and Mor, 1995; Baba and Schneewind, 1998; Hancock, 2001; Zasloff, 2002). Although debate continues over the specific killing mechanism of antimicrobial peptides, there is general consensus that microbial death initiated by the cationic peptides involves membrane disruption and subsequent unregulated ion exchange with the environment (Sitaram and Nagaraj, 2002; Mäntylä et al., 2005). This explanation is validated by the observation that antimicrobial peptides work very rapidly, apparently far too quickly for any process that involves translocation of the peptide across the cell membrane for binding to an intracellular target molecule (Sitaram and Nagaraj, 2002).

Antimicrobial peptides isolated from skin secretions produced by various amphibians include magainins from the African clawed frog, *Xenopus laevis* (Zasloff, 1987), bombinins from the European toad, *Bombina orientalis* (Gibson et al., 1991), and temporins and ranatuerins from North American ranids (Conlon et al., 2004a). One of the most striking characteristics of these peptides is the broad spectrum of antimicrobial activity they exhibit (Mangoni et al., 2003; Conlon, 2004b). Temporins isolated from *Rana temporaria*, for example, are effective individually against Gram-positive bacteria such as *Staphylococcus aureus* Cowan I. (Mangoni et al., 2000) and work synergistically against Gram-negative and mutant strains of bacteria by disrupting both the outer envelope and cell membrane (Rosenfield et al., 2006). Members of the temporin and brevinin families of peptides are represented in the current study and have been shown to inhibit growth of *Batrachochytrium dendrobatidis*, the pathogenic chytrid fungus associated with amphibian population declines worldwide (Berger et al., 1998; Rollins-Smith et al., 2002a; Rollins-Smith et al., 2003; Woodhams et al., 2005).

Peptides isolated from amphibian skin secretions are typically tested for antimicrobial activity against bacteria that are pathogenic to humans (e.g., *Escherichia coli* and *Staphylococcus aureus*), with less effort focused on strains that are endemic to the organism's natural habitat. Exceptions include antimicrobial assays on bacteria isolated from the natural flora of the frog's skin and oral cavity, such as *Aeromonas hydrophila* (e.g., Simmaco et al., 1998; Boman 2000; Mangoni et al., 2000; Rollins-Smith et al., 2002b). Strains endemic to and directly isolated from the organism's natural habitat have not been examined systematically. The current investigation takes this important and novel approach because amphibians necessarily are exposed to high concentrations of diverse microbial species in pond water and mud. Without an innate immunity to potential environmental pathogens, the amphibian lifestyle would be compromised. Understanding the breadth and intensity of this chemical defense mechanism is important given the vulnerable status of current amphibian populations (Pounds et al., 2006).

Given that one proposed function of amphibian skin secretions and their components is to protect an individual from adverse microbial opportunism and parasitism, we tested two peptides with antimicrobial properties produced in skin secretions of the Mink Frog, *Rana septentrionalis* (Bevier et al., 2004) for growth inhibition on 10 bacterial strains isolated

from either the frog skin surface or water and mud samples from the frog's habitat. These two peptides exhibit differential growth inhibition of both Gram-negative and Gram-positive laboratory standard microbial strains (Bevier et al., 2004). We report in this study the antimicrobial activity of these same two peptides on bacterial strains endemic to the natural habitat of *R. septentrionalis*.

Materials & Methods

Isolation of Bacterial Strains

Mud and water samples were collected in early June 2005 from a small pond (Somerset County, ME, USA) inhabited by a large population of *R. septentrionalis*. Water samples were collected from a slow moving stream which runs through the area by placing a sterile 50 mL graduated polypropylene tube 30 cm below the surface of the water. Mud samples were obtained from the bottom of the pond. A 500 μ L aliquot of the water sample was transferred and spread on the surface of a plate containing Brain Heart Infusion (BHI) solid plating media. One loop of soil was added to 40 mL of sterile deionized water, vortexed, then inoculated by streak plating on BHI solid plating media. One bacterial strain (RS12) was isolated from the dorsal area and foot webbing of an individual mink frog captured by hand from the pond. All cultures were incubated at room temperature. Once bacterial growth was observed, samples were streaked for isolation on BHI solid plating media. Purified stock isolates were maintained and routinely subcultured on BHI agar slant tubes at room temperature. Long-term stock cultures of each isolate were made by mixing 500 μ L each of fresh (24 h) BHI liquid culture and sterile glycerol in cryogenic vials and placing them immediately at -80°C .

Identification of endemic bacterial strains

Genomic DNA was isolated using E.Z.N.A. kits (Omega Bio-tek, Inc., Doraville, GA) and the manufacturer's protocol. All 10 isolates were identified based on 16S rRNA gene sequence similarities to known genera (Blastn against GenBank at <http://www.ncbi.nlm.nih.gov/BLAST>) using primers and cycling conditions described by Muyzer et al. (1993). Specifically, the variable V3 region of 16S rDNA was enzymatically amplified in the PCR with primers to conserved regions of the 16S rRNA genes. The nucleotide sequences of the primers were as follows: primer 1, 5'-CCTACGGGAGGCAGCAG-3'; primer 2, 5'-ATTACCGCGGCTGCTGG-3' (Muyzer et al., 1993). Reactions contained 50 to 100 ng of genomic DNA, 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X100, 0.2 mg mL⁻¹ bovine serum albumin, 1 mM dNTP, 0.5 pM of each primer, and 0.5 U QBioTaq polymerase (Qbiogene, Carlsbad, CA). PCR product sizes of the amplified 174-200 bp hypervariable region of 16S rDNA were confirmed on a 1.5% agarose gel containing 0.4 μ g mL⁻¹ ethidium bromide by UV transillumination (Foto/Analyst Investigator, Fotodyne Incorporated, Harland, WI). The 16S rDNA PCR products were then purified using the Omega Bio-tek E.Z.N.A. Cycle-Pure Kit (Doraville, GA). Forward and reverse sequencing of a 174-200 bp hypervariable region of 16S rDNA was accomplished using the Big Dye Terminator Reaction Kit (Applied Biosystems, Foster City, CA). All sample reactions were run in 10 μ L volumes and were then purified using Princeton Separations Centri-Sep Spin columns (Adelphia, NJ). After samples were purified and dried

they were reconstituted in 20 μ L of Applied Biosystems Template Suppression Reagent, heated for 2 min at 95° C and chilled on ice before sequencing. The sequencer used at the Colby College DNA Sequencing laboratory was an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). API 20E strips (BioMerieux, Marcy-L'Etoile, France) were used to classify an isolate to the genus level in the event of multiple similarly scored Blastn results.

Antimicrobial Assays

Two peptides were selected based on their broad-spectrum antimicrobial activity from Bevier et al. (2004) (Brevinin-2 related [NH₂-GIWDTIKSMGKVFAGKILQNL-CONH₂] and Temporin-1SPb [NH₂-FLSAITSLLGKLL-CONH₂]). These peptides were synthesized and purified (University of Pittsburgh's Molecular Medicine Institute- Peptide Synthesis Facility), then stored at -20°C. For the experiment, peptides were diluted in purified water to concentrations of 0.8, 1.6, 3.13, 6.25, 12.5, 25, 50, 100, 250, 500, and 1000 μ M, and were sterilized by filtration using 0.22 μ m pore size membrane filters.

Minimum inhibitory concentration (MIC) assays for the 10 endemic bacterial strains were measured by the standard microdilution method (National Committee for Clinical Laboratory Standards, 1997), using protocols adapted from Bevier et al. (2004) and Rollins-Smith et al. (2002a). Assays were run in triplicate for each strain, and MIC values were recorded as the lowest concentration of peptide where no visible growth was observed. Rather than culturing in liquid media, bacterial isolates were grown for 24 h on BHI solid plating media, and were diluted in sterile BHI broth to a 0.5 McFarland standard turbidity. Fifty μ L of each standardized culture was used to inoculate 96-well microtiter cell-culture plates containing 50 μ L of the appropriately diluted peptide. Plates were incubated at room temperature for 24 h, and they were visually scored for growth using a microtiter plate Manual Viewbox reader (Trek Diagnostics, Cleveland, OH). Positive control wells received 50 μ L of BHI broth without addition of peptide, and negative control wells received 50 μ L of heat killed (95°C; 10 min) cultures in addition to 50 μ L of the diluted peptides. Differences in MIC values between strains exposed to each peptide and among strains within each treatment were assessed using Wilcoxon rank-sum tests in Stata (version 8.0 for Macintosh).

Results

Ten culturable bacterial isolates were obtained from either frog skin or water and mud samples taken from the natural pond environment of *R. septentrionalis* in Somerset County, ME. After Blastn analysis, the 10 endemic isolates represented five Gram-negative genera, *Aeromonas*, *Burkholderia*, *Delftia*, *Pseudomonas*, *Serratia*, and two Gram-positive genera, *Microbacterium* and *Bacillus* (Table 1). Colonies of all Gram-negative strains and the Gram-positive *Micrococcus oxydans* strain were observed to produce capsular or exopolysaccharide (EPS) material when grown on BHI solid plating media. Notably, the two *Bacillus* isolates RS02 and RS03 exhibited the characteristic rough or non-mucoidal colonial morphologies when cultured on solid plating media.

Four of the isolates showed growth inhibition by temporin-1SPb at the concentrations used, and growth of all isolates, including a species of *Pseudomonas* (RS12) isolated directly from the skin of *R. septentrionalis*, was inhibited by the brevinin-2 related peptide within the range of peptide concentrations used (Table 1). MIC values were identical among the three replicates for each isolate and for each peptide. The brevinin-2-related peptide showed significantly higher antimicrobial activity against the isolated endemic strains compared to the same strains exposed to temporin-1SPb ($P < 0.001$). Both peptides showed particularly high potency against two of the Gram-positive species, *Bacillus psychrotolerans* and *B. fusiformis* (Table 1). MIC values for all other inhibited isolates were significantly higher, recorded as 100, 250 or 500 μM . For example, MIC values for *B. fusiformis* (25 μM) exposed to the brevinin-2 related peptide were significantly lower than those for *A. popoffi* (100 μM) ($P = 0.025$). Strains with MIC values greater than 500 μM were considered not to be susceptible to growth inhibition by the peptides under this study's experimental conditions.

Discussion

Our results contribute to the growing body of evidence that peptides isolated from amphibian skin secretions possess broad-spectrum antimicrobial activity (Bevins and Zasloff, 1990; Mangoni et al., 2003; Pukala et al., 2006). Using 16S ribosomal RNA gene sequencing techniques, 10 bacterial strains isolated primarily from water or mud samples were identified as belonging to the genera *Serratia*, *Bacillus*, *Aeromonas*, *Burkholderia*, *Pseudomonas*, *Microbacterium*, and *Delftia*. All of these genera are documented as occurring naturally in aquatic or soil habitats (Molin and Ternstrom, 1986; Wuthiekanun et al., 1996; Huys et al., 1997; Takeuchi and Hatano, 1998; Wen et al., 1999; Abd El-Rahman et al., 2002; Stock et al., 2003; Zhao et al., 2005). Only one bacterial strain in our study, *Pseudomonas* sp. RS12, was isolated from the skin of *R. septentrionalis*. Most importantly, we observed that growth of all 10 bacterial strains was inhibited by at least one peptide found in skin secretions produced by *R. septentrionalis* (Table 1).

Antimicrobial peptides in amphibians are synthesized in the multinucleated cells of the dermal glands of the skin and large amounts are stored in the secretory granules of these glands. These peptides are small (~12-46 amino acid residues), cationic, and hydrophobic (Zasloff, 2002). Most can adopt a α -helical conformation when bound to charged residues on target cell membranes. Resulting peptide complexes are thought to disturb the membrane, causing death of the target cell (Goraya et al., 2000; Sitaram and Naggaraj, 2002). This impressive peptide armament differs between frogs belonging to different taxa so that no two species have the same array of peptides. This striking divergence suggests that there could be a multitude of different peptides produced by the dermal glands of numerous amphibians.

Antimicrobial assays on laboratory standard strains are regularly included with the characterizations of such cationic peptides, but rarely on isolates taken from an amphibian's natural habitat. Here we show that a brevinin-2 related peptide and temporin-1SPb isolated from skin secretions produced by *R. septentrionalis* inhibited growth differentially of both Gram-negative and Gram-positive bacterial species endemic to the frog's natural habitat.

Results reported in Table 1 show that the brevinin-2-related peptide had significantly higher antimicrobial activity against the isolated endemic strains compared with the same strains exposed to temporin-1SPb. Both peptides showed high growth inhibition activity against two of the Gram-positive species, *B. psychrotolerans* (MIC = 6.25 μ M (temporin-1SPb) and 3.3 μ M (brevinin-2 related)) and *B. fusiformis* (MIC = 12.5 μ M (temporin-1SPb) and 25 μ M (brevinin-2 related)). Our observed MIC values for these two endemic isolates are similar to the MIC values of temporins obtained for the Gram-positive bacterial strains used in the study by Rosenfeld et al. (2006). For example, they found that temporins A, B, and L, had MICs of 3, 6, and 2.50 μ M, respectively, for *Bacillus megaterium* Bm11. Our results are also consistent with those reported by Mangoni et al., (2000); the brevinin peptide, brevinin-2T, was markedly more potent than the temporins A and B in growth inhibition studies on Gram-negative natural bacterial strains isolated from the skin of *Rana temporaria*.

Bacteria isolated directly from terrestrial or aquatic environments often possess an extensive extracellular polymeric matrix that is usually manifested as either wall polysaccharides (capsules) or as extracellular excretions into the surrounding environment referred to as exopolysaccharide (EPS) (Davey and O'Toole, 2000). The EPS plays an integral role in the formation of biofilms, broadly defined as assemblages of microorganisms and their associated extracellular products at an interface and typically attached to an abiotic or biotic surface. Biofilms can be composed of a population that developed from a single species or a community derived from multiple microbial species (Davey and O'Toole, 2000). Direct observation of different natural habitats has established that most microorganisms persist on surfaces within a structured biofilm ecosystem and not as free-floating or planktonic organisms (Costerton et al., 1995). In our study, we observed that all but the *Bacillus* spp. produced mucoidal colonies, and some strains, such as the pseudomonad isolates RS12 and RS04, had extensive EPS. While other studies on peptide antimicrobial activity specify liquid media for growing microorganisms in preparation for MIC analyses, we cultured our isolates on solid plating media in an effort to better simulate the abiotic or biotic surfaces from which our strains were isolated in the natural habitat (Bevier et al. 2004; Rosenfeld et al. 2006; Mangoni et al. 2000). Hence, upon inoculation in the MIC system employed, encapsulated isolates would most likely have preformed extracellular polymeric matrices while being exposed to peptides during the incubation period. Most importantly, the EPS matrix has the potential to physically prevent access of specific antimicrobial agents to cellular targets within the biofilm by acting as an ion exchanger, thereby restricting diffusion of compounds from the environment into the biofilm (Gilbert et al., 1997). Although most studies on biofilm based mechanisms of antimicrobial resistance involve antibiotics (Davey and O'Toole, 2000), EPS sequesters cations, metals, and toxins (Decho, 1990; Fleming, 1993). Notably, Lewis (2001) speculates that because they are enclosed within an exopolymer matrix, biofilms may restrict the diffusion of smaller antimicrobial peptides such as the numerous defensins and their analogs. Members of the temporin and brevinin families are cationic, which may explain the significant differences in the MIC values that we observed between the non-mucoidal *Bacillus* isolates RS02 and RS03 (MIC range from 3.13 to 12.5 μ M), and the other isolates, all of which produced capsule or EPS (MIC range from 100 to >500 μ M; Table 1).

The vast majority of studies involving naturally isolated antimicrobial peptides have examined biological activity on laboratory standard bacterial strains, but some have focused on the efficacy of peptide antibiosis on the natural flora of the amphibians producing the peptides. For example, Mangoni et al. (2000) isolated several Gram-negative bacterial isolates from the skin of *Bombina orientalis*, and Rosenfeld et al. (2006) isolated *Aeromonas hydrophila* from among the natural flora of *R. temporaria*. Both research groups subjected bacterial isolates to members of the temporin family of peptides in antimicrobial assays. Rosenfeld et al. (2006) reported MIC values of 400, 200, and 40 μM for temporins A, B, and L for their naturally isolated *Aeromonas hydrophila* RT-6 strain. Our study goes one step further, isolating bacteria from an amphibian's habitat, and we obtained a MIC of 500 μM for temporin-1SPb on both of our aeromonad natural isolates, RS06 and RS07 (Table 1).

Boman (2000) describes the role that natural flora may play in protecting the host from potential pathogens, and how antimicrobial peptides may participate in innate immunity. He suggests that these biological and chemical components of the innate defense system may be linked and function synergistically to protect the host from infectious disease agents. This proposed mechanism of innate defense is especially relevant for amphibians because of their constant exposure to high concentrations of diverse microbial species in their aquatic and terrestrial environments.

The two strains of *Aeromonas* that we isolated, RS06 and RS07, were susceptible to both brevinin-2 related and temporin-1SPb (Table 1). This finding is important because *A. hydrophila* is considered resistant to the majority of naturally occurring antimicrobial peptides and could have important implications for further investigations into cutaneous bacterial septicemia, or "red leg" disease; a syndrome commonly attributed to *A. hydrophila*, other Gram-negative and some Gram-positive bacteria (Pessier 2002; Rosenfeld et al., 2006).

Both pseudomonads in our study, *Burkholderia* sp RS04 and *Pseudomonas* sp RS12, were susceptible to the brevinin-2 related peptide (Table 1). *Burkholderia* spp. are known for their ability to resist growth inhibition by cationic peptides, as shown for polymyxin B, cathelicidin peptides, and human beta-defensins 1, 2 and 3 (Baird et al., 1999; Sahly et al., 2003), so this level of susceptibility is important to document. Species of *Pseudomonas* are commonly isolated from frog skin, as in our study, and have been implicated as both primary and secondary pathogens of anurans especially in cultured colonies (Cunningham et al., 1996; Taylor et al., 1999; Mauel et al., 2002). Interestingly, we found that *Pseudomonas* sp. RS12 showed growth inhibition by brevinin-2-related peptide (MIC=250 μM), but was not susceptible to inhibition by temporin-1SPb (Table 1). These results suggest that it is important to further examine the relationship that exists among a species complement of peptides, and the microbial species that exist naturally on host skin surfaces and in the amphibian's environment.

The presence of numerous antimicrobial peptides in frog skin, acting separately or synergistically, may enhance survival in habitats laden with opportunistic and pathogenic microorganisms. In many respects, these antimicrobial peptides with such diverse structures and modes of action can be viewed as the successful evolution of a multi-antimicrobial

defense system, minimizing the chances of microorganisms developing a resistance to any singular peptide (Nicolas and Mor, 1995; Zasloff, 2002; Vanhoye et al., 2003). Overall, a greater understanding of the variety and efficacy of the diverse peptide arsenal in amphibian skin secretions is essential and may be all that protects some amphibians from extinction.

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Table 1

Antimicrobial activity (MIC) of two peptides on bacterial strains isolated and identified from the natural environment of *R. septentrionalis*. Assays were run in triplicate for each isolate for each peptide, and identical MIC values were recorded at each observation.

Bacterial Isolate	Gram stain	Temporin -1SPb MIC (μM)	Brevinin-2 related MIC (μM)
<i>Bacillus fusiformis</i> RS02	positive	12.5	25
<i>Bacillus psychrotolerans</i> RS03	positive	6.25	3.13
<i>Burkholderia</i> sp. RS04	negative	>500	500
<i>Aeromonas popoffii</i> RS06	negative	500	100
<i>Aeromonas</i> sp. RS07	negative	500	100
<i>Serratia fonticola</i> RS09	negative	>500	250
<i>Delftia acidovorans</i> RS10	negative	>500	250
<i>Serratia</i> sp. RS11	negative	>500	250
<i>Pseudomonas</i> sp. RS12	negative	>500	250
<i>Microbacterium oxydans</i> RS14	positive	>500	250