Antimicrobial peptides with therapeutic potential from skin secretions of polyploid frogs of the Pipidae family

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To my family:

The ones who love, support, care and forgive

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General introduction

Milena Mechkarska

“I don’t see no p’ints about that frog that’s any better’n any other frog.”

Mark Twain, 1867, *The Celebrated Jumping Frog of Calaveras County*

Worldwide spread of infections caused by multidrug resistant bacteria

A global problem is rapidly evolving and expanding to the point of posing a serious threat to public health. This is the emergence of pathogenic bacteria and fungi resistant to commonly used antibiotics, which cause increased morbidity and mortality, and impact heavily on healthcare costs (Livermore, 2009).

Antibiotic resistance is a type of drug resistance in which a microorganism is able to survive exposure to an antibiotic. Antibiotics have been considered to be the single most significant discovery in medicine. However, even at the early stage after their discovery, antibiotic resistance had already begun to emerge **(**Rammelcamp and Maxon, 1942). Bacteria are either non-responsive or resistant to the action of antibiotics by a range of different mechanisms (Fig. 1).

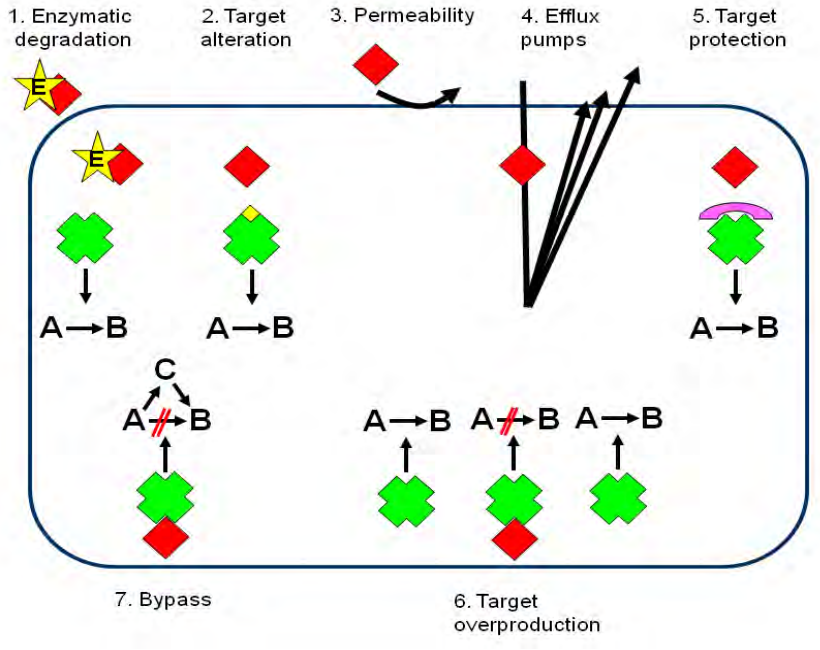

Fig. 1. Bacterial resistance mechanisms to antibiotics. (1) Enzymatic degradation as a result of production of proteases; (2) Target alteration such as composition of the membrane, LPS or intracellular molecules; (3) Reduction of permeability by changing trans-membrane potential or modification of membrane fluidity; (4) Over-expression of efflux pumps causing immediate export; (5) Protection of intracellular target; (6) Overproduction of target; and (7) Bypassing the action on target. Key: enzyme (proteases) ; antibiotic ; intracellular target ; protection ; alteration of target . Kindly provided by Prof. T. Pal (2012).

Resistance can arise either by mutations of bacterial genes, or even more frequently by horizontal gene transfer. Antibiotic resistance genes may be mobilized between the same or even different species by plasmids or transposons or acquired by natural competence for transformation by exogenous DNA. Thus, a gene for antibiotic resistance that evolves via natural selection may be shared in or across the population(s). The resistant bacteria are classified into multidrug-resistant (MDR), extensively drug-resistant (XDR) and pandrug-resistant (PDR) categories (Magiorakos et al., 2012). MDR is defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories, XDR is defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e. bacterial isolates remain susceptible to only one or two categories) and PDR is defined as non-susceptibility to all agents in all antimicrobial categories (Magiorakos et al., 2012). The genes for resistance to antibiotics, like the antibiotics themselves, are ancient (Donadio et al., 2010). Although there were low levels of pre-existing antibiotic-resistant bacteria (Nelson, 2009; Caldwell and Lindberg, 2011), the major factor in development and selection of MDR strains and in sharing of resistance between bacterial species is the widespread use of antibiotics (Hawkey and Jones, 2009). Examples of the misuse of antibiotics include their sales over the counter without a prescription; inappropriate prescription by doctors; addition to livestock feed; and unsound practices in the pharmaceutical manufacturing industry involving release of new antibiotics despite the fact that resistant strains have already been documented. The problem is made worse by the increasing trend for regional and international travel among human populations (Cohen, 1992; Tomasz, 1994; Swartz, 1997). This makes the emergence of antibiotic resistance “the most eloquent example of Darwin’s principle of evolution” (Livermore, 2009).

One of the major drug-resistant human pathogens is a strain of the Gram-positive aerobic, non-motile coccus: *Staphylococcus aureus*. This microorganism is carried by approximately one third of the human population. It causes local infections by colonizing mucous membranes and skin which can, in later stages, develop into systemic infections, such as bacteremia and sepsis in both adults and children. The second group of infections is toxin- and superantigen-mediated diseases (Kurlenda and Grinholc, 2012; and references therein). *S. aureus* possesses a wide spectrum of virulence factors (Feng et al., 2008; Plata et al., 2009) that enable it to bypass the barriers of the host defence system and it can survive hostile environmental conditions due to its extraordinary versatility and adaptability to antibiotic pressure (Lowy, 1998; Lowy, 2003).

The development of antibiotic resistance by *S. aureus* is summarized in Fig. 2. *S. aureus* was one of the first bacteria in which penicillin resistance was found (Rammelcamp and Maxon, 1942) and the specific role of penicillinase was subsequently identified (Kirby, 1944; Bondi and Dietz, 1945). This necessitated the clinical use of penicillinase-resistant derivatives such as methicillin as the antibiotics of choice. However, reports describing the emergence of methicillin-resistant *S. aureus* (MRSA) quickly

followed (Jevons, 1961). Since then, MRSA has become endemic in many countries and poses a significant problem worldwide (Kreiswirth et al., 1993). The subsequent usage of newer classes of antibiotics also quickly caused appearance of resistant *S. aureus* strains (Bozdogan et al., 2003). Thus, apart from being resistant to all β-lactam antibiotics, MRSA may not be responsive to macrolides, clindamycin, fluoroquinolones, tetracycline and gentamicin (Feng et al., 2008).

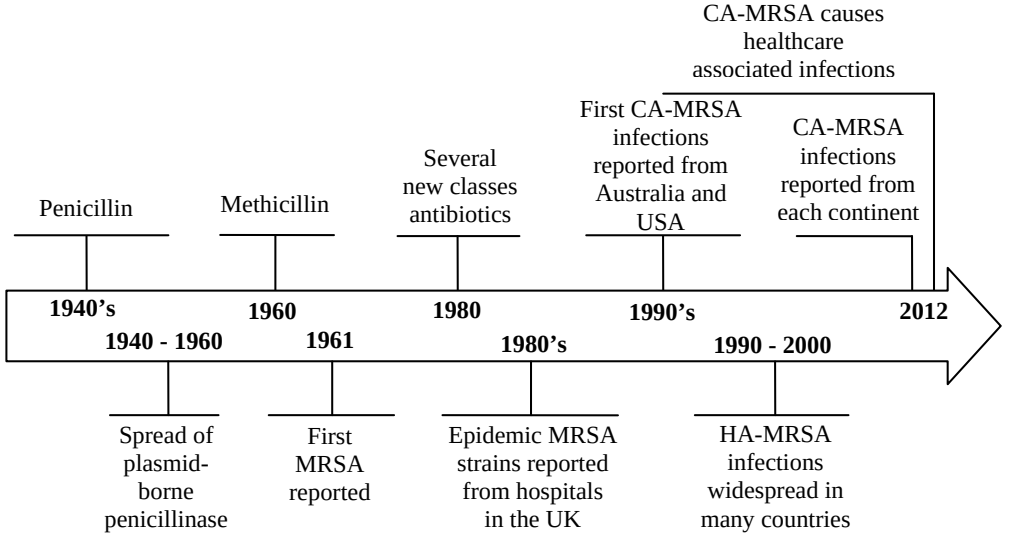

Fig. 2. Timeline for the introduction of antibiotics into medical practice. The history of the emergence of methicillin-resistant *S. aureus* (MRSA) with the subsequent change in epidemiology of MRSA infections is presented in parallel. CA – community-acquired; HA – hospital-acquired. Kindly provided by Dr. A. Sonnevend (2012).

The epidemiology of infections caused by MRSA is rapidly changing. Originally, MRSA was associated predominantly with hospital-acquired infections (HA-MRSA) (Chambers and Deleo, 2009; Rosenthal et al., 2010); however, in the past 10 years, community-acquired MRSA (CA-MRSA) has become prevalent. CA-MRSA infections occur in otherwise healthy people who have not been recently hospitalized or had a medical procedure (e.g. dialysis, surgery, catheter) and usually involve skin infections, such as abscesses, boils, and other pus-filled lesions. Frequently though, CA-MRSA is responsible for rapidly progressive, fatal diseases, including necrotizing pneumonia, severe sepsis and necrotizing fasciitis (Boyle-Vavra and Daum, 2007; Woodford and Livermore, 2009).

Although effective new types of antibiotics against multidrug-resistant Gram-positive bacteria such as MRSA have been introduced or are in clinical trials (Kurlenda and

Grinholc, 2012), the situation regarding new treatment options for infections produced by MDR Gram-negative pathogens such as extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae (primarily *Escherichia coli and Klebsiella pneumoniae*), *Pseudomonas aeruginosa* and *Acinetobacter baumannii* is less encouraging (Giamarellou and Poulakou, 2009). In general, drug-resistant Gram-negative bacteria threaten mainly hospitalized patients whose immune systems are weak. These bacteria can survive for a long time on surfaces in the hospital and enter the body through wounds, catheters and ventilators. Pulmonary infections, particularly hospital-acquired pneumonia, caused by these carbapenemase-producing bacteria that are resistant to all β-lactam antibiotics are particularly difficult to treat (Kollef, 2010). Gram-negative bacteria have a great ability for exchanging genetic material among strains of the same species and even among different species.

The most common mechanism of resistance among *E. coli*, *K. pneumoniae*, and other Enterobacteriaceae is through the production of β-lactamases which, depending on the enzyme, inactivate a wide range of β-lactam antibiotics (Jacoby and Munoz-Price, 2005). The ESBLs are a heterogeneous group of β-lactamase enzymes that are mostly encoded by plasmid-bourne genes and hydrolyse the majority of β-lactam antibiotics with the exception of carbapenems. ESBLs now number 532 distinct enzymes and convey varying degrees of resistance to cephalosporins, penicillins, β-lactamase inhibitors, and monobactams (Jacoby and Munoz-Price, 2005; Bhavnani et al., 2006). The prevalence of ESBL-producing strains varies by geography (particularly in urban areas), type of hospital, and patient age. Importantly, there have been reports of ESBL-producing strains that also produce carbapenemases [*K. pneumoniae* carbapenemase (KPC), metallo-β-lactamase (VIM type and NDM1 type) and oxacillinase (OXA) carbapenemases] (Bradford et al., 2004) and thus became resistant to all β-lactam antibiotics.

One of the emerging multidrug resistant HA- and CA-pathogens is *A. baumannii*, which was long thought to be relatively avirulent (Talbot et al., 2006). It is an increasingly common cause of ventilator-associated pneumonia. This microorganism has rapidly developed resistance to currently available antimicrobials via a wide range of mechanisms, including production of aminoglycoside-modifying enzymes, ESBLs, carbapenemases, and through changes in outer membrane proteins, penicillin-binding proteins, and topoisomerases (Gales et al., 2001; Bonomo and Szabo, 2006). It is not surprising, therefore, that *Acinetobacter* spp. have emerged as an important pathogen in the hospital settings and even in war zones. In many regions it is common to find strains of *Acinetobacter* spp. that are resistant to all aminoglycosides, cephalosporins, and fluoroquinolones (Landman et al., 2002; Vila and Pachón, 2008). During the last decade, MDR *A. baumanii* (MDRAB) have emerged to become a major phenotype in hospitals worldwide with a high mortality rate (Dijkshoorn et al., 2007; Peleg et al., 2008).

P. aeruginosa is a highly prevalent, opportunistic, invasive Gram-negative pathogen that is responsible for a wide range of severe HA-infections, including pneumonia, urinary tract infections, and bacteremia (Talbot et al., 2006). Importantly, this pathogen is intrinsically susceptible to only a limited number of antibacterial agents because of the low permeability of its cell wall (Lambert, 2002). Consequently, infections are often difficult to treat and may be life-threatening, particularly if the causative strain is drug resistant. In addition to its intrinsic resistance, *P. aeruginosa* has also acquired resistance via multiple mechanisms, including production of β-lactamases and carbapenemases, upregulation of multidrug efflux pumps, and cell wall mutations leading to a reduction in porin channels (Lambert, 2002). Recent studies have shown that phenotypic resistance associated with biofilm formation or the emergence of small-colony-variants may be important in the response of *P. aeruginosa* populations to antibiotic treatments (Cornelis, 2008; McCollister, 2011).

Another important opportunistic human pathogen, an inhabitant of the oral cavity and the gastrointestinal and the genitourinary tracts of most healthy people, is *Candida albicans*. This microorganism is a polymorphic fungus, which transitions from budding yeast, pseudohyphae to true hyphae (Whiteway and Bachewich, 2007). The major virulence factors of *C. albicans* are proteinase secretion, hyphal formation, and adhesion and phenotype switching between two distinct types of cells, white and opaque (Yang, 2003). This switching of phenotypes contributes to a better adaptation of *C. albicans* to new host niches (Soll, 1988). White cells are much more virulent than opaque cells after intravenous infection. In a healthy host, *C. albicans* persists in equilibrium with the host's microflora (McCallum, 2010). However, it can cause serious disseminated infections in critically ill or immunocompromised patients (Eggiman et al., 2003). Although resistance of *C. albicans* towards antifungal agents is less frequent than in many other species, an increasing number of resistant strains are emerging (Slutsky et al., 1985; Soll, 2002).

In the past decade, the situation regarding antimicrobial resistance has worsened significantly. Increasing numbers of patients with immune deficiencies, such as HIV infection or receiving immunosuppressive therapy for cancers and organ transplants, mean that there is an ever increasing population at risk of invasive bacterial and fungal infection. In view of the successful spread and the notable virulence capacity of the MDR, XDR and PDR pathogens combined with their antibiotic resistance, novel treatment approaches are urgently required in order to control the infections.

Innate immunity and antimicrobial peptides (AMPs)

Innate immunity is the initial response of a host in defending against pathogen invasion (Janeway and Medzhitov, 2002; Beutler, 2004). The key features of an innate immune

response are that it is non-specific (broad-spectrum), rapid (often within minutes to hours) and conserved in its pattern of recognition, meaning that this system responds to pathogens in a generic way. Innate immunity can be constitutively expressed or be inducible and is characterized by mechanisms that adapt on an evolutionary timescale, rather than over a single lifespan.

One of the central components of the innate immune response is the production of antimicrobial peptides (AMPs). AMPs are host-defense effector molecules present in virtually all life forms, including vertebrates, invertebrates, and plants (Zasloff, 2002), emphasizing their evolutionary importance in host defense.

General characteristics of AMPs

AMPs are typically small molecules generally composed of 8 - 50 amino acid residues, mostly in the usual L configuration, with a net positive charge of +2 to +6 (reviewed in Pukala et al., 2006; Hancock and Sahl, 2006; Conlon, 2011). They are cationic due to a high percentage of the basic amino acids, arginine, lysine, and histidine (Hancock and Diamond, 2000; Boman, 1998; Boman, 2003). Typically, more than 50% of an AMP's amino acids are hydrophobic (Scott and Hancock, 2000).

Structures of AMPs

Although exceptions exist, the AMPs can be divided into three major classes based on their three dimensional structure (Boman, 2003):

i) cationic peptides with and without cysteine amino acids that adopt an amphipathic α–helical conformation;

ii) peptides with three disulfide bonds adopting a flat β-sheet conformation;

iii) peptides with a high percentage of certain amino acids particularly proline, arginine and tryptophane.

The α-helical peptides are the most common in nature and the most intensively studied of the three classes.

Interaction of α-helical AMPs with biological membranes

AMPs target the cell membranes and their action ultimately leads to bacterial death (Matsuzaki et al., 1989; Williams et al., 1990; Matsuzaki et al., 1991; Grant et al., 1992; Vaz Gomez et al., 1993; Blazyk et al., 2001; Yeaman and Yount, 2003). Membranes are the fundaments maintaining life through compartmentalization of cells and homeostasis. The

lipid headgroups vary in size, charge, and polarity and their composition constitutes the most important difference between eukaryotic and prokaryotic membranes (Fahy et al., 2005) (Fig. 3).

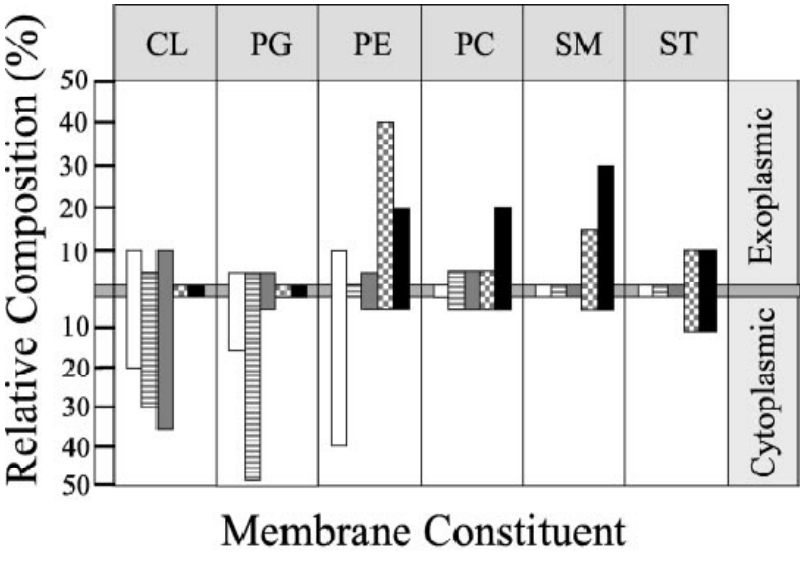

Fig. 3. Asymmetric lipid composition of cytoplasmic membranes from several bacteria compared with that of human erythrocytes. Membrane constituents ranging from anionic (left) to zwitterionic or neutral (right) are cardiolipin (CL), glycerophosphoglycerol (PG), glycerophosphoethanolamine (PE), glycerophosphocholine (PC), sphingomyelin (SM) and sterols (cholesterol or ergesterol) (ST). Key: open, *E. coli*; horizontal hatching, *S. aureus*; shaded, *B. subtilis*; checkered, *C. albicans*; solid, human erythrocyte (from Yeaman and Yount, 2003).

Prokaryotic cytoplasmic membranes contain a high proportion of anionic lipids, such as glycerophosphoglycerols and cardiolipins, whereas these lipids are rarely found in mammalian cytoplasmic membranes. On the other hand, eukaryotic membranes possess a high proportion of zwitterionic lipids like glycerophosphoethanolamines, glycerophosphocholines, sphingomyelin (or ceramide phosphocholines) and the sterol lipids which are important for the dynamics and integrity of the lipid layers (de Almeida et al., 2003; Koronkiewicz and Kalinowski, 2004).

The mode of action of AMPs on membranes is dictated by their physicochemical properties, mainly their cationic and amphipathic nature (Fig. 4) (Chapter **8**), as well as by the intrinsic characteristics of the pathogens (Fig. 5).

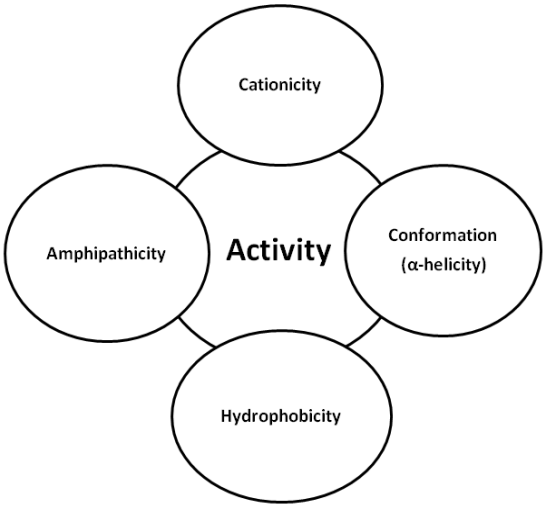

Fig. 4. Interrelationship among structural determinants in AMPs. The activity and selectivity of the peptides on phospholipid membranes originates from several interdependent physicochemical parameters such as charge (cationicity), conformation (α-helicity), amphipathicity and hydrophobicity (modified from Yeaman and Yount, 2003).

Two membrane architectures prevail in bacteria (Fig. 5, step 1). In Gram-negative bacteria, an outer membrane, rich in PG, lipopolysaccharide (LPS) and magnesium ions (Mg^{2+}) is followed by a 2 nm thick peptidoglycan wall that protects the cytoplasmic membrane. In Gram-positive bacteria, the cytoplasmic membrane is directly surrounded by a 20 to 40 nm thick peptidoglycan wall featuring anionic lipoteichoic acid groups that provide a negatively charged surface. The relevant target of AMPs is the cytoplasmic membrane (Fig. 5, step 3).

Bacterial membranes are characterized by a highly asymmetric lipid charge distribution generating a transmembrane potential. This electrical potential is necessary for several energy-dependent catalytic and transport processes. Thus, the presence of large inside-negative transmembrane potentials in respiring prokaryotic cells but not in erythrocytes (Matsuzaki et al., 1995a) leads to selective electrostatic interactions between bacterial membranes and AMPs.

The AMPs are usually unstructured in an aqueous environment but adopt a stable α-helical conformation upon contact with the lipid bilayer. At low concentrations the AMPs lie on the membrane surface. It has been reported that under certain conditions, the AMPs can aggregate or assemble together even before binding to the membrane (Urrutia et al.,

1989). After reaching a “threshold” concentration of peptide (Fig. 5, step 4), defects in the membrane structure appear and leakage occurs through the membrane in different manners according to three models:

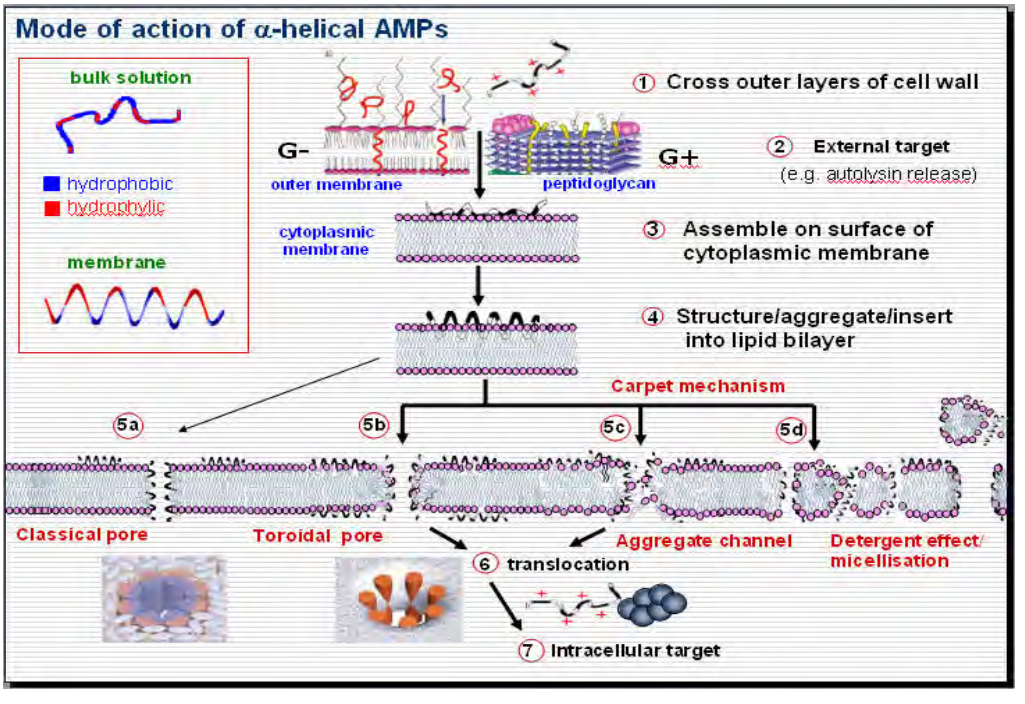

Fig. 5. Proposed mechanisms of action for α-helical AMPs on Gram-negative (G-) and Gram-positive (G+) bacteria: peptide insertion and membrane permeability models. Kindly provided by Prof. A. Tossi, University of Trieste, Italy.

The barrel-stave pore model (Fig. 5, step 5a): This was the first mechanism proposed to explain killing of bacteria by AMPs (Ehrenstein and Lehar, 1977). Initially, the AMPs accumulate as monomers alongside the bacterial surface. When concentration is increased, the peptide assembly changes its orientation to transmembrane thus forming a classical pore. The tightly packed peptide assembly forms a barrel-like structure spanning the membrane, in which the hydrophobic regions of the inserted peptides align with the lipid core region of the bilayer and the hydrophilic peptide regions constitute the pore lining. Progressively, new AMPs are recruited and, through a process of self-aggregation, the pore size increases.

The carpet model (Fig. 5, steps 5c and 5d): This model was proposed on the basis of studies with dermaseptin S (a cationic amphipathic α-helical frog peptide from *Phyllomedusa sauvagei*) (Pouny et al., 1992). Locally, AMPs accumulate on the lipid bilayer surface, in an orientation that is parallel to the membrane during the process. When a high peptide concentration is attained, the membrane is weakened by unfavorable energetic influences and thinning of the membranes occurs. The AMPs then re-orient and form peptide aggregates that intercalate into the membrane and disrupt it by producing micelles in a detergent like manner.

The toroidal wormhole model (Fig. 5, step 5b): This mechanism combines components of the barrel-stave (Fig. 5, steps 5c and 5d) and carpet models (Fig. 5, step 5a) (Hancock and Chapple, 1999). The AMPs assemble with intercalated anionic lipid headgroups to compensate for the charge repulsion (Wieprecht et al., 2000) and form pores (Duclohier, 1994; Matsuzaki et al., 1995b; Ludtke et al., 1996). The direct involvement of lipids in the pore structure leads to a toroidal-shaped hole. The pores have a larger radius than the classical pore (Fig. 5, step 5a). As a consequence of this pore formation, the peptides enhance the lipid translocation rate (Matsuzaki et al., 1996). This can dissipate the electric potential and uncouple the respiration of cells (Westerhoff et al., 1989; Wieprecht et al., 2000; Glaser et al., 2005).

Although disruption of membrane integrity is the principal mechanism of action of AMPs, some members can cross the cell membrane without causing damage to the lipid bilayer (Fig. 5, step 6). For example, AMPs rich in arginine, such as buforin II isolated from *Bufo bufo gargarizans* (Park et al., 1996), are reported to translocate across both the cellular and nuclear membranes (Futaki et al., 2001). Once inside the cell, the AMPs can bind to DNA and RNA targets resulting in inhibition of cell-wall synthesis and enzymatic activity (Fig. 5, step 7) (Brogden, 2005).

The AMPs, due to their markedly different and highly destructive mode of action, are usually active against microorganisms that are resistant to currently licensed antibiotics. The plethora of cationic AMPs is a consequence of a co-evolution between host organisms and microbes which has shaped the diversity of the AMPs (Peschel and Sahl, 2006). Despite the fact that bacteria have had a huge time-span to adapt to their killing mechanisms, the development of resistance against AMPs has been shown to be a much slower process than against conventional antibiotics (Ge et al., 1999; Perron et al., 2006). Since the primary target of AMPs is the cell membrane, the pathogen would have to redesign its whole cell membrane – a very lengthy process – in order to develop a resistance against their action. Therefore, it is very unlikely for a resistance phenomenon to arise rapidly.

AMPs in frogs

Anurans (frogs and toads) are confined, for at least part of their life cycle, to a warm and moist environment that is conductive to the growth of potentially harmful bacteria and fungi. Therefore, these animals have evolved innate defenses to protect the skin of the organism from invasion by pathogenic microorganisms. Frogs have the full complement of the immune systems found in higher vertebrates including both adaptive and innate immunity (Du Pasquier et al., 1989; Woodhams et al., 2007). They are poikilothermic organisms whose internal temperature relies heavily upon the temperature of their environment. In addition to being activated too slowly to provide sufficient protection for infections in the skin (Simmaco et al., 1998), the adaptive immune system of frogs is temperature-dependent and suppressed if temperature decreases. Similarly, humoral innate immunity, including complement mediated effects, is also temperature-dependent (Green and Cohen, 1977; Simmaco et al., 1998). Therefore, the specialized innate immune defenses of skin (being relatively temperature-independent) are essential in protecting the frogs against various pathogens. Because it is the first to respond, the innate immune system plays a key role in the host's ability to avoid the progression of a potential pathogen from a threat to an infection (Scott and Hancock, 2000).

Innate immune defenses in amphibians include: pattern-recognition receptors (PRRs), AMPs (Simmaco et al., 1998; Rollins-Smith et al., 2005), a complement lytic system (Green and Cohen, 1977), cytokines and various types of cells, such as phagocytic macrophages, dendritic cells and granulocytes (Manning and Horton, 1982), natural killer cells (NK cells) (Horton et al., 1996), neutrophils, eosinophils, mast cells, basophils and epithelial cells (Hancock and Diamond, 2000) (Fig. 6).

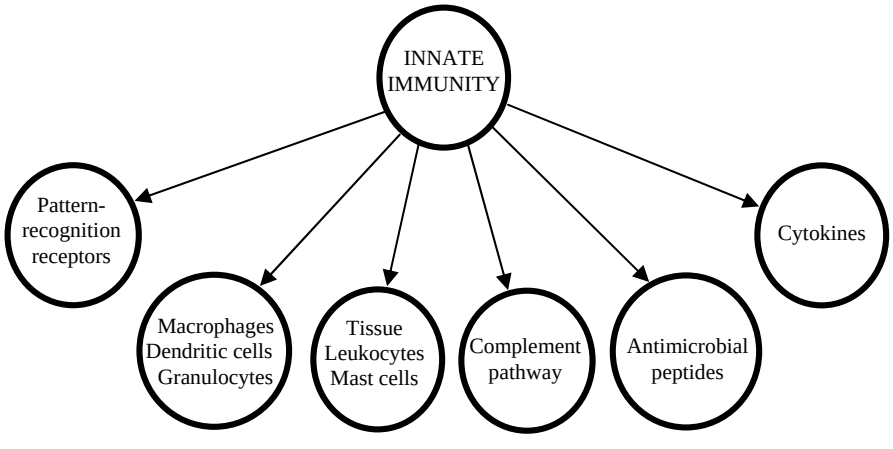

Fig. 6. Components of the innate immunity in amphibians.

In general, AMPs are encoded by single genes or homologous gene families. These gene families are located in clustered arrangements in the genome and mapped to syntenic (co-localized genetic loci) chromosomal regions, which suggests co-evolution of different subclasses of AMPs (Scott and Hancock, 2000; Bals, 2000). The expression of the AMPs during amphibian development correlates with metamorphic changes and is induced by thyroid hormone treatment (Reilly et al., 1994; Ohnuma et al., 2006). AMPs are generally found in cells of the granular glands in adult frog skin (Fig. 7) (Hoffmann et al., 1983a; Hoffmann et al., 1983b), and sometimes in the mucosa of the gastrointestinal tract (Moore et al., 1991; Wang et al., 1998). Larval skin is devoid of granular glands able to synthesize and secrete AMPs and there is no evidence for the expression of AMPs during pre and pro-metamorphic stages (Pollet, 2010). Afterwards, these glands develop rapidly and become functional at the climax of metamorphosis.

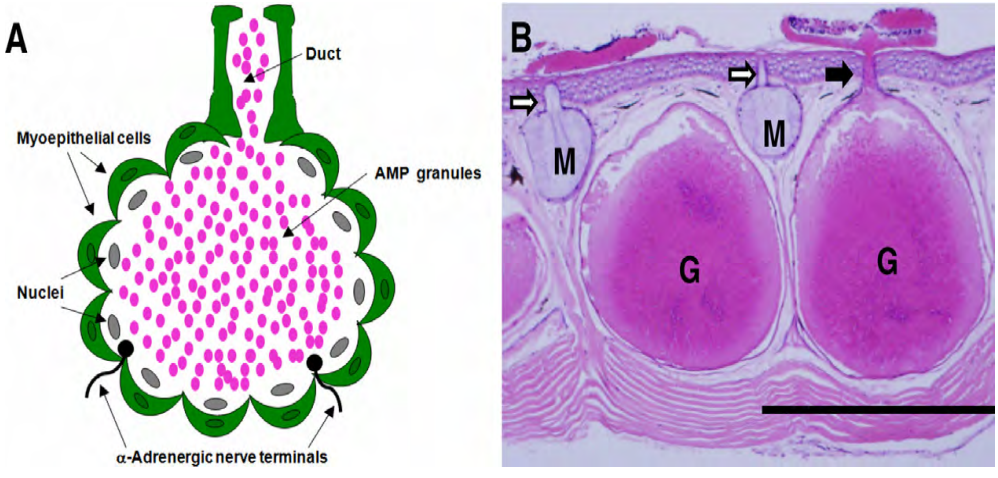

Fig. 7. Stimulated amphibian granular glands secrete AMPs. Dermal granular glands are surrounded by myoepithelial cells that contract upon stimulation of local α-adrenergic nerve terminals, causing secretion of protective AMPs through a gland duct onto the epidermal surface. (A) Schematic depiction of an amphibian dermal granular gland filled in with AMP granules (depicted in pink); (B) A hematoxylin and eosin stained skin section of *X. laevis* 24h after injection with amphibian phosphate buffer saline with active secretion of one granular gland (G) and two mucus glands (M) through gland ducts (indicated by solid and open arrows, respectively). The scale bar is equivalent to 500 µm (Gammill et al., 2012).

Adult frogs have two types of skin glands that are located in the dermis: granular and mucus glands (Duellman and Trueb, 1986; Stebbins and Cohen, 1995; Shepherd et al.,

1998). Both types of glands may occur in aggregations, or as macroglands visible as raised areas of skin, described variously as parotid glands or dorsolateral folds in some species (Duellman and Trueb, 1986; Tyler, 1987). Many frog species lack these aggregations, and in these species glands are distributed evenly throughout the integument (Duellman and Trueb, 1986; Steinborner et al., 1997). The mucus glands are mainly responsible for secreting components to keep the skin continuously moist (Duellman and Trueb, 1986; Stebbins and Cohen, 1995), which is vital for efficient respiration and thermoregulation. The granular glands are large multinucleated granule-filled cells, surrounded by myocytes and innervated by sympathetic fibers (Simmaco et al., 1998). They are the main anatomical structures in which a variety of biologically-active AMPs are stored (Duellman and Trueb, 1986; Erspamer, 1994; Shepherd et al., 1998).

AMPs from the different frog families are synthesized as large precursor peptides (prepropeptides). All have a common “signal-spacer-bioactive peptide” organization with a conserved signal peptide followed by acidic spacer sequences flanking the AMPs sequences (Vanhoye et al., 2003). After synthesis, the signal sequence is removed from the precursor peptides by an endoprotease. Adrenergic stimulation of myocytes, such as by electrical excitation, administration of norepinephrine, injury, predation, handling or other stressors (Tyler et al., 1992; Rollins-Smith et al., 2002; Woodhams, 2003), causes compression of the peptide-containing serous cells. The cells discharge the contents of the storage vesicles by a holocrine-like mechanism involving the rupture of the glands (Moore et al., 1992; Flucher et al., 1986).

As a result, secretions contain not only AMPs but also cytosolic components and intact polyadenylated mRNAs encoding the peptides (Chen et al., 2003). Within 15-30 minutes, the peptides are cleaved and deactivated on the skin surface (Resnick et al., 1991; Steinborner et al., 1997; Bowie et al., 1999). This is assumed to be a means of detoxification in order to prevent self-injury, as AMPs are also cytotoxic at higher concentrations. Some microorganisms, which are an abundant component of the skin microbiota of healthy frogs such as *Proteus mirabilis* and *Aeromonas hydrophilia*, secrete proteolytic enzymes that inhibit the antimicrobial activity by degrading the skin peptides (Schadich, 2009; Schadich and Cole, 2009). After depletion of the granular glands, the deposits of skin peptides may be restored within 6 days to 2 weeks (Erspamer, 1994; Rollins-Smith et al., 2005).

Distribution of AMPs in different frog families

In the Anuran Defense Peptide Database, the AMPs constitute the most common class based on the classification of peptides by biological function (Novkovic et al., 2012). AMPs have been isolated from skin secretions and/or skin extracts from frog species

belonging to both the primitive Archeobatrachia and the more highly-derived Neobatrachia (Pukala at el., 2006; Conlon, 2011) (Fig. 8).

However, not all frogs synthesize and release cytotoxic peptides into their skin secretions (Conlon, 2010) making the distribution of dermal AMPs among anuran families sporadic. In some species the production of AMPs is seasonal and influenced by thyroid hormone status (Ohnuma et al., 2006), and may be either stimulated by exposure to bacteria (Matutte et al., 2000) or inhibited by environmental factors such as contact with pesticides (Davidson et al., 2007).

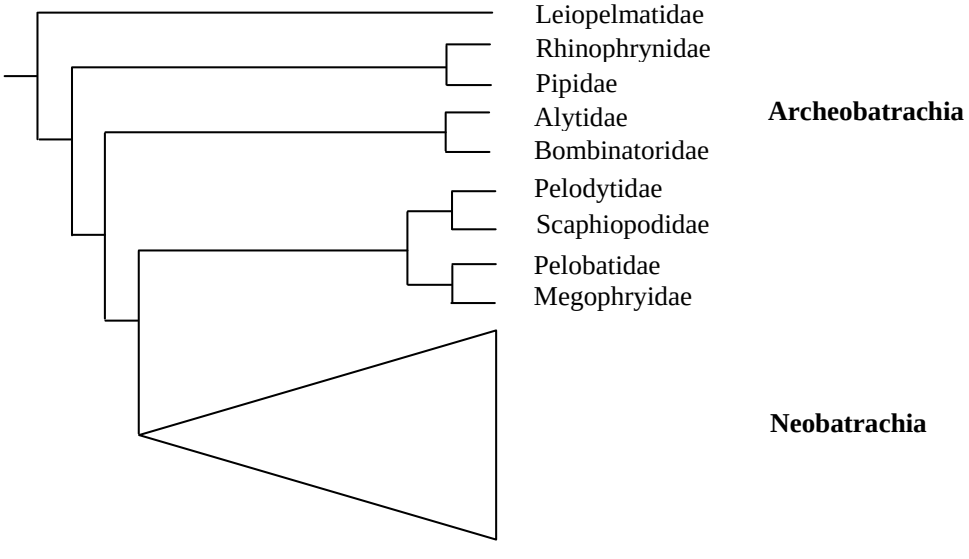

Fig. 8. Phylogeny of anuran amphibians (modified from Konig et al., 2012).

Among the more ancient Archeobatrachia, α-helical peptides with antimicrobial activity have been identified in species belonging to the families Leiopelmatidae, Alytidae, Bombinatoridae, and Pipidae (Table 1), but are not present in those species examined to date that belong to the families Pelobatidae and Scaphiopodidae (Conlon et al., 2009; Conlon, 2011).

Table 1. Examples of AMPs from skin secretions of frogs of the Archaeobatrachia.

Family	Genus	Species	Peptides
Leiopelmatidae	*Ascaphus*	*Ascaphus truei*	ascaphins
Alytidae	*Alytes*	*Alytes obstetricans*	alyteserins
Bombinatoridae	*Bombina*	*Bombina bombina*	bombinins
Pipidae	*Xenopus*	*Xenopus laevis*	magainins* PGLa* CPF* XPF*

*- described in section 6

A similar sporadic distribution of AMPs is found in the more highly derived Neobatrachia. Species, belonging to the six families Discoglossidae, Hyperoliidae, Hylidae, Myobatrachidae, Ranidae, and Leptodactylidae release AMPs into their skin secretions. However, AMPs have not been detected in those species analysed to date belonging to the families Bufonidae, Ceratophyridae, Eleutherodactylidae, Microhylidae, Pyxicephalidae and Rhacophoridae in the Neobatrachia (Conlon et al., 2009; Conlon, 2011).

Frogs of the Pipidae family

Pipid frogs are indisputably basal, belonging to the phylogenetically more ancient Archaeobatrachia (Cannatella and de Sa, 1993) (Fig. 8) but show multiple adaptations befitting their habitat – webbed feet, flattened body, and a lateral line organ system, visible as stitches on the body, to detect wave motion in water (Cannatella and Trueb, 1988). The frogs in the family Pipidae are exclusively aquatic. However, they need to rise to the surface to breathe air because they have lungs and not gills. Pipids are called clawed frogs, because they are the only amphibians to have actual claws. Pipid frogs have powerful legs for swimming and lunging after food and also use the claws on their feet to tear pieces of

large food. These frogs lack a tongue and vocal cords, instead having bony rods in the larynx that help produce clicking sound in a radically different way from the other frogs (Rabb, 1960; Yager, 1996).

The extant clawed frogs of the family Pipidae comprise five genera. *Pipa* is found in South America with the other four genera *Hymenochirus, Pseudhymenochirus, Silurana*, and *Xenopus* in sub-Saharan Africa. They are proposed to have diverged approximately 100 million years ago (MYA) (Cannatella and de Sa, 1993; Roelants et al., 2007).

Pipa, Pseudhymenochirus and *Hymenochirus* are united in the sub-family *Pipinae*. There are 7 species in genus *Pipa,* a single species in the genus *Pseudhymenochirus* – *P. merlini*, and 4 species – *H. boettgeri*, *H. boulengeri*, *H. curtipes* and *H. feae* in genus *Hymenochirus*. The number of chromosomes in *Pipinae* varies (2n = 20 for *P. carvalhoi*; 2n = 30 for *P. parva* and 2n = 24 for *H. boettgeri*) (Canatella and Trueb, 1988; Cannatella and de Sa, 1993).

S*ilurana* and *Xenopus* are united in the subfamily Xenopodinae that is monophyletic based upon nucleotide sequences of the mitochondrial genes. However, the use of two genera "underscores trenchant biological and historical differences between the two clades" (Evans et al., 2004). The cytogenetics and evolutionary history of this taxon are complex (de Sa and Hillis, 1990; Kobel and Du Pasquier, 1991; Kobel et al., 1996; Evans, 2008). The species distribution can best be explained if it is assumed that two different modes of speciation have acted on the Xenopodinae (Fig. 9). One is bifurcating speciation such as allopatric speciation when two populations of an ancestral species become geographically separated (de Queiroz, 1998). The other one is reticulate sympatric speciation involving genome duplication by allopolyploidization, in which genetic combination between two ancestral species results in a descendant with the full complement of chromosomes from both ancestors (Kobel, 1996).

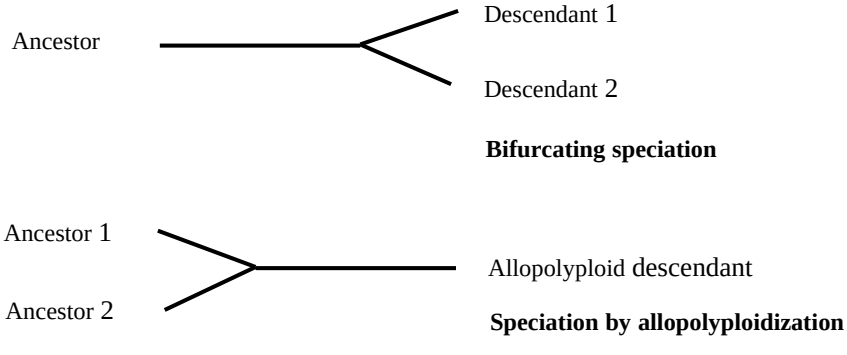

Fig. 9. Modes of speciation acting on subfamily Xenopodinae (Conlon et al., 2012).

An evolutionary scenario is proposed in which a diploid ancestor diverged into *Silurana* and *Xenopus* between 53 and 64 MYA (Fig. 10).

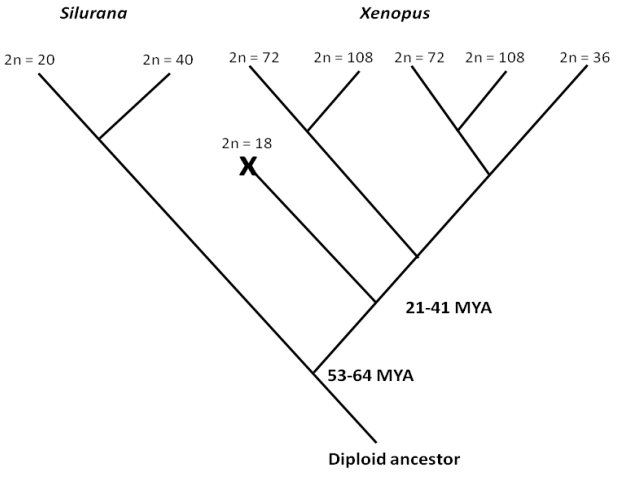

Fig. 10. An evolutionary scenario for subfamily Xenopodinae, including species with different ploidy level (2n). The diploid *Xenopus* species (2n = 18) has either disappeared or has not yet been discovered (indicated by **X**). The figure is based upon the results of comparisons of the nucleotide sequences of mitochondrial genes provided by Evans et al. (2004).

The genus *Silurana* comprise at least three extant species – *S. tropicalis* has retained diploid status with chromosome number 2n = 20, whereas more recent polyploidization events have produced *S. epitropicalis* and at least two other incompletely characterised tetraploid species with 2n = 40 chromosomes (Tymowska, 1991; Tymowska and Fischberg, 1982). Between 21 and 41 MYA, a genome duplication event in the *Xenopus* lineage produced the ancestor of all the extant tetraploid *Xenopus* species with 2n = 36 chromosomes (Fig. 10). These species have been divided further into three groups on the basis of their morphology, advertisement calls, and proposed evolutionary history (Evans, 2008; Evans et al., 2004; Tobias et al., 2011): the ***laevis*** group includes *X. laevis*, *X. gilli*, *X. largeni*, *X. petersii*, and *X. victorianus*; the ***muelleri*** group includes *X. muelleri*, *X. borealis* and *X. clivii*; and the ***fraseri*** group includes *X. fraseri* and *X. pygmaeus*. It would appear that the original *Xenopus* diploid species (2n = 18) has either disappeared or has not yet been discovered (Fig. 10).

At least two further allopolyploidization events produced two groups octoploid frogs comprising 7 species and in a similar fashion, genetic combination between the octoploid (2n = 72) and the tetraploid species produced the 2 dodecaploid (2n = 108) species (Fig. 11).

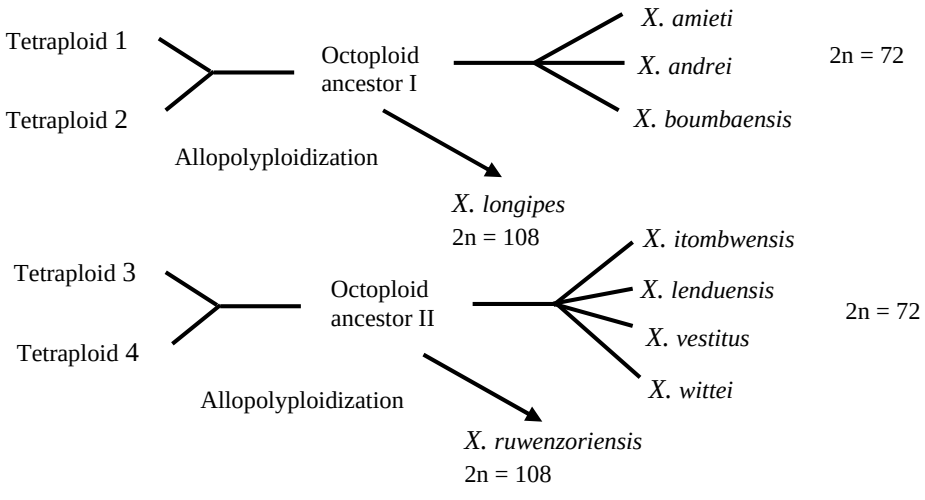

Fig. 11. A simplified schematic representation of the origin of the octoploid and dodecaploid species in the genus *Xenopus*. The figure is based upon the results of comparisons of the nucleotide sequences of mitochondrial genes provided by Evans et al. (2004) (Conlon et al., 2012).

AMPs in *X. laevis*

X. laevis was the first species in which amphibian AMPs were unambiguously identified (Zasloff, 1987). These AMPs provide a basis for comparison of the amino acid sequences of novel AMPs isolated from other species belonging to Pipidae family (Table 2).

Table 2. Peptides isolated from *X. laevis.*

Peptide name	Peptide sequence
Magainin 1	GIGKFLHSAGKFGKAFVGEIMKS
Magainin 2	GIGKFLHSAKKFGKAFVGEIMNS
PGLa	GMASKAGAIAGKIAKVALKAL[a]
CPF-1	GLASFLGKALKAGLKIGAHLLGGAPQQ
XPF-1	GWASKIGQTLGKIAKVGLQGLMQPK

C-terminal α-amidation is denoted by [a]

The AMP genes in *X. laevis* display the stereotypical structure “signal-spacer-bioactive peptide” with a partially conserved 20 amino acid signal peptide (Hunt and Barker, 1988; Konig and Bininda-Emonds, 2011) (Fig. 12).

Magainin	**MFKGLFICSLIAVICANALP**
XPF-1	**MYKGIFLCVLLAVICANSLA**
PGLa	**MYKQIFLCLIIAALCATIMA**

Fig. 12. Alignment of the AMP signal peptides in *X. laevis*. The shaded residues have been conserved in the three signal peptides.

The prepropeptide for PGLa is one of the simplest comprising only 64 amino acids. In contrast, the genes for magainin- and caerulin-precursor in *X. laevis* show tandem repeats of the spacer-peptide subunits, a notable feature for many archaeobatrachian AMP genes (Zasloff, 1987; Hunt and Barker, 1988; Gibson et al., 1991). The significant structural similarity in the N-terminal pro-regions of the precursor, as well as the repeats of the spacer-peptide segments, suggests that the AMPs in *X. laevis* may have evolved from a common ancestral gene by a series of duplication events and exon shuffling (Hunt and Barker, 1988; Kuchler et al., 1989).

Magainins

The name *magainin* comes from Hebrew word *magain* which means “a shield”. The magainin prepropeptide is predicted to be 303 residues long. It contains one magainin 1 sequence and five magainin 2 sequences flanked by almost identical acidic spacers (Fig. 13). The two closely related peptides – magainin 1 and 2, differ by two residues at positions 10 and 22 (Table 2) and exhibit a net charge of +4 at neutral pH (Zasloff, 1987; Giovannini et al, 1987). Biophysical studies have shown that these peptides are unstructured in solution, form an antiparallel dimer of amphipathic helices upon binding to the bacterial cell membrane, and disrupt the membrane via toroidal-type pore formation (described in Chapter **1**, *Interaction of α-helical AMPs with biological membranes*; Fig. 5, step 5b) (Matsuzaki, 1999).

Both magainin 1 and 2 have low haemolytic activity, inhibit growth of numerous species of bacteria and fungi and induce osmotic lysis of protozoa (Zasloff, 1987). Amino acid deletions in the N-terminal region (residues 1-14) result in the complete loss of

antimicrobial activity (Cuervo et al., 1988). In contrast, analogues with deletions in the C-terminal region (Ala-15, Cly-18 or Clu-19) have equal or even increased antimicrobial activity.

```
                                MFKGLFICSLIAVICANALPQPEASA   26
DEDMDEREVRGIGKFLHSAGKFGKAFVGEIMKSKRDAEAVGPEAFA              72
DEDLDEREVRGIGKFLHSAKKFGKAFVGEIMNSKRDAEAVGPEAFA              128
DEDLDEREVRGIGKFLHSAKKFGKAFVGEIMNSKRDAEAVGPEAFA              174
DEDLDEREVRGIGKFLHSAKKFGKAFVGEIMNSKRDAEAVGPEAFA              210
DEDFDEREVRGIGKFLHSAKKFGKAFVGEIMNSKRDAEAVGPEAFA              256
DEDLDEREVRGIGKFLHSAKKFGKAFVGEIMNSKRDAEAVDDRRWVE             303
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Fig. 13. Prepropetide sequence for magainin 1 and 2 peptides deduced from *X. laevis* skin cDNA library. Magainin 1 peptide is underlined. Spacers with almost identical sequences are flanking the sequences of the magainin 1 (one copy) and magainin 2 (five copies) depicted in bold (Terry et al., 1988).

Because magainins were among the first amphibian AMPs to be identified, there has been considerable research associated with their structure and mechanism of action. Thus, magainin 2 shows potent *in vitro* activity against *E. coli* (Zasloff et al., 1988) and *S. aureus* (Giacometti et al., 2000), and is also active, but at higher concentrations, against *P. aeruginosa* (Zasloff et al., 1988; Giacometti et al., 2000). Synthetic analogues have been developed in order to maximize the antimicrobial effects and minimize cytotoxicity. An extensive structure-activity investigation on magainin 2 resulted in the development of the 22 amino-acid, lysine-containing, linear analogue MSI-78 or pexiganan acetate with broad-spectrum antibacterial activity against Gram-positive and Gram-negative, anaerobic and aerobic bacteria but low cytotoxicity to mammalian cells (Ge et al., 1999). The bactericidal activity of pexiganan against aerobes and anaerobes is equivalent to that of ofloxacin (Fuchs et al., 1998). In addition, it is active against some clinical isolates of the opportunistic yeast pathogen *C. albicans*. Pexiganan showed distinct promise as a topical anti-infective agent for treatment of infected foot ulcers in diabetic patients and as a possible treatment for impetigo (Ge et al., 1999; Lipsky et al., 2008) but did not receive FDA approval.

Peptidyl-glycine-leucine-amide (PGLa)

Initially, peptidyl-tyrosine-leucine-amide (PYLa) was predicted from the screening of a *X. laevis* skin cDNA library for clones encoding the precursor of caerulein (Hoffmann et al., 1983a). It was furthermore shown that in total polyadenylated mRNA isolated from *X. laevis*, the PYL precursor mRNA is one of the most abundant components (Hoffman et al., 1983b). It was concluded that PYLa could form a membrane-active amphipathic helix similar to peptides with bacteriostatic, cytotoxic and/or lytic properties. However, the natural counterpart of PYLa named PGLa was isolated two years later from skin secretions of *X. laevis* by the same group (Andreu et al., 1985). PGLa is a 21-residue peptide, lacking the N-terminal tripeptide of PYLa, with a net charge of +5 at neutral pH because of four lysine residues and the amino group at the N-terminal glycine (Table 2). It also has an amidated C-terminus that provides resistance to the action of carboxypeptidases (Lohner and Prossnigg, 2009). Biophysical studies indicate that PGLa is unstructured in aqueous solution but is predominantly α-helical in the presence of negatively-charged lipid bilayers or micelles (Jackson et al., 1992; Latal et al., 1997; Bechinger et al., 1998).

PGLa exhibits broad spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi and protozoa but has minimal toxicity against eukaryotic cells (Bevins and Zasloff, 1990). In particular, PGLa shows potent *in vitro* activity against *E. coli*, *S. aureus*, and *S. pyogenes*, and is also active, but at higher concentrations, against *P. aeruginosa*, *Saccharomyces cerevisiae*, and *C. albicans* (Soravia et al., 1988). PGLa forms heterodimers with magainin 2 resulting in synergistic membrane disruption and antimicrobial activities (Westerhoff et al., 1995; Matsuzaki et al., 1998). Interestingly, PGLa adopts different orientations in the bacterial cell membrane, from a surface aligned monomeric state, via tilted dimers to an upright inserted state (described in Chapter **1**, *Interaction of α-helical AMPs with biological membranes*), depending on peptide concentration and interactions with magainin 2 (Tremouilhac et al., 2006a; Tremouilhac et al., 2006b).

Xenopsin precursor fragment (XPF)

The octapeptide xenopsin and the related XPF are bioactive peptides derived from a processing of a single precursor molecule (Gibson et al., 1986) (Table 2). The precursor protein contains xenopsin at the COOH terminus (Sures and Crippa, 1984). Both components can be isolated from extracts of *X. laevis* skin (Araki et al., 1973), as well as from specific granular cells in the lower esophagus and stomach. Xenopsin-like immunoreactivity, but not XPF, was also detected in tall, thin cells of the duodenum and in club-shaped cells of the large intestine (Sadler et al., 1992). XPF exhibits antimicrobial

activities against Gram-positive and Gram-negative bacteria comparable to those of the magainins (Soravia et al., 1988).

Caerulein-precursor fragment (CPF)

Caerulein is a decapeptide whose amino acid sequence shows a close resemblance to the C-terminal bioactive sites of the mammalian gastrointestinal hormone cholecystokinin (CCK). It was originally isolated from the skin of the Australian frog *Litoria caerulea*, formerly *Hyla caerulea* (Anastasi et al., 1967). Caerulein-related peptides have been identified in the skins of *X. laevis* as well as in multiple species in the families Hylidae, Leptodactylidae, Hyperoliidae, and Ranidae (Bowie and Tyler, 2006). Although the amino acid sequence at N-terminal region of the caeruleins is variable, most of the peptides contain a C-terminal sequence that is common to the mammalian peptides CCK and gastrin (Gly-Trp-Met-Asp-Phe.NH_2) and so exert similar biological effects (Erspamer and Melchiorri, 1983). It has been proposed that the genes encoding caeruleins in *Xenopus* and *Litoria* arose independently with the former arising from a duplication of the CCK gene and the latter from a duplication of the gastrin gene (Roelants et al., 2010). As with proxenopsin, the biosynthetic precursor of caerulein contains the additional bioactive peptide CPF. The structure of a CPF was predicted in cloning experiments using a cDNA library from *X. laevis* skin (Wakabayashi et al., 1985) (Table 2). Subsequent cDNA cloning studies demonstrated that the *X. laevis* genome contains multiple caerulein/CPF genes, containing one, three, or four copies of caerulein (Richter et al., 1986; Gibson et al., 1986; Giovannini et al., 1987). The antimicrobial activity of CPF has not been studied in detail.

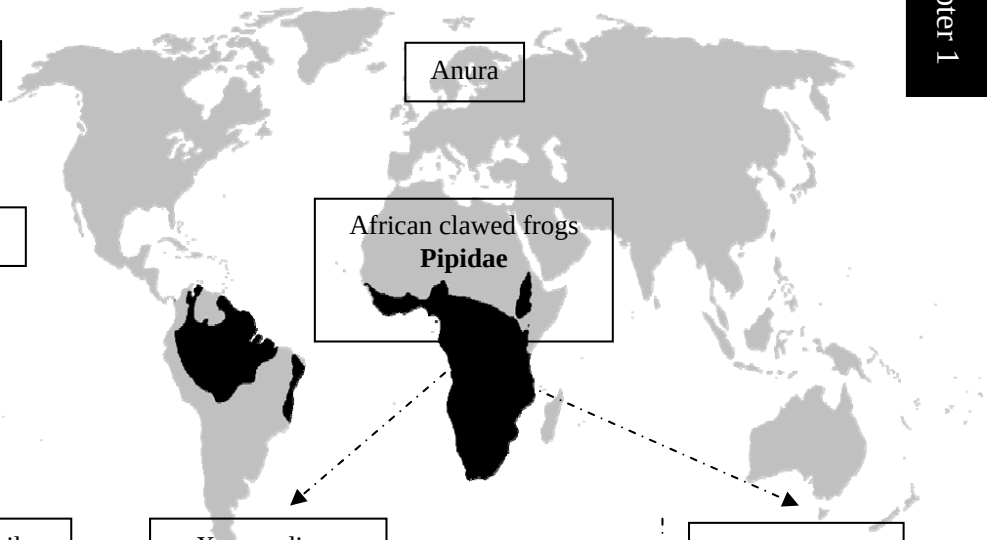
Research aim, objectives and thesis outline

Until recently, the diploid frog *S. tropicalis* (Ali et al., 2001), the tetraploid frog *X. laevis* (Zasloff, 1987; Giovannini et al., 1987), and the octoploid frog *X. amieti* (Conlon et al., 2010) were the only representatives of the Pipidae family from which dermal AMPs had been isolated and characterized. The aim of this thesis was to isolate systematically, purify and characterize AMPs with therapeutic potential from skin secretions of species of African clawed frogs with different level of polyploidy belonging to the Pipidae family.

The specific objectives of the study were:

- To isolate and purify AMPs from skin secretions of various polyploid frog species from the Pipidae family.
- To determine the structures of the newly discovered AMPs by automated Edman degradation combined with mass spectrometry and amino acid composition analysis.
- To characterize the *in vitro* activity of the purified AMPs against various reference strains and clinical isolates of a range of microorganisms.
- To investigate the possible application of the AMPs as classification tools to study the taxonomic relation of different frog species and genera.
- To analyse whether genome duplications affect the multiplicity of the AMPs in the skin secretions.
- To determine the extent to which interspecies hybridization affects the number of the AMPs in skin secretions from hybrid frogs.

The following species have been studied:



order	Anura			
family	African clawed frogs **Pipidae**			
sub-family	Xenopodinae			Pipinae
genus	***Silurana***	***Xenopus***		***Hymenochirus***
ploidy	2n = 40	2n = 36	2n = 72	2n = 20
species	"*S.* new tetraploid 1"	*X. clivii*	*X. andrei*	*H. boettgeri*
		X. borealis		
		X. muelleri West		
		X. muelleri		
interspecies hybrid		*X. muelleri* x *X. laevis*		

The isolation and structural characterization of AMPs in norepinephrine-stimulated skin secretions of *X. borealis* and *X. clivii* is described in Chapter **2** and Chapter **3** respectively. Additionally, the effect of the purified peptides on the growth and viability of reference strains of clinically relevant microorganisms was investigated.

Chapter **4** presents results from a comparative analysis of skin secretions from the incompletely characterized "*Silurana* new tetraploid 1" and the octoploid *X. andrei*. The primary structures and physicochemical properties of the AMPs are determined and the antimicrobial activities are tested against a range of microorganisms. The multiplicity of AMPs in each species was compared to those described in Chapters **2** and **3**, and in *X. laevis*.

A comparative study of the AMPs in skin secretions from two discrete populations of *X. muelleri* is presented in Chapter **5**. The antimicrobial potential of the newly identified peptides was evaluated using reference bacterial strains. In addition, a comparison of the primary sequences of the AMPs sets was performed to clarify the taxonomic relationship between the two frog populations.

The AMP profile from *Hymenochirus boettgeri*, a species in the subfamily Pipinae, was studied in Chapter **6**. A novel family of five structurally-related peptides, termed the hymenochirins, is described and their antimicrobial activities were determined against a range of reference strains of microorganisms.

The study in Chapter **7** was focused on F1 hybrid *X. laevis* x *X. muelleri* frogs. The AMP profile in skin secretions from female hybrid frogs is compared with the corresponding profiles from both parent species *X. laevis* and *X. muelleri* (Chapter **5**) in order to determine whether hybridization affects AMP multiplicity.

This thesis is completed by a general discussion (Chapter **8**) of the results reported in Chapters **2-7**. The potential of the newly characterised AMPs as candidates for development into therapeutic agents, as well as their application as reliable markers to elucidate the evolutionary relations among frogs from different genera and species is outlined. In addition, the fate of the duplicated AMPs genes is discussed. Moreover, the suitability of the interspecies hybrids as a model to study the mode of inheritance of the AMPs genes is described.

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Chapter 2

Antimicrobial peptides with therapeutic potential from frog skin secretions of the Marsabit clawed frog *Xenopus borealis* (Pipidae)

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ABSTRACT

Nine peptides with differential growth inhibitory activity against *Escherichia coli* and *Staphylococcus aureus* were isolated from norepinephrine-stimulated skin secretions of the tetraploid frog *Xenopus borealis* Parker, 1936 (Pipidae). Structural characterization of the peptides demonstrated that they were orthologous to magainin-2 (1 peptide), peptide glycine-leucine-amide, PGLa (2 peptides), caerulein-precursor fragments, CPF (4 peptides) and xenopsin-precursor fragments, XPF (2 peptides), previously isolated from *X. laevis* and *X. amieti*. In addition a second magainin-related peptide (G**KFLHSAGKFGKAFLGEVMIG) containing a two amino acid residue deletion compared with magainin-2 was identified that had only weak antimicrobial activity. The peptide with the greatest potential for development into a therapeutically valuable anti-infective agent was CPF-B1 (GLGSLLGKAFKIGLKTV GKMMGGAPREQ) with MIC = 5 µM against *E.coli*, MIC = 5 µM against *S. aureus*, and MIC = 25 µM against *Candida albicans*, and low hemolytic activity against human erythrocytes (LC_{50} >200 µM). This peptide was also the most abundant antimicrobial peptide in the skin secretions. CPF-B1 was active against clinical isolates of the nosocomial pathogens, methicillin-resistant *S. aureus* (MRSA) and multidrug-resistant *Acinetobacter baumannii* (MDRAB) with MIC values in the range 4 - 8 µM.

1. Introduction

The emergence of pathogenic strains of bacteria and fungi with resistance to commonly used antibiotics has necessitated a search for new types of antimicrobial agents (Norrby et al., 2005; Livermore, 2009). Peptides with broad spectrum antimicrobial activity synthesized in granular glands in the skin of several, although by no means all, species of Anura (frogs and toads) represent a source of potential therapeutically valuable anti-infective drugs (Rinaldi, 2002; Conlon et al., 2007). Despite the fact that the African clawed frog *Xenopus laevis* was the first amphibian species in which such peptides were identified (Zasloff, 1987), relatively little work has been done to exploit other species belonging to the family Pipidae as a source of pharmaceutically useful antimicrobial compounds. Analysis of *X. laevis* skin secretions has led to the isolation and characterization of magainin-1 and -2, peptides that are encoded by the same gene (Giovannini et al., 1987; Zasloff, 1987) and multiple antimicrobial peptides with varying potencies and specificities that are derived from the post-translational processing of the biosynthetic precursors of, caerulein, xenopsin, and peptide glycine-leucine-amide (Gibson et al., 1986; Richter et al., 1986; Soravia et al., 1988; James et al., 1994) . A comparison of the amino acid sequences of the biosynthetic precursors of procaerulein, promagainin, and proxenopsin, deduced from the nucleotide sequences of cDNAs, reveals significant structural similarity in the N-terminal regions of the precursors suggesting that the peptides may have evolved from a common ancestral gene by a series of duplication events (Hunt and Barker, 1988; Kuchler et al., 1989). In common with the vast majority of frog skin antimicrobial peptides, all the active components isolated from *X. laevis* have the propensity to form an amphipathic α-helical conformation over at least part of their structures in a membrane-mimetic solvent such as 50% trifluoroethanol-water or in the presence of an anionic phospholipid vesicle (Powers and Hancock, 2003).

The clawed frogs comprise 32 species distributed in five genera (*Hymenochirus*, *Pipa*, *Pseudhymenochirus*, *Silurana*, and *Xenopus*) within the family Pipidae (Frost, 2010). The taxonomic status of *Silurana* is controversial as cladistic analyses based upon nucleotide sequences of mitochondrial genes strongly support the monophyly of *Xenopus* + *Silurana* but it was pointed out that the use of two genera "underscores trenchant biological and historical differences between the two clades" (Evans et al., 2004). The genus *Xenopus* currently contains 18 species although several additional, as yet unnamed, species have been reported (Evans et al., 2004). The cytogenetics of the African clawed frogs is complex (Kobel and Du Pasquier, 1991). *S. tropicalis* with 2n = 20 chromosomes is the only diploid species (de Sa and Hillis, 1990). A putative whole genome duplication within the *Silurana* lineage has given rise to the tetraploid species *S. epitropicalis* (Tymowska and Fischberg, 1982), and a duplication within the *Xenopus* lineage has produced tetraploid species that include *X. laevis* and *Xenopus borealis* with 2n = 36 chromosomes (Evans et al., 2004). At least one further genome duplication event within the tetraploid lineage has given rise to

octoploid species that include *X. amieti*, and further independent polyploidizations involving the combination of tetraploid and octaploid genomes have produced the dodecaploid species *X. ruwenzoriensis* and *X. longipes* (2n = 108) (Evans et al., 2004).

In addition to *X. laevis*, antimicrobial peptides have been isolated only from *S. tropicalis* (Ali et al., 2001) and *X. amieti* (Conlon et al., 2010). Consequently, the aim of the present study was to identify novel peptides with therapeutic potential as anti-infective agents and to gain insight into the molecular evolution of the diverse array of *Xenopus* antimicrobial peptides by isolating and characterizing the components that are present in norepinephrine-stimulated skin secretions of the Marsabit clawed frog *X. borealis* (also known as the Kenya clawed frog). *X. borealis* is widely distributed in central and northern Kenya and northern Tanzania and may also be found in Uganda. It is an aquatic species whose natural habitats are high altitude (above 1500 m) freshwater ponds and slow-flowing streams in grasslands and pasturelands. It is currently listed as a species of least concern in the IUCN Red List of Threatened Species (Tinsley et al., 2004).

The nomenclature used for designating the antimicrobial peptides identified in this study is that used previously for peptides from *X. laevis* (James et al., 1994) and *X. amieti* (Conlon et al., 2010). The magainin, peptide glycine-leucine amide (PGLa), caerulein-precursor fragment (CPF), and xenopsin-precursor fragment (XPF) peptide families are recognized. The species origin is denoted by B and paralogs are differentiated by numerals e.g. CPF-B1 and CPF-B2.

2. Experimental

2.1. Collection of skin secretions

All procedures with live animals were approved by the Animal Research Ethics committee of U.A.E. University (Protocol No. A21-09) and were carried out by authorized investigators. Adult specimens of *X. borealis* (n = 2; male 23 g, female 33 g) were supplied by Xenopus Express Inc. (Brooksville, FL, USA). The frogs were collected in Kenya (2°20′N, 37°59′E). Each animal was injected at two sites in the dorsal lymph sac with norepinephrine bitartrate (40 nmol/g body mass) and immersed in water for 15 min. The solution containing the secretions was acidified with trifluoroacetic acid (TFA) (final concentration 1% v/v) and stored at -20^{o} C. The pooled solution was passed at a flow rate of 2 mL/min through 8 Sep-Pak C-18 cartridges (Waters Associates, Milford, MA, USA) connected in series. Bound material was eluted with acetonitrile/water/ TFA (70.0:29.9:0.1, v/v/v; 10 mL) and freeze-dried.

2.2. Antimicrobial assays

Reference strains of *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), and *Candida albicans* (ATCC 90028) were purchased from the American Type Culture Collection (Rockville, MD, USA). Six strains of methicillin-resistant *S. aureus* (MRSA) strains and five multi-drug resistant *Acinetobacter baumannii* (MDRAB) strains were isolated from patients at four different hospitals in Abu Dhabi Emirate. The origin, spectrum of resistance to antibiotics, and the clonal lineage of the strains have been described previously (Pál et al., 2006; Conlon et al., 2009).

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) from a log-phase culture of reference strains of *S. aureus* and *E. coli* in 96-well microtiter cell-culture plates for 18 h at 37 ^{o}C in a humidified atmosphere of air. Incubations with *C. albicans* were carried out in RPMI 1640 medium for 48 h at 35 ^{o}C with an inoculum of 5 x 10^4 CFU/mL. After incubation, the absorbance at 630 nm of each well was determined using a microtiter plate reader. Minimum inhibitory concentration (MIC) of the peptides was measured in the concentration range of 3 - 200 μM by standard double dilution methods (Clinical and Laboratory Standards Institute, 2008a,b) and was taken as the lowest concentration of peptide where no visible growth was observed. This value was confirmed by measurement of absorbance at 630 nm. In order to monitor the validity and reproducibility of the assays, incubations with bacteria were carried out in parallel with increasing concentrations of ampicillin and incubations with *C. albicans* with amphotericin as previously described (Conlon et al., 2007, 2010).

Hemolytic activity against human erythrocytes taken from a healthy donor was measured as previously described (Conlon et al., 2010). The LC_{50} value was taken as the mean concentration of peptide producing 50% hemolysis in two independent experiments.

2.3. Peptide purification

The skin secretions, after partial purification on Sep-Pak cartridges, were redissolved in 0.1% (v/v) TFA/water (4 mL) and injected onto a (2.2 x 25-cm) Vydac 218TP1022 (C-18) reversed-phase HPLC column (Grace, Deerfield, IL, USA) equilibrated with 0.1% (v/v) TFA/water at a flow rate of 6.0 mL/min. 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 nm and 280 nm and fractions (1 min) were collected. The abilities of freeze-dried aliquots (50 μL) of the fractions to inhibit the growth of *S. aureus* and *E. coli* were determined as described in the previous section. Fractions

associated with antimicrobial activity were successively chromatographed on a (1 x 25-cm) Vydac 214TP510 (C-4) column, a (1 x 25-cm) Vydac 208TP510 (C-8) column, and a (1 x 25-cm) 238EV510 Everest (C-18) 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.

2.4. Structural characterization

The primary structures of the peptides were determined by automated Edman degradation using an Applied Biosystems model 494 Procise sequenator (Foster City, CA, USA). MALDI-TOF mass spectrometry was carried out using a Voyager DE-PRO instrument (Applied Biosystems) that was operated in reflector mode with delayed extraction and the accelerating voltage in the ion source was 20 kV. The instrument was calibrated with peptides of known molecular mass in the 2- 4 kDa range. The accuracy of mass determinations was ± 0.02%. Amino acid composition analyses were performed by the University of Nebraska Medical Center Protein Structure Core Facility (Omaha, NE, USA).

3. Results

3.1. *Purification of the peptides*

The pooled skin secretions from *X. borealis*, after partial purification on Sep-Pak C-18 cartridges, were chromatographed on a Vydac C-18 preparative reversed-phase HPLC column (Fig. 1). The prominent peaks designated 1-8 were collected and subjected to further purification. Under the conditions of assay, peaks 1-4 and 8 were associated with the ability to inhibit the growth of *E. coli* only whereas peaks 5-7 inhibited the growth of both *E. coli* and *S. aureus*. Subsequent structural analysis demonstrated that peak 1 contained magainin-B1, peak 2: magainin-B2, peak 3: XPF-B1, peak 4: XPF-B2, peak 5: PGLa-B1, peak 6: a mixture of CPF-B1, CPF-B2, and PGLa-B2, peak 7: CPF-B3, and peak 8 CPF-B4.

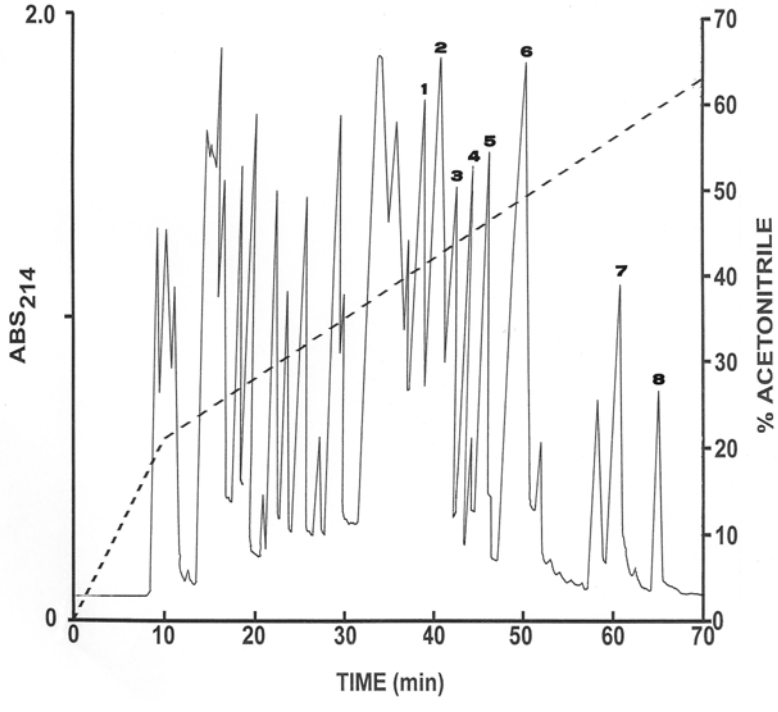

Fig. 1. Reversed-phase HPLC on a preparative Vydac C-18 column of skin secretions from *X. borealis*, after partial purification on Sep-Pak cartridges. The peaks designated 1-8 displayed varying degrees of antimicrobial activity and were purified further. The dashed line shows the concentration of acetonitrile in the eluting solvent.

The peptides were purified to near homogeneity, as assessed by a symmetrical peak shape and mass spectrometry, by further chromatography on semipreparative Vydac C-4, Vydac C-8, and Everest C-18 columns. The methodology is illustrated by the partial separation of CPF-B1, CPF-B2, and PGLa-B2 on a Vydac C-4 column (Fig. 2A), followed by purification to near homogeneity of CPF-B1 by successive chromatographies on a Vydac C-8 column (Fig. 2B) and an Everest C-18 column (Fig. 2C).

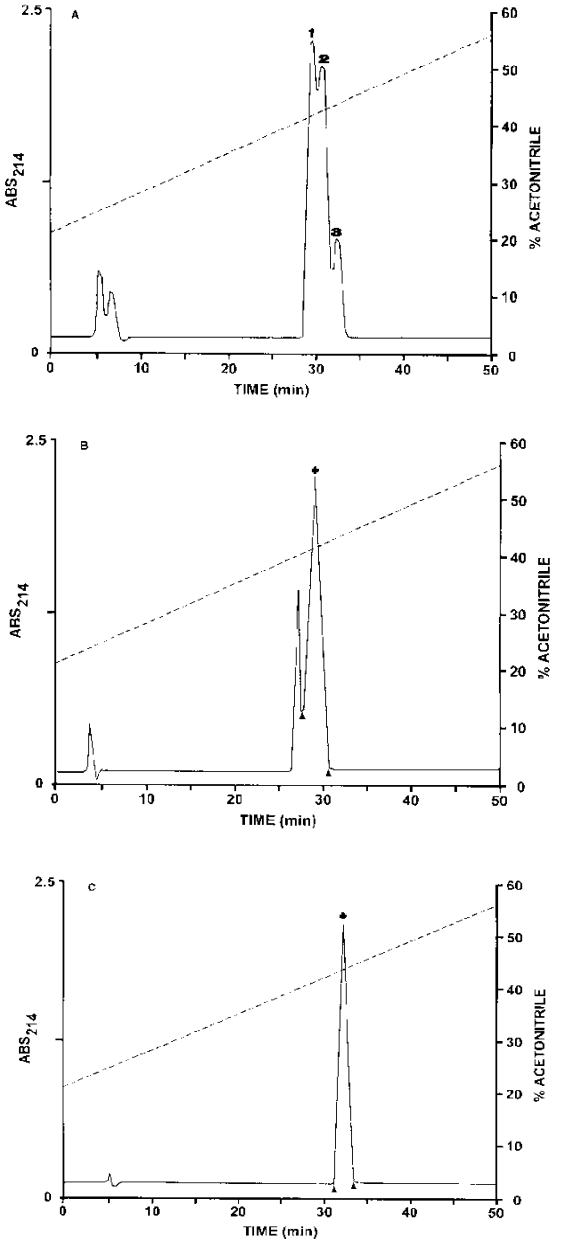

Fig. 2. Purification to near homogeneity of CPF-B1 on semipreparative (a) Vydac C-4, (b) Vydac C-8, and (c) Everest C-18 columns. The arrowheads show where peak collection began and ended. In panel A, peak 1 contains CPF-B1, peak 2 contains PGLa-B2, and peak 3 contains CPF-B3. In panels B and C, the peak containing CPF-B1 is denoted by the symbol +.

The approximate final yields of purified peptides (nmol) were magainin-B1 120, magainin-B2 590, XPF-B1 25, XPF-B2 80, PGLa-B1 35, PGLa-B2 510, CPF-B1 720, CPF-B2 20, CPF-B3 55, and CPF-B4 35.

3.2. *Structural characterization*

The primary structures of the antimicrobial peptides isolated from *X. borealis* skin secretions were established by automated Edman degradation. As the C-terminal regions of the magainin-B1, PGLa-B1, PGLa-B2, and CPF-B4 are strongly hydrophobic, it was not possible to determine their C-terminal amino acid residue because of wash-out from the sequencer disk. However, this ambiguity was resolved by determining the amino acid compositions of the peptides and their complete primary structures are shown in Fig. 3. MALDI-TOF mass spectrometry was used to confirm the proposed structures and to demonstrate that PGLa-B1 and PGLa-B2 are C-terminally α-amidated.

		$M+H_{obs}$	$M+H_{calc}$
Magainin-B1	GKFLHSAGKFGKAFLGEVMIG	2194.0	2194.2
Magainin-B2	GIGKFLHSAGKFGKAFLGEVMKS	2409.5	2409.3
XPF-B1	GFKQFVHSMGKFGKAFVGEIINPK	2666.7	2666.4
XPF-B2	GWASKIGTQLGKMAKVGLKEFVQS	2563.6	2563.4
PGLa-B1	GMASKAGTIAGKIAKTAIKLAL.NH_2	2113.1	2113.3
PGLa-B2	GMASKAGSIVGKIAKIALGAL.NH_2	1955.2	1955.2
CPF-B1	GLGSLLGKAFKIGLKTVGKMMGGAPREQ	2844.5	2844.6
CPF-B2	GLGSLLGKAFKIGLKTVGKMMGGAPR	2587.6	2587.5
CPF-B3	GLGSLLGSLFKFIPKLLPSIQQ	2356.2	2356.4
CPF-B4	GLLTNVLGFLKKAGKGVLSGLLPL	2408.2	2408.5

Fig. 3. Amino acid sequences, observed molecular masses (M_r obs), and calculated molecular masses (M_r calc) of the antimicrobial peptides isolated from skin secretions of *X. borealis*.

3.3. *Antimicrobial activities*

The abilities of the most abundant of the peptides in the *X. borealis* skin secretions to inhibit the growth of reference strains of the Gram-positive bacterium *S. aureus*, the Gram-negative bacterium *E. coli*, and the opportunistic yeast pathogen *C. albicans* are compared in Table 1. The MIC values for CPF-B1 against clinical isolates of MRSA and MDRAB were within the range 4-8 μM for all strains tested. Magainin-B2, PGLa-B2, and CPF-B1 displayed very low hemolytic activity (LC_{50} > 200 μM) against human erythrocytes. There was insufficient pure material to determine the antimicrobial activities of PGLa-B1, CPF-B2, CPF-B3, CPF-B4, and XPF-B1.

Table 1. Minimum inhibitory concentrations against microorganisms and hemolytic activity against human erythrocytes (HC_{50}) of the endogenous peptides isolated from skin secretions of *X. borealis*

	E. coli	***S.aureus***	***C. albicans***	**HC_{50}**
Magainin-B1	>100	>100	ND	ND
Magainin-B2	50	>100	100	>200 (0%)
PGLa-B2	25	50	25	>200 (27%)
CPF-B1	5	5	25	>200 (18%)
XPF-B2	100	25	ND	ND

Data are expressed as μM. The values in parentheses represent the % hemolysis at 200 μM.

4. Discussion

The present study has identified ten peptides with varying degrees of antimicrobial activity in norepinephrine-stimulated skin secretions of the tetraploid frog *X. borealis*. As shown in Fig. 4, a comparison of their primary structures reveals that they represent orthologs of previously described peptides (magainin, PGLa, CPF, and XPF) isolated from the diploid frog *S. tropicalis* (Ali et al., 2001), the tetraploid frog *X. laevis* (Gibson et al., 1986; James et al., 1994), and the octoploid frog *X. amieti* (Conlon et al., 2010). It is unclear whether CPF-B2, which is identical to residues (1 -26) of CPB-B1, represents an alternative post-translational processing product of the CPB-B1 precursor arising from cleavage at Arg^{26} or, less probably, is the product of a separate gene. In addition, a peptide (magainin-B1) was isolated in relatively low yield that is clearly a member of the magainin family on the basis of structural similarity but contains a two amino acid residue (Ile^2-Gly^3)

deletion compared with magainin-B2 (Fig. 3). This peptide had only very weak antimicrobial activity (MIC > 100 µM against *E. coli* and *S. aureus*). A Schiffer-Edmundson wheel (1967) representation of the magainin-B1 structure (not shown) demonstrates that the peptide retains the propensity to form an amphipathic α-helix so that the reduced antibacterial activity is probably a consequence of the decreased positive charge on the peptide produced by the substitution of the Lys^{22} residue in magainin-B2 by Ile (Fig. 3). Studies with analogs of magainin-2 have demonstrated that increasing the positive charge on the peptide resulted in an increase in antimicrobial activity until a limit was reached, whereupon further increases in cationicity did not result in any further increase in activity (Bessalle et al., 1992; Dathe et al., 2001).

Evolutionary pressure to conserve the primary structures of the antimicrobial peptides from the clawed frogs has not been particularly strong (Fig. 4).

Magainin-related peptides

X. borealis	G**KFLHSAGKFGKAFLGEVMIG
X. borealis	GIGKFLHSAGKFGKAFLGEVMKS
X. amieti	GIKEFAHSLGKFGKAFVGGILNQ
X. amieti	GVSKILHSAGKFGKAFLGEIMKS
X. laevis	GIGKFLHSAGKFGKAFVGEIMKS
X. laevis	GIGKFLHSAKKFGKAFVGEIMNS

PGLa-related peptides

X. borealis	GMASKAGTIAGKIAKTAIKLALa
X. borealis	GMASKAGSIVGKIAKIAL*GALa
X. amieti	GMASKAGSVLGKVAKVALKAALa
X. amieti	GMASTAGSVLGKLAKAVAIGALa
X. laevis	GMASKAGAIAGKIAKVALK*ALa
S. tropicalis	GMATKAGTALGKVAKAVIGAALa

Procaerulein-derived peptides

X. borealis	GLLTNVLGFLKKAGKGVLSGLLPL
X. amieti	GL*GSVLGKILKMGANLLGGAPKGA
X. amieti	GL*GSVLGKALKIGANLLa
X. amieti	GL*GSLVGNALRIGAKLLa
S. tropicalis	GLLGPLLKIAAKVGSNLLa
S. tropicalis	GFLGSLLKTGLKVGSNLLa

X. borealis	GL*GSLLGKAFKIGLKTVGKMMGGAPREQ
X. borealis	GLGSLLGSLF*KFIPK******LLPSIQQ
X. laevis	GLASFLGKAL*KAGLK*IGAHLLGGAPQQ
X. laevis	GFASFLGKAL*KAALK*IGANMLGGTPQQ
X. laevis	GFGSFLGKAL*KAALK*IGANALGGSPQQ
X. laevis	GLASLLGKAL*KAGLK*IGTHFLGGAPQQ
S. tropicalis	GFLGPLLKLAAKGVAK**VIPHLIPSRQQ

Proxenopsin-derived peptides

X. borealis	GFKQFVH*SMGKFGKAFVGEIINPK
X. borealis	GWASKIGTQLGKMAKVGLKEFVQS
X. amieti	GWASKIAQTLGKMAKVGLQELIQPK
X. laevis	GWASKIGQTLGKIAKVGLQGLMQPK
X. laevis	GWASKIGQTLGKIAKVGLKELIQPK
S. tropicalis	GLASTLGSFLGKFAKGGAQAFLQPK

Fig. 4. A comparison of the primary structures of the antimicrobial peptides from *X. borealis* with orthologous peptides from the octoploid frog *X. amieti*, the tetraploid frog *X. laevis*, and the diploid frog *S. tropicalis*. Structural similarity is emphasized by the shading. (*) denotes a residue deletion and ([a]) indicates that the peptide is C-terminally α-amidated.

In peptides belonging to the magainin family from the four species studied to-date, 10 out of 23 amino acids are invariant and in the case of peptides from the PGLa family, 11 out of 22 residues are invariant. In contrast, sequences of the procaerulein- and proxenopsin-related peptides are highly variable. Only 5 residues out of 24 are conserved in the XPF family. A comparison of the primary structures of the CPF family peptides suggests that they divide naturally into two sub-groups but only 3 amino acids in the first group and 5 amino acids in the second group are invariant, even after deletions have been introduced into some sequences in order to maximize structural similarity.

The most abundant antimicrobial peptide in the *X. borealis* skin secretions was the component with the greatest potential for development into a therapeutically valuable anti-infective agent. CPF-B1 showed potent, broad spectrum growth-inhibitory activity against reference strains of Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacteria, and against the opportunistic yeast pathogen *C. albicans* but only very low hemolytic activity against human erythrocytes (Table 1). In addition, the peptide displayed high potency (MIC = 4 - 8 μM) against isolates of two groups of clinically important pathogens for which antibiotic resistance has become a serious problem, notably methicillin-resistant

Staphylococcus aureus (MRSA) and multidrug-resistant *Acinetobacter baumannii* (MDRAB). During the last decade, MRSA (Chambers and Deleo, 2009) and MDRAB (Dijkshoorn et al., 2007; Peleg et al., 2008) have emerged to become a major phenotype in hospitals worldwide with a high rate of mortality. More recently, new strains of the pathogens have emerged in the community, causing infections in young, otherwise healthy, people (Gootz and Marra, 2008; Cooke and Brown, 2010; Deleo et al., 2010). In addition to β-lactam resistance, MRSA strains may exhibit multi-drug resistance including non-susceptibility to quinolones, macrolides and sulfonamides (Garau et al., 2009). Similarly, among strains causing nosocomial outbreaks, resistance of MDRAB to fluoroquinolones, aminoglycosides, sulphonamides, third-generation cephalosporins and even carbapenems is common (Vila and Pachón, 2008). Treatments of MDRAB with alternative drugs such as polymyxins, particularly colistin (polymyxin E), and the glycylcycline, tigecycline are far from optimal due to concerns with nephrotoxicity regarding colistin and the bacteriostatic nature of tigecycline (Karageorgopoulos and Falagas, 2008). There is clearly a need for new anti-infective agents to which these pathogens have not been exposed and CPF-B1 represents a promising candidate.

A major obstacle to the development of peptide-based anti-infective drugs, particularly if they are to be administered systemically, is their short half-lives in the circulation (Zhang and Falla, 2010). However, peptides applied to infected skin or skin lesions in the form of sprays or ointments can penetrate into the *stratum corneum* to kill microorganisms.

MRSA is a serious problem in infections of the superficial epidermis such as impetigo in infants and children (Geria and Schwartz, 2010) and in colonization of surface lesions such as the foot ulcers of diabetic patients (Bowling et al., 2009). Similarly, MDRAB infection of wounds, particularly associated with natural disasters and conflicts in war zones, is becoming increasingly common and can lead to necrotizing lesions that do not heal (Sebeny et al., 2008). The high potency of CPF-B1 against MRSA and MDRAB together with its low toxicity suggests that topical applications of the peptide may have a role to play as a treatment option in combating skin infections caused by these pathogens and in therapeutic regimes to promote wound healing.

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Chapter 3

Purification and properties of antimicrobial peptides from skin secretions of the Eritrea clawed frog *Xenopus clivii* (Pipidae)

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ABSTRACT

Five peptides with antimicrobial activity were isolated from norepinephrine-stimulated skin secretions of the tetraploid frog *Xenopus clivii* Peracca, 1898 (Pipidae). Characterization of the peptides demonstrated that they are structurally similar to magainins (2 peptides), caerulein-precursor fragments, CPF (2 peptides), and xenopsin-precursor fragments, XPF (1 peptide) that have been previously isolated from other species of the genus *Xenopus*. The magainins and the XPF peptide were active only against the Gram-negative microorganism *Escherichia coli* whereas the CPF peptides were also active against the Gram-positive *Staphylococcus aureus*. The most abundant antimicrobial peptide in the secretions, CPF-C1 (GFGSLLGKALRLG ANVL.NH_2) inhibited the growth of the Gram-negative bacteria *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* (MIC ≤ 25 µM) suggesting potential for development into an anti-infective agent for use against these emerging antibiotic-resistant pathogens.

1. Introduction

The skin secretions of many species of frogs contain biologically active peptides with the potential for development into pharmaceutically useful compounds (Clarke, 1997). The family Pipidae is composed of 32 species in 5 genera (*Hymenochirus*, *Pipa*, *Pseudhymenochirus*, *Silurana*, and *Xenopus*) (Frost, 2010). All are found in Africa except for members of the genus *Pipa* which are found in South America. The genus *Xenopus* (commonly known as African clawed frogs) currently contains 18 species although several additional, as yet unnamed, species have been reported (Evans et al., 2004). Examples of therapeutically valuable peptides that have been isolated from skin secretions of the extensively studied species *X. laevis* include caerulein, used to restore peristalsis in intestinal paralysis after abdominal surgery and as a diagnostic aid in x-ray examination of the gallbladder and small bowel (Vincent et al., 1982), and xenopsin, shown to inhibit vascular leakage after tissue injury (Gao and Wei, 1993).

Analysis of *X. laevis* skin secretions has led to the isolation and characterization of a range of peptides with growth inhibitory activity against bacterial and fungal pathogens. These include magainin-1 and -2, encoded by the same gene (Giovannini et al., 1987; Zasloff, 1987), and multiple antimicrobial peptides that are derived from the post-translational processing of the biosynthetic precursors of caerulein (Gibson et al., 1986; Richter et al., 1986), xenopsin (Gibson et al., 1986; Soravia et al., 1988), and peptide glycine-leucine-amide (Andreu et al., 1985). Although magainin-2 has only modest antimicrobial potency, a lysine-substituted C-terminally α-amidated analog, termed pexiganan, has shown potential as a topical anti-infective agent for the treatment of infected foot ulcers in diabetic patients and as a possible treatment for impetigo (Lipsky et al., 2008; Gottler and Ramamoorthy, 2009).

Despite the fact that *X. laevis* was the first amphibian species in which cutaneous antimicrobial peptides were unambiguously identified (Zasloff, 1987), frogs of the Pipidae family have not been extensively studied as a source of potential anti-infective agents. Besides *X. laevis*, antimicrobial peptides have been isolated only from the diploid frog *Silurana tropicalis* (formerly *Xenopus tropicalis*) (Ali et al., 2001), the tetraploid frog *X. borealis* (Mechkarska et al., 2010), and the octoploid frog *X. amieti* (Conlon et al., 2010b). Consequently, the aim of the present study was to identify novel peptides with therapeutic potential in norepinephrine-stimulated skin secretions of the Eritrea clawed frog *Xenopus clivii* (also known as Peracca's clawed frog). Structural characterization of these peptides allows insight into the molecular evolution of the diverse array of *Xenopus* antimicrobial peptides. *X. clivii* is a tetraploid frog (chromosome number 2n = 36) with a distribution centered on the Ethiopian plateau that is also found in northwestern Kenya and adjacent Sudan (Frost, 2010). It is a wholly aquatic species whose wide-ranging natural habitats are relatively high altitude (820 - 2745 m elevation), still and slow-flowing

permanent water bodies in montane forest and grassland, *Acacia* woodland and savannah. It is a resilient species and is currently listed as a species of least concern in the IUCN Red List of Threatened Species (Measey et al., 2004). Although still relatively abundant, populations have declined due to habitat loss.

The nomenclature used for designating the antimicrobial peptides identified in this study is that used previously for peptides from *X. laevis* (James et al., 1994), *X. amieti* (Conlon et al., 2010b), and *X. borealis* (Mechkarska et al., 2010). The magainin, caerulein-precursor fragment (CPF), and xenopsin-precursor fragment (XPF) peptide families are recognized. The species origin is denoted by C and paralogs are differentiated by numerals e.g. CPF-C1 and CPF-C2.

2. Experimental

2.1. Collection of skin secretions

All procedures with live animals were approved by the Animal Research Ethics committee of U.A.E. University (Protocol No. A21-09) and were carried out by authorized investigators. Two specimens of *X. clivii* (male 41 g, female 15 g) were supplied by Xenopus Express Inc. (Brooksville, FL, USA). The animals were collected in Ethiopia (09°02′N, 38°42′E). Each animal was injected at two sites in the dorsal lymph sac with norepinephrine bitartrate (40 nmol/g body mass) and immersed in water (100 ml) for 15 min at room temperature. The solution containing the secretions was acidified with trifluoroacetic acid (TFA) (final concentration 1% v/v) and stored at -20 ^{o}C. The pooled solution was passed at a flow rate of 2 mL/min through 6 Sep-Pak C-18 cartridges (Waters Associates, Milford, MA, USA) connected in series. The bound material was eluted with acetonitrile/water/TFA (70.0:29.9:0.1, v/v/v; 10 mL) and freeze-dried.

2.2. Antimicrobial and hemolytic assays

Reference strains of *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25726), *Klebsiella pneumoniae* (ATCC 700603), and *Pseudomonas aeruginosa* (ATCC 27853) were purchased from the American Type Culture Collection (Rockville, MD, USA). The Euroclone I NM8 strain of *Acinetobacter baumannii* has been described previously (Conlon et al., 2010a). 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) from a log-phase culture of reference strains of *S. aureus* and *E. coli* in 96-well microtiter cell-culture plates for 18 h at 37 ^{o}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 concentration (MIC) of the peptides was measured in the concentration range of 3 - 100 μM by a standard double dilution method (Clinical and Laboratory Standards Institute, 2008) and was taken as the lowest concentration of peptide where no visible growth was observed. This value was confirmed by measurement of absorbance at 630 nm. In order to monitor the validity and reproducibility of the assays, incubations were carried out in parallel with increasing concentrations of antibiotics (ampicillin for *S. aureus*, *E. coli*, and *A. baumannii*, and ciprofloxacin for *K. pneumoniae* and *P. aeruginosa*) as previously described (Mechkarska et al., 2010).

Hemolytic activity against human erythrocytes taken from a healthy donor was measured as previously described (Conlon et al., 2010b). The LC_{50} value was taken as the mean concentration of peptide producing 50% hemolysis in two incubations.

2.3. Peptide purification

The skin secretions, after partial purification on Sep-Pak cartridges, were redissolved in 0.1% (v/v) TFA/water (4 mL) and injected onto a (2.2 x 25-cm) Vydac 218TP1022 (C-18) reversed-phase HPLC column (Grace, Deerfield, IL, USA) equilibrated with 0.1% (v/v) TFA/water at a flow rate of 6.0 mL/min. 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 nm and 280 nm and fractions (1 min) were collected. The abilities of freeze-dried aliquots (50 μL) of the fractions to inhibit the growth of *S. aureus* and *E. coli* were determined as described in the previous section. Fractions associated with antimicrobial activity were successively chromatographed on (1 x 25-cm) Vydac 214TP510 (C-4) and (1 x 25-cm) Vydac 208TP510 (C-8) columns. 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.

2.4. Structural characterization

The primary structures of the peptides were determined by automated Edman degradation using an Applied Biosystems model 492 Procise sequenator (Foster City, CA, USA). MALDI-TOF mass spectrometry was carried out using a Voyager DE-PRO instrument (Applied Biosystems) that was operated in reflector mode with delayed extraction and the accelerating voltage in the ion source was 20 kV. The instrument was calibrated with peptides of known molecular mass in the 2 - 4 kDa range. The accuracy of mass determinations was ± 0.02%.

2.5. Secondary structure prediction

Prediction of secondary structure and determination of helicity per residue for the peptides were performed using the AGADIR program (Muñoz and Serrano 1994). AGADIR is a prediction algorithm based on the helix/coil transition theory which predicts the helical behaviour of monomeric peptides. Calculations were performed at pH 7 and 298 K. A minimum percentage of 1% helicity/residue was considered to predict the presence of a helix.

3. Results

3.1. Purification of the peptides

The pooled skin secretions from *X. clivii*, after partial purification on Sep-Pak C-18 cartridges, were chromatographed on a Vydac C-18 preparative reversed-phase HPLC column (Fig. 1).

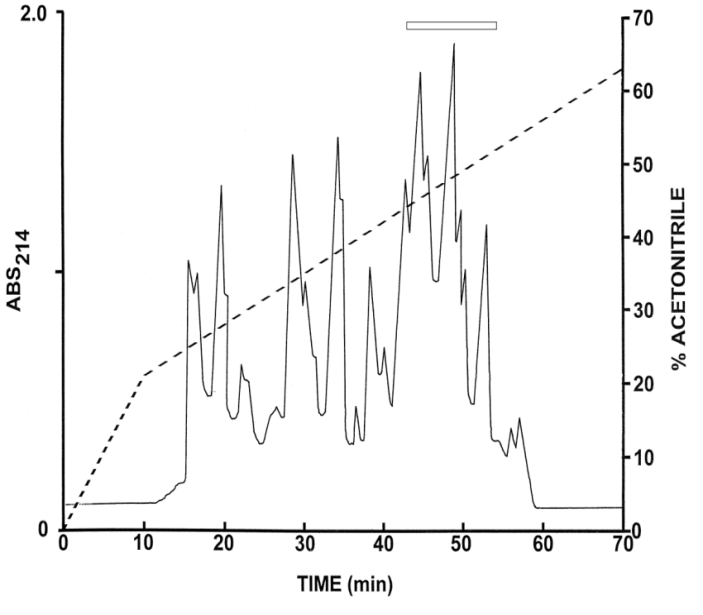

Fig. 1. Reversed-phase HPLC on a preparative Vydac C-18 column of skin secretions from *X. clivii*, after partial purification on Sep-Pak cartridges. The fractions designated by the horizontal bar inhibited the growth of *E. coli* and *S.aureus* and were purified further. The dashed line shows the concentration of acetonitrile in the eluting solvent.

Compared with chromatograms from the related species *X. amieti* (Conlon et al., 2010b) and *X. borealis* (Mechkarska et al., 2010) generated under the same conditions, the resolution was poor and relatively broad peaks were obtained. The fractions with retention times between 39 and 51 min (denoted by the bar in Fig. 1) contained peptides that inhibited the growth of both *E. coli* and *S. aureus*. These fractions were individually subjected to further purification on a semipreparative Vydac C-4 column and those components with growth-inhibitory activity were purified to near homogeneity, as assessed by a symmetrical peak shape and mass spectrometry, by a final chromatography on a semipreparative Vydac C-8 column. The methodology is illustrated by the purification of CPF-C1 (Fig. 2). The approximate final yields of purified peptides (nmol) were magainin-C1 330, magainin-C2 290, XPF-C1 195, CPF-C1 760, and CPF-C2 255.

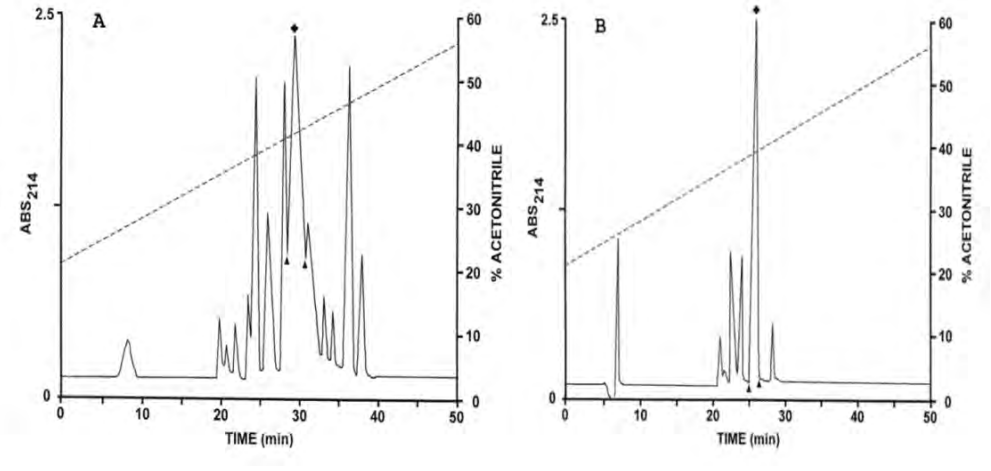

Fig. 2. Purification to near homogeneity of CPF-C1 on semipreparative (A) Vydac C-4, and (B) Vydac C-8 columns. The arrowheads show where peak collection began and ended. The peak containing CPF-C1 is denoted by the symbol + and inhibited the growth of both *E. coli* and *S. aureus*.

3.2. Structural characterization

The primary structures of the antimicrobial peptides isolated from *X. clivii* skin secretions were established by automated Edman degradation and are shown in Fig. 3. MALDI-TOF mass spectrometry was used to confirm the proposed structures and to demonstrate that CPF-C1 is C-terminally α-amidated.

		$M+H_{obs}$	$M+H_{calc}$
Magainin-C1	GVGKFLHSAKKFGQALASEIMKS	2433.4	2433.3
Magainin-C2	GVGKFLHSAKKFGQALVSEIMKS	2461.5	2461.4
XPF-C1	GWASKIGQALGKVAKVGLQQFIQPK	2651.7	2651.5
CPF-C1	GFGSLLGKALRLGANVL.NH_2	1684.0	1684.0
CPF-C2	GLGSLLGKALKFGLKAAGKFMGGEPQQ	2702.5	2702.5

Fig. 3. Amino acid sequences, observed molecular masses ($M+H_{obs}$), and calculated molecular masses ($M+H_{calc}$) of the antimicrobial peptides isolated from skin secretions of *X. clivii*.

3.3. Antimicrobial activities

The abilities of the peptides in the *X. clivii* skin secretions to inhibit the growth of reference strains of the Gram-positive bacterium *S. aureus* and the Gram-negative bacterium *E. coli* are compared in Table 1. Only CPF-C1 displayed appreciable hemolytic activity against human erythrocytes (LC_{50} = 140 µM) at concentrations up to 200 µM (Table 1).

Table 1. Minimum inhibitory concentrations against microorganisms and hemolytic activity against human erythrocytes (LC_{50}) of the endogenous peptides isolated from skin secretions of *X. clivii.*

Peptide	***E. coli***	***S. aureus***	**LC_{50}**
Magainin-C1	100	>100	>200
Magainin-C2	50	>100	>200
XPF-C1	12.5	>100	ND
CPF-C1	6	6	140
CPF-C2	25	25	>200

Data are expressed as µM. ND: not determined.

The MIC values for CPF-C1 and the orthologous peptides CPF-AM1 from *X. amieti* (Conlon et al., 2010b) and XT-7 from *S. tropicalis* (Ali et al., 2001) against reference strains of *A. baumannii*, *K. pneumoniae*, and *P. aeruginosa* are shown in Table 2.

Table 2. Minimum inhibitory concentrations of peptides belonging to the CPF family against reference strains of Gram-negative bacteria

Peptide	***K. pneumoniae***	***P. aeruginosa***	***A. baumannii***
CPF-C1	25	25	3
CPF-AM1	25	25	6
Peptide XT-7	50	50	12.5

Data are expressed as μM.

4. Discussion

The present study has identified five peptides with varying degrees of antimicrobial activity in norepinephrine-stimulated skin secretions of the tetraploid frog *X. clivii*. As shown in Fig. 4, a comparison of their primary structures reveals that they represent orthologs of previously described peptides (magainin, CPF, and XPF) isolated from the diploid frog *S. tropicalis* (Ali et al., 2001), the tetraploid frogs *X. laevis* (Gibson et al., 1986; James et al., 1994) and *X. borealis* (Mechkarska et al., 2010), and the octoploid frog *X. amieti* (Conlon et al., 2010b).

Application of the AGADIR algorithm to predict helical content of peptides indicates that magainin-C1 has only a weak propensity to adopt an α-helical conformation between residues 13 and 18 (% helicity/residue between 1.0 and 1.40) and the substitution $Ala^{17} \rightarrow$ Val in magainin-C2 results in a further destabilization of the helix. Similarly, XPF-C1 is predicted to adopt a α-helical conformation between residues 6 and 16 but the value for the % helicity per residue is also low (between 1.0 and 1.6). It has been shown using model peptides that a stabilized amphipathic α-helical conformation is an absolute requirement for cytolytic activity against Gram-positive bacteria whereas the structural requirements for activity against Gram-negative bacteria are less stringent (Giangaspero et al., 2001; Powers and Hancock, 2003). Provided that cationicity and hydrophobicity are maintained, peptides with impaired helicity are still active. Consistent with this hypothesis, the magainin peptides and XPF-C1 were active against the Gram-negative *E. coli* but showed only very weak activity against the Gram-positive *S. aureus*.

Magainin-related peptides

X. clivii-C1	GVGKFLHSAKKFGQALASEIMKS
X. clivii-C2	GVGKFLHSAKKFGQALVSEIMKS
X. borealis-B1	G**KFLHSAGKFGKAFLGEVMIG
X. borealis-B2	GIGKFLHSAGKFGKAFLGEVMKS
X. amieti-AM1	GIKEFAHSLGKFGKAFVGGILNQ
X. amieti-AM2	GVSKILHSAGKFGKAFLGEIMKS
X. laevis-1	GIGKFLHSAGKFGKAFVGEIMKS
X. laevis-2	GIGKFLHSAKKFGKAFVGEIMNS

Procaerulein-derived peptides

X. clivii-C1	GF*GSLLGKALRLGANVL[a]
X. borealis-B4	GLLTNVLGFLKKAGKGVLSGLLPL
X. amieti-AM1	GL*GSVLGKALKIGANLL.NH_2
X. amieti-AM2	GIGSALAKAAKLVAGIV.NH_2
X. amieti-AM3	GL*GSVLGKILKMGANLLGGAPKGA
X. amieti-AM4	GL*GSLVGNALRIGAKLL[a]
S. tropicalis XT-6	GFLGSLLKTGLKVGSNLL[a]
S. tropicalis XT-7	GLLGPLLKIAAKVGSNLL[a]

X. clivii-C2	GLGSLLGKAL*KFGLKAAGKFM*GGEPQQ
X. borealis-B1	GLGSLLGKAF*KIGLKTVGKMMGGAPREQ
X. borealis-B2	GLGSLLGKAF*KIGLKTVGKMMGGAPR
X. borealis-B3	GLGSLLGSLF*KFIPK******LLPSIQQ
X. laevis 1	GLASFLGKAL*KAGLK*IGAHLLGGAPQQ
X. laevis 2	GFASFLGKAL*KAALK*IGANMLGGTPQQ
X. laevis 3	GFGSFLGKAL*KAALK*IGANALGGSPQQ
X. laevis 4	GLASLLGKAL*KAGLK*IGTHFLGGAPQQ
S. tropicalis XT-1	GFLGPLLKLAAKGVAK**VIPHLIPSRQQ

Proxenopsin-derived peptides

X. clivii-C1	GWASKIGQALGKVAKVGLQQFIQPK
X. borealis-B1	GFKQFVH*SMGKFGKAFVGEIINPK
X. borealis-B2	GWASKIGTQLGKMAKVGLKEFVQS
X. amieti-AM1	GWASKIAQTLGKMAKVGLQELIQPK
X. laevis 1	GWASKIGQTLGKIAKVGLQGLMQPK
X. laevis 2	GWASKIGQTLGKIAKVGLKELIQPK
X. tropicalis XT-2	GVWSTVLGGLKKFAKGGLEAIVNPK
S. tropicalis XT-3	GLASTLGSFLGKFAKGGAQAFLQPK
S. tropicalis XT-4	GVFLDALKKF***AKGGMNAVLNPK

Fig. 4. A comparison of the primary structures of the antimicrobial peptides from *X. clivii* with orthologous peptides from the octoploid frog *X. amieti*, the tetraploid frogs *X. laevis* and *X. borealis*, and the diploid frog *S. tropicalis*. Conservation of amino acid residues is emphasized by the shading. In order to maximize sequence similarity, residue deletions denoted by (*) have been introduced in some sequences. ([a]) indicates that the peptide is C-terminally α-amidated.

In contrast, the CPF family peptides showed broad-spectrum activity and the most abundant antimicrobial peptide in the *X. clivii* skin secretions, CPF-C1 was also the most potent in inhibiting the growth of bacteria (MIC = 6 μM against both *E. coli* and *S. aureus*) (Table 1). The AGADIR algorithm predicts that CPF-C1 has a high probability of adopting a stable α-helical conformation between residues 4 and 14 (% helicity/residue between 1.0 and 4.2). Similarly, CPF-C2 has a high probability of adopting a stable α-helical conformation between residues 2 and 18 (% helicity/residue between 1.0 and 5.8). The higher potency of the arginine-containing CPF-C1 is probably a consequence of its increased cationicity (isoelectric point of 11.49 compared with 10.55 for CPF-C2). Previous studies with a range of naturally occurring and model peptides (reviewed in Conlon et al., 2007) have shown that an increase in peptide cationicity promotes interaction with the negatively charged bacterial cell membrane and so increases antimicrobial potency. On the other hand, similar studies using naturally occurring (Dathe et al., 1997) and model peptides (Stark et al., 2002) have shown that increasing overall hydrophobicity increases hemolytic activity. The calculated mean hydrophobicities of the peptides based upon the hydrophobicity scales for amino acids of Kyte and Doolittle (1982) are +15.3 for CPF-C1 and +0.6 for CPF-C2. Thus, the increased hemolytic activity of CPF-C1 (LC_{50} = 140 μM) compared with CPF-C2 (LC_{50} > 200 μM) is consistent with its appreciably greater hydrophobicity.

The study supports an earlier conclusion (Mechkarska et al., 2010) that evolutionary pressure to conserve the primary structures of the antimicrobial peptides from the clawed frogs has not been strong (Fig. 4). In peptides belonging to the magainin family from the four species studied to-date, only 7 out of 23 amino acids are invariant and conservation of amino acid sequence in peptides of the CPF and XPF families is even less. A comparison of the primary structures of peptides of the CPF family peptides suggests that they divide naturally into two sub-groups but, even after deletions have been introduced into some sequences in order to maximize structural similarity, only the N-terminal glycine residue in the first group and 5 amino acids in the second group are invariant. Nevertheless, amino acid substitutions are almost always conservative and Schiffer-Edmundson (1967) helical wheel representations of the structures indicate that the amphipathic character of the peptides has been maintained (data not shown).

Antimicrobial peptides belonging to the peptide glycine-leucine-amide family have been isolated from skin secretions of *X. laevis* (PGLa), *X. amieti* (PGLa-AM1 and PGLa-AM2), *X. borealis* (PGLa-B1 and PGLa-B2), and *S. tropicalis* (peptide XT-5) [reviewed in (Mechkarska et al., 2010)] but an ortholog was not identified in the *X. clivii* secretions. Similarly, as shown in Fig. 4, three XPF paralogs (XT-2, XT-3, and XT-4) were identified in skin secretions of the diploid frog *S. tropicalis* (Ali et al., 2001) whereas only one XPF peptide was identified in the *X. clivii* secretions. One possibility is that more peptides belonging to these families are present in the *X. clivii* secretions but their activity is so low that they have escaped detection. Alternatively, the gene(s) encoding the peptides have been deleted from the *X. clivii* genome during the course of evolution. This explanation is consistent with the conclusion of a previous study with the octoploid frog *X. amieti* that nonfunctionalization, involving deletion of a duplicated gene or its degeneration to a pseudogene, is the most common outcome following polyploidization events in the *Xenopus/Silurana* genera (Conlon et al., 2010b).

Extended-spectrum β-lactamase (ESBL)-producing Gram-negative bacteria are becoming increasingly prevalent and their antibiotic resistance necessitates novel therapeutic intervention. Bacteria which express ESBL genes have the capacity to hydrolyse a broad spectrum of β-lactam antibiotics, including carbapenems (Bush, 2010). Treatment of patients with bacterial infections caused by such multi-resistant pathogens is challenging as antibiotic options are becoming increasingly limited. Although some effective new types of antibiotics against multidrug-resistant Gram-positive bacteria, such as methicillin-resistant *S. aureus* (MRSA), have been introduced or are in clinical trials, the situation regarding new treatment options for infections produced by emerging multidrug-resistant Gram-negative pathogens such as *A. baumannii*, *K. pneumoniae*, and *P.aeruginosa* is less encouraging (Norrby et al., 2005; Livermore, 2009). The present study has demonstrated CPF-C1 and other members of the CPF family [CPF-AM1 from *X. amieti* (Conlon et al., 2010b) and XT-7 from *S. tropicalis* (Ali et al., 2001)] show relatively potent

growth-inhibitory activities against reference strains of these Gram-negative bacteria. Future studies will investigate their effectiveness against ESBL-producing clinical isolates. Previous work from the laboratory has demonstrated the feasibility of using peptides against ESBL-producing pathogens (Eley et al., 2008). The frog skin peptide, ascaphin-8 and its lysine-substituted analogs were effective against a range of clinical isolates of ESBL- producing strains of *E. coli* and *K. pneumoniae*. Like ascaphin-8, CPF-C1 shows moderate hemolytic activity against human erythrocytes (LC_{50} = 140 μM) which limits its applications for systemic use but a strategy has been developed for design of analogs of naturally occurring frog skin antimicrobial peptides that retain activity against microorganisms but are non-hemolytic (Conlon et al., 2007). For example, the LC_{50} value against human erythrocytes for the potent analog [Lys^{10}]ascaphin-8 was > 500 μM compared with 55 μM for the native peptide (Eley et al., 2008). Future studies will investigate structure-activity relationships for CPF-C1 with a view to the design of non-toxic analogs with enhanced potency against microorganisms.

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Chapter 4

Genome duplications within the *Xenopodinae* do not increase the multiplicity of antimicrobial peptides in *Silurana paratropicalis* and *Xenopus andrei* skin secretions

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ABSTRACT

A putative genome duplication event within the *Silurana* lineage has given rise to the tetraploid frog *S. paratropicalis* and a second polyploidization within the *Xenopus* lineage has produced the octoploid frog *X. andrei*. Peptidomic analysis of norepinephrine-stimulated skin secretions of *S. paratropicalis* and *X. andrei* led to identification of multiple peptides with growth-inhibitory activity against *Escherichia coli* and *Staphylococcus aureus*. Structural characterization demonstrated that the *S. paratropicalis* components comprised three peptides belonging to the caerulein-precursor fragment family (CPF-SP1, - SP2 and -SP3), two peptides from the xenopsin-precursor fragment family (XPF-SP1 and -SP2), and one peptide orthologous to peptide glycine-leucine-amide (PGLa-SP1). The CPF peptides showed potent, broad-spectrum antimicrobial activity. The *X. andrei* components comprised two peptides from the magainin family, (magainin-AN1 and -AN2), two from the XPF family (XPF-AN1 and -AN2), two from the PGLa family (PGLa-AN1 and -AN2), and one caerulein-precursor fragment (CPF-AN1). The primary structures of these peptides indicate a close phylogenetic relationship between *X. andrei* and the octoploid frog *X. amieti*. Under the same experimental conditions, seven orthologous antimicrobial peptides were previously isolated from the diploid frog *S. tropicalis*, nine from the tetraploid frog *X. borealis*, and five from the tetraploid frog *X. clivii*. The data indicate, therefore, that nonfunctionalization (gene deletion) has been the most common fate of duplicated antimicrobial peptide genes following polyploidization events in the *Silurana* and *Xenopus* lineages.

1. Introduction

The clawed frogs comprise at least 33 species distributed in five genera *Hymenochirus*, *Pipa*, *Pseudhymenochirus*, *Silurana*, and *Xenopus* within the family Pipidae (Frost, 2011). The genus *Xenopus* has a complex evolutionary history and its members have undergone at least one polyploidization event such that all species share either a tetraploid (2n = 36) karyotype or euploid multiples (2n = 72 or 2n = 108) (Kobel and Du Pasquier, 1991). Although several additional, as yet unnamed, species have been reported (Evans et al., 2004; Evans et al., 2008), a single polyploidization event in an ancestral *Xenopus* species has generated eight well characterized tetraploid species (*X. borealis, X. clivii, X. fraseri, X. gilli, X. laevis, X. largeni, X. muelleri, X. pygmaeus*) (Evans, 2008; Frost, 2011; Evans et al., 2011). The taxonomic status of *X. laevis petersii* and *X. laevis victorianus* as separate species is unclear (Frost, 2011). Subsequent independent genome duplication events have given rise to at least seven octoploid species with 2n = 72 (*X. amieti, X.andrei, X. boumbaensis, X. itombwensis, X. lenduensis, X. vestitus,* and *X. wittei*) (Evans et al., 2005). Two further independent polyploidizations involving the combination of tetraploid and octoploid genomes have produced the dodecaploid species *X. ruwenzoriensis* and *X. longipes* (2n = 108) (Evans et al., 2005; Du Pasquier et al., 2009). Allopolyploidization, in which two species hybridize and the descendant inherits the complete genome of both ancestors, is considered to be the principal mode of genome duplication in the *Xenopus* genus (Kobel, 1996).

The taxonomic status of *Silurana* is controversial. Traditionally, the single diploid species that has retained the ancestral chromosome number for pipid frogs of 2n = 20 was termed *Xenopus tropicalis* but the species has now been reclassified in the genus *Silurana* (Cannatella and Trueb, 1988). Cladistic analysis based upon nucleotide sequences of mitochondrial genes strongly supports the monophyly of *Xenopus* + *Silurana* which are united in the Xenopodinae (de Sá and Hillis, 1990) but the use of two genera "underscores trenchant biological and historical differences between the two clades" (Evans et al., 2004). A putative genome duplication event within the *Silurana* lineage has given rise to *S. epitropicalis* and *S. paratropicalis* with 2n = 40 as well as at least two further unnamed tetraploid species (Tymowska, 1991; Tymowska and Fischberg, 1982). The difference in chromosome number (36 and 40) in the two tetraploid lineages suggests that divergence of *Xenopus* from *Silurana* predates the polyploidization event that occurred between 21 and 41 million years ago in a putative diploid (2n = 18) *Xenopus* ancestor (Kobel, 1996; Evans, 2007).

Peptides with antimicrobial activity are present in skin secretions of many, although by no means all, species of Anura (frogs and toads) and have excited interest as a source of potential therapeutically valuable anti-infective drugs (Zhang and Falla, 2010) and for the insight that they provide into the evolutionary history of the species (Conlon, in press).

Skin secretions of *X. laevis* have proved to be a rich source of such peptides. Following the initial discovery of the magainins (Giovanni et al., 1987; Zasloff, 1987), multiple antimicrobal peptides derived from the post-translational processing of the biosynthetic precursors of caerulein, xenopsin, and peptide glycine-leucine-amide (PGLa) have been described [reviewed in (Mechkarska et al., 2010)]. A comparison of the nucleotide sequences of cDNAs encoding the biosynthetic precursors of procaerulein, promagainin, and proxenopsin reveals significant structural similarity in the signal peptide and N-terminal regions of the precursors suggesting that the peptides may have evolved from a common ancestral gene by a series of duplication events (Hunt and Barker, 1988). These studies with *X. laevis* have since been extended to include the characterization of the antimicrobial peptides present in skin secretions of *S. tropicalis* (Ali et al., 2001), *X. amieti* (Conlon et al., 2010), *X. borealis* (Mechkarska et al., 2010), and *X. clivii* (Conlon et al., 2011).

The Gabon clawed frog *Silurana paratropicalis* is closely related to, but less well characterized than, the Cameroon clawed frog *S. epitropicalis* with which it is sympatric. Both species occur in the Congo Basin encompassing Gabon, Cameroon, western Democratic Republic of Congo and extreme northeastern Angola (Frost, 2011). *S. paratropicalis* was first recognized as tetraploid (2n = 40) by Tymowska (1991) who referred to the species as *Xenopus* nova VII. Although morphologically very similar, *S. paratropicalis*, *S. epitropicalis*, and *S. tropicalis* may be distinguished on the basis of their mating calls. Andre's clawed frog *Xenopus amieti* is widely distributed in coastal Cameroon, northern Gabon and western Central African Republic and probably occurs in Equatorial Guinea and the Republic of Congo (Frost, 2011). Its natural habitats are shady swamps and small ponds in lowland forest. The species is adaptable and its population stable. Its IUCN Red List status is 'Least Concern' but numbers in certain areas have declined due to harvesting for human consumption (Tinsley and Measey, 2004).

The present study is part of an on-going program of peptidomic analysis using MALDI-TOF mass spectrometry coupled with reversed-phase HLPC that aims to gain insight into the diversity and evolutionary history of *Silurana* and *Xenopus* host-defense peptides. There is no universally accepted nomenclature for designating antimicrobial peptides from frogs of the Pipidae family and so the peptides described in this study are classified according to the terminology used previously for peptides from *X. laevis*, *X. borealis*, and *X. clivii* (Conlon et al., 2011). The magainin, peptide glycine-leucine-amide (PGLa), caerulein-precursor fragment (CPF), and xenopsin-precursor fragment (XPF) peptide families are recognized. The species origin is denoted by SP (*S. paratropicalis*) or AN (*X. andrei*) and paralogs are differentiated by numerals e.g. magainin-AN1 and magainin-AN2. To maintain consistency, the peptides from *X. tropicalis* that were originally termed XT-1, XT-6, and XT-7 (Ali et al., 2001) are now termed CPF-ST1, CPF-ST2, and CPF-ST-3 respectively, the peptides XT-2, XT-3, and

XT-4 are now termed XPF-ST1, XPF-ST2, and XPF-ST3 respectively, and XT-5 is now termed PGLa-ST1.

2. Materials and methods

2.1. Collection of skin secretions

All experiments with live animals were approved by the Animal Research Ethics committee of U.A.E. University (Protocol No. A21-09) and were carried out by authorized investigators. Adult *S. paratropicalis* (n = 2; male 8 g, female 18 g) were collected in Cameroon (02°48′ N, 11°08′E). Adult *X. andrei* (n = 2; male 15 g, female 22 g) were collected in Cameroon (03°04′ N, 09°58′E). Both species were supplied by Xenopus Express Inc. (Brooksville, FL, USA). Each animal was injected at two sites in the dorsal lymph sac with norepinephrine bitartrate (80 nmole per g body weight) and immersed in water for 15 min. The solution containing the secretions was acidified with trifluoroacetic acid (TFA) (final concentration 1% v/v) and stored at -20^{o} C. The pooled solutions from each species were separately passed at a flow rate of 2 mL/min through 6 Sep-Pak C-18 cartridges (Waters Associates, Milford, MA, USA) connected in series. Bound material was eluted with acetonitrile/water/ TFA (70.0:29.9:0.1, v/v/v) and freeze-dried.

2.2. Antimicrobial assays

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) from a log-phase culture of reference strains of *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25726) in 96-well microtiter cell-culture plates for 18 h at 37 ^{o}C in a humidified atmosphere of air. After incubation, the absorbance at 630 nm of each well was determined using a microtiter plate reader. In order to monitor the validity and reproducibility of the assays, incubations with bacteria were carried out in parallel with increasing concentrations of ampicillin. Minimum inhibitory concentrations (MIC) of the peptides against reference strains of *S. aureus* (ATCC 25923), *E. coli* (ATCC 25726), *Klebsiella pneumoniae* (ATCC 700603), *Pseudomonas aeruginosa* (ATCC 27853), and *Candida albicans* (ATCC 90028), and a clinical isolate of *Acinetobacter baumannii* (Euroclone I NM8 strain) were measured in the concentration range of 3 - 100 μM by standard double dilution methods (Clinical Laboratory Standards Institute, 2008a,b) and were taken as the lowest concentration of peptide where no visible growth was observed. The values were confirmed by measurement of absorbance at 630 nm. In order to monitor the validity and reproducibility of the assays, incubations were carried out in parallel with increasing concentrations of antibiotics

(ampicillin for *S. aureus* and *E. coli*; ciprofloxacin for *K. pneumoniae* and *P. aeruginosa;* and amphotericin for *C. albicans*) as previously described (Mechkarska et al., 2010).

2.3. Peptide purification

The lyophilized skin secretions from both species, after partial purification on Sep-Pak cartridges, were redissolved in 0.1% (v/v) TFA/water (4 mL) and separately injected onto a (2.2 x 25 cm) Vydac 218TP1022 (C-18) reversed-phase HPLC column (Grace, Deerfield, IL, USA) equilibrated with 0.1% (v/v) TFA/water at a flow rate of 6.0 mL/min. 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 nm and 280 nm and fractions (1 min) were collected. The abilities of freeze-dried aliquots (50 μL) of the fractions to inhibit the growth of *S. aureus* and *E. coli* were determined as described in the previous section. Fractions associated with antimicrobial activity were successively chromatographed on a (1 x 25 cm) Vydac 214TP510 (C-4) column and a (1 x 25 cm) Vydac 208TP510 (C-8) 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.

2.4. Structural characterization

The primary structures of the peptides were determined by automated Edman degradation using an Applied Biosystems model 494 Procise sequenator (Foster City, CA, USA). MALDI-TOF mass spectrometry was carried out using a Voyager DE-PRO instrument (Applied Biosystems) that was operated in reflector mode with delayed extraction and the accelerating voltage in the ion source was 20 kV. The instrument was calibrated with peptides of known molecular mass in the 2 - 4 kDa range. The accuracy of mass determinations was ± 0.02%.

2.5. Secondary structure prediction

Prediction of secondary structure and determination of helicity per residue for the peptides were performed using the AGADIR program (Muñoz and Serrano 1994). AGADIR is a prediction algorithm based on the helix/coil transition theory which predicts the helical behaviour of monomeric peptides. Calculations were performed at pH 7 and 278 K. A minimum percentage of 1% helicity/residue was considered to predict the presence of a helix. Calculated mean hydrophobicities of the peptides were based upon the hydrophobicity scales for amino acids of Kyte and Doolittle (1982).

3. Results

3.1 Purification of the peptides from S. paratropicalis

The pooled skin secretions from *S. paratropicalis*, after partial purification on Sep-Pak C-18 cartridges, were chromatographed on a Vydac C-18 preparative reversed-phase HPLC column (Fig. 1).

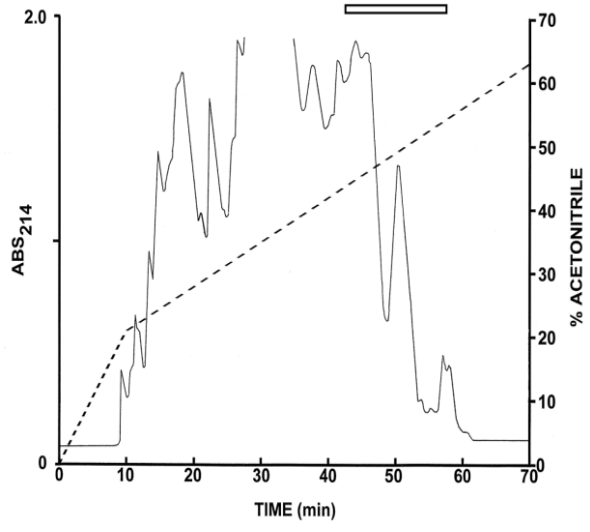

Fig. 1. Reversed-phase HPLC on a preparative Vydac C-18 column of skin secretions from *S. paratropicalis*, after partial purification on Sep-Pak cartridges. The fractions designated by the bar displayed growth inhibitory activity against *E. coli* and *S. aureus* and were purified further. The dashed line shows the concentration of acetonitrile in the eluting solvent. Only absorbance at 214 nm is shown.

The fractions with retention times between 44 and 57 min (denoted by the bar in Fig. 1) contained peptides that inhibited to varying degrees the growth of either *E. coli* or *S. aureus.*

These fractions were individually subjected to further purification on a semipreparative Vydac C-4 column and those components with growth-inhibitory activity were purified to near homogeneity (> 98% purity), as assessed by a symmetrical peak shape and mass spectrometry, by a final chromatography on a semipreparative Vydac C-8 column. The methodology is illustrated by the purification of the most abundant peptide in the

secretions, CPF-SP3 by sequential chromatography on the Vydac C-4 column (Fig. 2A) and Vydac C-8 column (Fig. 2B).The approximate final yields of purified peptides (nmol) were CPF-SP1 320, CPF-SP2 235, CPF-SP3 775, XPF-SP1 155, XPF-SP2 190, and PGLa-SP1 230.

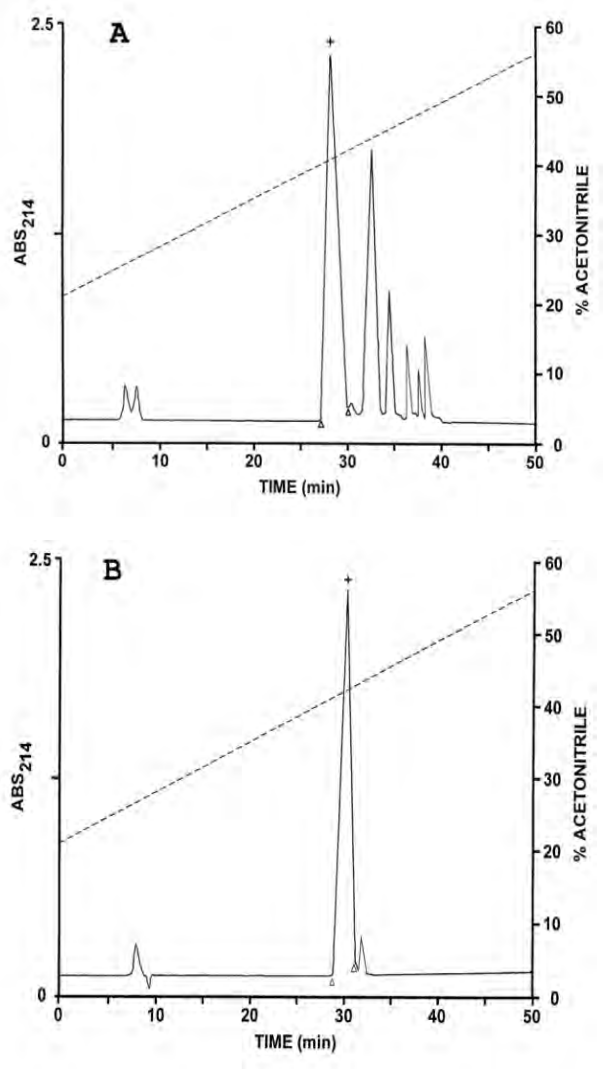

Fig. 2. Purification to near homogeneity of CPF-SP3 from *S. paratropicalis* on semipreparative (A) Vydac C-4, and (B) Vydac C-8 columns. The arrowheads show where peak collection began and ended. The peak denoted by the cross inhibited the growth of *E. coli* and *S. aureus*.

3.2. Purification of the peptides from X. andrei

The pooled skin secretions from *X. andrei*, after partial purification on Sep-Pak C-18 cartridges, were chromatographed on a Vydac C-18 preparative reversed-phase HPLC column under the same conditions used for purification of the *S. paratropicalis* peptides (Fig. 3).

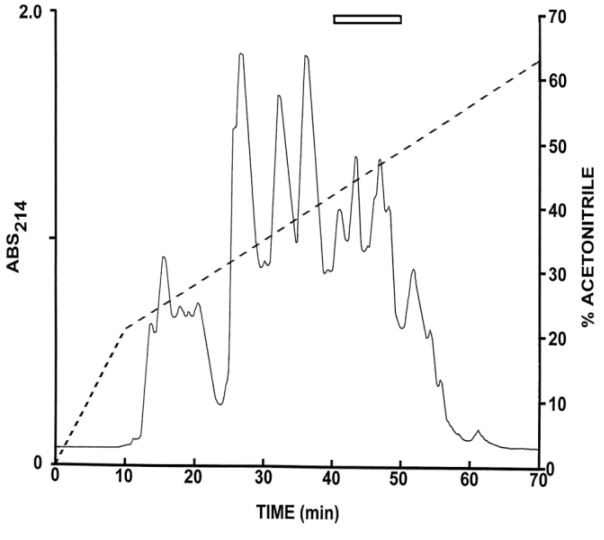

Fig. 3. Reversed-phase HPLC on a preparative Vydac C-18 column of skin secretions from *X. andrei*, after partial purification on Sep-Pak cartridges. The fractions designated displayed growth inhibitory activity against *E. coli* and *S. aureus* and were purified further. The dashed line shows the concentration of acetonitrile in the eluting solvent.

Consistent with previous studies with skin secretions from *X. clivii* (Conlon et al., 2011) and with the *S. paratropicalis* secretions (Fig. 1), the resolution on this column was poor and relatively broad peaks were obtained. The fractions with retention times between 39 and 51 min (denoted by the bar in Fig. 3) contained peptides with antimicrobial activity. These fractions were individually subjected to further purification on a semipreparative Vydac C-4 column and those components with growth-inhibitory activity were purified to near homogeneity, as assessed by a symmetrical peak shape and mass spectrometry, by a final chromatography on a semipreparative Vydac C-8 column. Chromatography on these columns resulted in sharp, well-defined peaks. The approximate final yields of purified peptides (nmol) were magainin-AN1 25, magainin-AN2 370, PGLa-AN1 20, PGLa-AN2 85, CPF-AN1 20, XPF-AN1 70, and XPF-AN2 15.

3.3. Structural characterization

The primary structures of the antimicrobial peptides isolated from *S. paratropicalis* and *X. andrei* skin secretions were established by automated Edman degradation and are shown in Fig. 4. MALDI-TOF mass spectrometry was used to confirm the proposed structures and to demonstrate that CPF-SP3, PGLa-SP1, CPF-AN1, PGLa-AN1 and PGLa-AN2 are C-terminally α-amidated.

		$M_r$obs	$M_r$calc
S. paratropicalis			
XPF-SP1	GFWSSALEGLKKFAKGGLEALTNPK	2649.7	2648.4
XPF-SP2	GLASTIGSLLGKFAKGGAQAFLQPK	2460.3	2459.4
PGLa-SP1	GMATKAGTALGKVAKAVIGAAL.NH_2	1997.3	1997.2
CPF-SP1	GFLGPLLKLGLKGVAKVLPHLIPSRQQ	2878.9	2878.8
CPF-SP2	GFLGPLLKLGLKGAAKLLPQLLPSRQQ	2857.5	2856.8
CPF-SP3	GFLGSLLKTGLKVGSNLL.NH_2	1815.4	1815.1
X. andrei			
Magainin-AN1	GIKEFAHSLGKFGKAFVGGILNQ	2417.3	2417.4
Magainin-AN2	GVSKILHSAGKFGKAFLGEIMKS	2404.9	2404.4
XPF-AN1	GWASKIGQTLGKMAKVGLQELIQPK	2680.6	2680.5
XPF-AN2	GWVSKIGQTLGKMAKVGLQELIQPK	2708.4	2708.5
PGLa-AN1	GMASKAGSVLGKVAKVALKAAL.NH_2	2068.2	2068.2
PGLa-AN2	GMASKAGSVLGKLAKVAIGAL.NH_2	1940.2	1940.2
CPF-AN1	GFASVLGKALKLGANLL.NH_2	1669.9	1670.0

Fig. 4. Amino acid sequences, observed molecular masses (M_r obs), and calculated molecular masses (M_r calc) of the antimicrobial peptides isolated from skin secretions of *S. paratropicalis* and *X. andrei*. The calculated masses of CPF-SP3, PGLa-SP1, CPF-AN1, PGLa-AN1 and PGLa-AN2 assume that the peptides are C-terminally α-amidated.

3.4. Antimicrobial activities

The abilities of the purified peptides in the *S. paratropicalis* skin secretions to inhibit the growth of a range of clinically relevant bacteria are compared in Table 1. In view of the low amounts of purified peptides obtained, it was not possible to determine the growth inhibitory potencies of all the *X. andrei* peptides but limited data were obtained for the more abundant magainin-AN2, XPF-AN1, PGLa-AN2.

Table 1. Minimum inhibitory concentrations (μM) against microorganisms of the endogenous peptides isolated from skin secretions of *Silurana paratropicalis* and *Xenopus andrei*

	E. coli	***S. aureus***	***A. baumannii***	***K. pneumoniae***	***P. aeruginosa***	***C. albicans***
CPF-SP1	25	6	12.5	25	6	25
CPF-SP2	50	6	6	50	25	12.5
CPF-SP3	50	25	6	50	50	50
XPF-SP1	50	>100	12.5	>50	ND	ND
XPF-SP2	100	>100	25	>50	ND	>50
PGLa-SP1	100	>100	25	>50	ND	50
Magainin-AN2	100	>100	ND	100	200	ND
XPF-AN1	50	50	ND	ND	ND	ND
PGLa-AN2	25	100	ND	ND	ND	ND

Data are expressed as μM. ND: not determined

4. Discussion

This study has described the purification and characterization of six peptides with varying degrees of antimicrobial activity in norepinephrine-stimulated skin secretions of the tetraploid frog *S. paratropicalis* and seven antimicrobial peptides in skin secretions of the octoploid frog *X. andrei*. As shown in Figs. 5 and 6, a comparison of their primary structures reveals that they represent orthologs of previously described dermal peptides (magainin, PGLa, CPF, and XPF) isolated from the octoploid frog *X. amieti* (Conlon et al., 2010) and the tetraploid frogs *X. laevis* (Gibson et al., 1986), *X. borealis* (Mechkarska et al., 2010), and *X. clivii* (Conlon et al., 2011).

Procaerulein-derived peptides

S. paratropicalis CPF-SP1	GFLGPLLKLGLKGVAKVLPHLIPSRQQ
S. paratropicalis CPF-SP2	GFLGPLLKLGLKGAAKLLPQLLPSRQQ
S. tropicalis CPF-ST1	GFLGPLLKLAAKGVAKVIPHLIPSRQQ
S. paratropicalis CPF-SP3	GFLGSLLKTGLKVGSNLL.NH_2
S. tropicalis CPF-ST2	GFLGSLLKTGLKVGSNLL.NH_2
S. tropicalis CPF-ST3	GLLGPLLKIAAKVGSNLL.NH_2

Proxenopsin-derived peptides

S. paratropicalis XPF-SP1	GFWSSALEGLKKFAKGGLEALTNPK
S. paratropicalis XPF-SP2	GLASTIGSLLGKFAKGGAQAFLQPK
S. tropicalis XPF-ST1	GVWSTVLGGLKKFAKGGLEAIVNPK
S. tropicalis XPF-ST2	GLASTLGSFLGKFAKGGAQAFLQPK
S. tropicalis XPF-ST3	GVFLDALKKF***AKGGMNAVLNPK

PGLa-related peptides

S. paratropicalis PGLa-SP1	GMATKAGTALGKVAKAVIGAAL.NH_2
S. tropicalis PGLa-ST1	GMATKAGTALGKVAKAVIGAAL.NH_2

Fig. 5. A comparison of the primary structures of the antimicrobial peptides from the tetraploid frog, *S. paratropicalis* with orthologous peptides from the diploid frog *S. tropicalis*. (*) denotes a residue deletion.

Magainin-related peptides

X. andrei-AN1	GIKEFAHSLGKFGKAFVGGILNQ
X. andrei-AN2	GVSKILHSAGKFGKAFLGEIMKS
X. amieti-AM1	GIKEFAHSLGKFGKAFVGGILNQ
X. amieti-AM2	GVSKILHSAGKFGKAFLGEIMKS
X. clivii-C1	GVGKFLHSAKKFGQALASEIMKS
X. clivii-C2	GVGKFLHSAKKFGQALVSEIMKS
X. borealis-B1	G**KFLHSAGKFGKAFLGEVMIG
X. borealis-B2	GIGKFLHSAGKFGKAFLGEVMKS
X. laevis-1	GIGKFLHSAGKFGKAFVGEIMKS
X. laevis-2	GIGKFLHSAKKFGKAFVGEIMNS

Procaerulein-derived peptides

X. andrei-AN1	GFASVLGKALKLGANLL.NH_2
X. amieti-AM1	GLGSVLGKALKIGANLL.NH_2
X. amieti-AM2	GIGSALAKAAKLVAGIV.NH_2
X. amieti-AM4	GLGSLVGNALRIGAKLL.NH_2
X. clivii-C1	GFGSL*GKALRLGANVL.NH_2
X. amieti-AM3	GLGSVLGKILKMGANLLGGAPKGA
X. clivii-C2	GLGSLLGKALKFGLKAAGKFM*GGEPQQ
X. borealis-B1	GLGSLLGKAFKIGLKTVGKMMGGAPREQ
X. borealis-B2	GLGSLLGKAFKIGLKTVGKMMGGAPR
X. borealis-B3	GLGSLLGSLFKFIPK******LLPSIQQ
X. borealis-B4	GLLTNVLGFLKKAGKGVLSGLLPL
X. laevis 1	GLASFLGKALKAGLK*IGAHLLGGAPQQ
X. laevis 2	GFASFLGKALKAALK*IGANMLGGTPQQ
X. laevis 3	GFGSFLGKALKAALK*IGANALGGSPQQ
X. laevis 4	GLASLLGKALKAGLK*IGTHFLGGAPQQ

Proxenopsin-derived peptides

X. andrei-AN1	GWASKIGQTLGKMAKVGLQELIQPK
X. andrei-AN2	GWVSKIGQTLGKMAKVGLQELIQPK
X. amieti-AM1	GWASKIAQTLGKMAKVGLQELIQPK
X. clivii-C1	GWASKIGQALGKVAKVGLQQFIQPK
X. borealis-B1	GFKQFVH*SMGKFGKAFVGEIINPK
X. borealis-B2	GWASKIGTQLGKMAKVGLKEFVQS
X. laevis 1	GWASKIGQTLGKIAKVGLQGLMQPK
X. laevis 2	GWASKIGQTLGKIAKVGLKELIQPK

PGLa-related peptides

X. andrei-AN1	GMASKAGSVLGKVAKVALKAAL.NH_2
X. andrei-AN2	GMASKAGSVLGKLAKVAI*GAL.NH_2
X. amieti-AM1	GMASKAGSVLGKVAKVALKAAL.NH_2
X. amieti-AM2	GMASTAGSVLGKLAKAVAIGAL.NH_2
X. borealis-B1	GMASKAGTIAGKIAKTAIKLAL.NH_2
X. borealis-B2	GMASKAGSIVGKIAKIAL*GAL.NH_2
X. laevis	GMASKAGAIAGKIAKVALK*AL.NH_2

Fig. 6. A comparison of the primary structures of the antimicrobial peptides from the octoploid frog *X. andrei* with orthologous peptides from the octoploid frog *X. amieti*, and the tetraploid frogs, *X. borealis*, *X. clivii*, and *X. laevis*. (*) denotes a residue deletion.

Consistent with previous work with the diploid frog *S. tropicalis* (Ali et al., 2001), peptides orthologous to the magainins were not detected in the *S. paratropicalis* secretions. The distribution of the antimicrobial peptides in the skin secretions of *X. andrei* is atypical (Fig. 7).

Most abundant → Least abundant

S. tropicalis	CPF-ST1	CPF-ST3	CPF-ST2	PGLa-ST1	XPF-ST3	XPF-ST1	XPF-ST2		
S. paratropicalis	CPF-SP3	CPF-SP1	CPF-SP2	PGLa-SP1	XPF-SP2	XPF-SP1			
X. borealis	CPF-B1	Magainin-B2	PGLa-B2	Magainin-B1	XPF-B2	CPF-B3	CPF-B4	PGLa-B1	XPF-B1
X. clivii	CPF-C1	Magainin-C1	Magainin-C2	CPF-C2	XPF-C1				
X. amieti	Magainin-AM2	CPF-AM1	PGLa-AM1	CPF-AM2	PGLa-AM2	CPF-AM4	Magainin-AM1	XPF-AM1	CPF-AM3
X. andrei	Magainin-AN2	PGLa-AN2	XPF-AN1	Magainin-AN1	PGLa-AN1	CPF-AN1	XPF-AN2		

Fig. 7. Relative abundance of the antimicrobial peptides isolated from skin secretions of the diploid frog *S. tropicalis*, the tetraploid frogs *S. paratropicalis*, *X. borealis* and *X. clivii*, and the octoploid frogs *X. amieti* and *X. andrei.*

The potent and broad spectrum CPF antimicrobial peptides are present in high abundance and in multiple molecular forms in the secretions of all other species studied to-date, including *S. paratropicalis*, whereas only a single CPF peptide was isolated from the *X. andrei* secretions and this in low yield.

As well as their potential for development into therapeutically valuable anti-infective agents, the antimicrobial peptides in frog skin secretion can provide insight into the evolutionary history of closely related species. The primary structures of the *Xenopus* antimicrobial peptides have generally been poorly conserved both within and between species (Mechkarska et al., 2010; Conlon et al., 2011). However, the primary structures of the PGLa peptides from *S. paratropicalis* and *S. tropicalis* are the same and CPF-SP3 is identical to CPF-ST2 (Fig. 5) suggesting that the tetraplodization event within the *Silurana* lineage that produced *S. paratropicalis* was relatively recent. The data in Fig. 6 indicate a particularly close phylogenetic relationship between the octoploid frogs, *X. andrei* and *X. amieti* as the amino acid sequences of the magainins and the PGLa-1 peptides are identical. This conclusion is consistent with data from comparisons of nucleotide sequences of mitochondrial DNA (Evans et al., 2004; Evans, 2008) and the RAG1 gene (Evans, 2007). It was proposed that *X. amieti*, *X. andrei*, and *X. boumbaensis* share a common octoploid ancestor whereas the octoploid frogs, *X. vestitus* and *X. wittei* arose from independent polyploidization events.

The antimicrobial potencies and specificities of the peptides isolated in this study varied appreciably (Table 1). CPF-SP1 represents the component with the greatest potential for development into a therapeutically valuable anti-infective agent, displaying relatively high potency (MIC $\leq$ 25 μM) against reference strains of the Gram-negative bacteria *E. coli*, *A. baumannii*, *K. pneumonia*, and *P. aeruginosa*. The peptide was also active against a reference strain of the Gram-positive *S. aureus* (MIC = 6 μM) and against the opportunistic yeast pathogen *C. albicans* (MIC = 25 μM). There was insufficient material to determine accurately the hemolytic activity of the peptide against human erythrocytes but the LC_{50} value was > 100 μM. The emergence of multidrug-resistant Gram-negative pathogens poses a particularly serious threat to public health and the situation regarding new treatment options for infections produced by these microorganisms is not encouraging (Livermore, 2009). There is clearly an urgent need for new types of antimicrobial agents to which the pathogens have not been exposed.

The observed potencies of the *S. paratropicalis* peptides correlate moderately well with their physicochemical properties (Table 2). The antimicrobial potency of a peptide against microorganisms is determined by complex interactions among cationicity, hydrophobicity, α-helicity and amphipathicity [reviewed in (Conlon et al. 2007)]. Studies using model α-helical peptides have demonstrated that an increase in cationicity promotes interaction with the negatively charged phospholipids in the bacterial cell membrane and

thereby increases antimicrobial activity. Consistent with this observation, CPF-SP1 and CPF-SP2 with a molecular charge of +4 at pH 7 and a predicted α-helical domain between residues 5 - 11, are more potent than CPF-SP3 which has a charge of +3 and a low propensity to adopt a α-helical conformation.

Table 2. Physicochemical properties of the antimicrobial peptides isolated from skin secretions of *Silurana paratropicalis* and *Xenopus andrei*

Peptide	Mean hydrophobicity	Charge at pH 7	α-helical domain
CPF-SP1	+10.5	+4	5-11
CPF-SP2	+6.7	+4	5-11
CPF-SP3	+14.6	+3	Non-helical
XPF-SP1	-6.3	+2	5-15,18-22
XPF-SP2	+7.9	+3	6-14
PGLa-SP1	+20.3	+4	9-19
Magainin-AN1	+4.6	+3	Non-helical
Magainin-AN2	+5.6	+2	Non-helical
CPF-AM1	+18.1	+3	5-17
XPF-AN1	-5.1	+3	10-16, 18-22
XPF-AN2	-2.7	+3	10-16, 18-22
PGLa-AN1	+18.4	+5	9-18
PGLa-AN2	+20.3	+4	9-18

Mean hydrophobicities (H) of the peptides are calculated using the hydrophobicity scales for amino acid residues of Kyte and Doolittle. Predictions of helical domains were made using the AGADIR program.

The low activity of XPF-SP1 is consistent with its lower charge (+2 at pH 7) and low mean hydrophobicity. The reduced potency of PGLa-SP1 is more difficult to explain as it possesses a charge of +4 at pH 7 and can adopt a stable, amphipathic α-helical conformation. The peptide is strongly hydrophobic and it is possible that its activity is

reduced by low solubility and a tendency to oligomerize. Magainin-2 and PGLa from *X. laevis* act synergistically so that the peptides in combination show more potent growth inhibitory activity against *E. coli* than either peptide tested alone (Westerhoff et al., 1995). It is probable, therefore, that the mixture of antimicrobial peptides in the *S. paratropicalis* and *X. andrei* skin secretions provides greater protection for the animal against pathogenic microorganisms in the environment than inferred from data obtained by measuring the MIC of each individual peptide.

Polyploidization events involving duplication of the complete genome, are believed to have occurred several times during vertebrate evolution – twice during the very early history of the chordates (2R hypothesis) and once in fish immediately before the radiation of the teleost lineage (Lundin et al., 2003). Subsequent genome duplications are believed to have occurred sporadically in individual species among the Anura but it is only in the Xenopodinae that polyploid species predominate (Otto and Whitton, 2000). A duplicated gene has three possible evolutionary fates – nonfunctionalization involving deletion of the gene or its degeneration to a pseudogene; subfunctionalization in which the mother and daughter genes share functionality; and neofunctionalization in which the duplicated gene evolves to adopt a new biological role (Lynch and Katju, 2004). Analysis of large numbers of duplicate gene pairs in tetraploid *Xenopus* and *Silurana* species has shown that both copies of the duplicate gene are subject to strong purifying selection (a greater number of synonymous substitutions per site than nonsynonymous substitutions per site) (Chain and Evans, 2006; Chain et al., 2008), and it has been estimated that only 23% of the duplicate gene copies in *X. laevis* have been lost following tetraploidization (Hughes and Hughes, 1993). Consistent with this estimate, electrophoretic analysis of serum proteins from 20 *Silurana/Xenopus* species revealed that *S. paratropicalis*, *S. epitropicalis* and all tetraploid *Xenopus* species displayed two albumin bands whereas *S. tropicalis* gave only one (Graf and Fischberg, 1986). However, in the case of the octoploid and dodecaploid species, the rate of duplicate gene loss following the second and third polyploidization events appears to be greater. The serum of the octaploid species *X. andrei* contained only three albumins instead of the expected four and the dodecaploid species *X. ruwenzoriensis* contained three albumins instead of the expected six. A similar high level of gene deletion and/or gene silencing in octoploid and dodecaploid species was seen in the case of genes encoding RAG1 and RAG2, proteins crucial to the process of somatic rearrangement of DNA in adaptive immunity (Evans, 2007) and among major histocompatibility complex genes (Sammut et al., 2002; Du Pasquier et al., 2009). Studies of duplicated RAG-1 genes in *Xenopus* and *Silurana* species have indicated that degeneration of paralogs occurs via incorporation of premature stop codons and frameshift mutations (Evans et al., 2005).

It is not claimed that the antimicrobial peptides isolated from *S. paratropicalis* and *X. andrei* skin secretions in this study necessarily represent the full complement of expressed antimicrobial gene products as components present in low abundance and/or with

very weak growth-inhibitory activity may have been missed. However, it is fair to claim that the multiplicity of antimicrobial peptides in the skin secretions of the tetraploid species *S. paratropicalis* is not appreciably greater that in the skin secretions of the diploid species *S. tropicalis* (Ali et al., 2001). Similarly, the multiplicity of the peptides in the secretions of the octoploid frogs *X. andrei* and *X. amieti* (Conlon et al., 2010) is not appreciably different from that in the tetraploid species *X. borealis* (Mechkarska et al., 2010) and *X. clivii* (Conlon et al., 2011) when analyzed using exactly the same experimental protocol. It is concluded, therefore, that nonfunctionization is the most common fate of antimicrobial peptide genes following the putative genome duplication event in the *Silurana* lineage and the second polyploidization in the *Xenopus* lineage. The existence of multiple biologically active CPF peptides in the *S. paratropicalis* secretions appears to be a clear example of subfunctionalization. In view of the very low antimicrobial potencies of the XPF and PGLa peptides in *S. paratropicalis*, it is tempting to speculate that they are evolving towards a new, as yet undetermined function (neofunctionalization).

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Chapter 5

Peptidomic analysis of skin secretions demonstrates that the allopatric populations of *Xenopus muelleri* (Pipidae) are not conspecific

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ABSTRACT

Mueller's clawed frog *Xenopus muelleri* (Peters 1844) occupies two non-contiguous ranges in east and west Africa. The phylogenetic relationship between the two populations is unclear and it has been proposed that the western population represents a separate species. Peptidomic analysis of norepinephrine-stimulated skin secretions from *X. muelleri* from the eastern range resulted in the identification of five antimicrobial peptides structurally related to the magainins (magainin-M1 and -M2), xenopsin-precursor fragments (XPF-M1) and caerulein-precursor fragments (CPF-M1 and -M2) previously found in skin secretions of other *Xenopus* species. A cyclic peptide (WCPPMIPLCSRF.NH_2) containing the RFamide motif was also isolated that shows limited structural similarity to the tigerinins, previously identified only in frogs of the Dicroglossidae family. The components identified in skin secretions from *X. muelleri* from the western range comprised one magainin (magainin-MW1), one XPF peptide (XPF-MW1), two peptides glycine-leucine amide (PGLa-MW1 and -MW2), and three CPF peptides (CPF-MW1, -MW2 and -MW3). Comparison of the primary structures of these peptides suggest that western population of *X. muelleri* is more closely related to *X. borealis* than to *X. muelleri* consistent with its proposed designation as a separate species. The CPF peptides showed potent, broad-spectrum activity against reference strains of bacteria (MIC 3 - 25 μM), but were hemolytic against human erythrocytes.

1. Introduction

Peptides with broad-spectrum antibacterial and antifungal activities and with the ability to permeabilize mammalian cells are found in skin secretions from a variety of species of Anura (frogs and toads). It is proposed that they constitute a component of the system of innate immunity that defends the animal against invasion by pathogens in the environment [3]. Such peptides have attracted interest for their possible clinical applications in treating infections produced by antibiotic-resistant microorganisms [39]. With few exceptions, the frog skin antimicrobial peptides are cationic and lack stable secondary structure in aqueous solutions but have the propensity to form an amphipathic α-helix in the environment of a phospholipid vesicle or in a membrane-mimetic solvent such as 50% trifluoroethanol-water [4,30]. The peptides may be grouped together in families on the basis of limited similarities in amino acid sequence and skin secretions from a single species frequently contain peptides from different families as well as several members of a particular peptide family. Their primary structures are hypervariable and it is rare that orthologous peptides from two different species have the same amino acid sequence even when those species are closely related phylogenetically. Consequently the antimicrobial peptides in skin secretions may serve as valuable phylogenetic markers for gaining insight into the evolutionary history of the different frog families [6,7,9].

The clawed frogs comprise at least 33 species distributed in five genera *Hymenochirus*, *Pipa*, *Pseudhymenochirus*, *Silurana*, and *Xenopus* within the family Pipidae [15]. All are found in Africa south of the Sahara except for members of the genus *Pipa* which are found in South America. The genera *Silurana* and *Xenopus* are united in the Xenopodinae [14] and have a complex evolutionary history characterized by both bifurcating and reticulating modes of speciation [11]. Allopolyploidization events, in which two species hybridize and the descendant inherits the complete genome of both ancestors, have given rise to tetraploid, octoploid, and dodecaploid species with only a single species, *S. tropicalis* retaining the diploid status that is thought to be related to the ancestral state existing prior to one or more whole genome duplications [12-14,21,22]. The 10 tetraploid *Xenopus* species have been divided into three species groups on the basis of similarities in morphology, advertisement calls, and/or nucleotide sequences of mitochondrial genes: the *laevis* group includes *X. laevis, X. gilli, X. largeni, X. petersii, and X. victorianus*; the *muelleri* group includes *X. muelleri, X. borealis* and *X. clivii*; and the *fraseri* group includes *X. fraseri* and *X. pygmaeus* [14,16,35].

Mueller's clawed frog *Xenopus muelleri* Peters, 1844 (also known as Mueller's Platanna) is a relatively large aquatic species with females reaching up to 9 cm snout-to-vent length and males about 20% smaller. It occupies both temporary and permanent ponds, and also streams and rivers in the dry season, preferring agricultural and other altered habitats and is seldom found in forests. In certain regions, *X. muelleri*, like *X. borealis* and

X. gilli, is known to hybridize with *X. laevis* revealing that reproductive isolation is not complete [15,25]. In view of its very wide distribution and tolerance of a broad range of habitats, its current IUCN (Red List) status is 'Least Concern' but numbers in certain areas are declining due to loss of habitat and harvesting for human consumption [25].

X. muelleri occupies two discontinuous ranges. In the eastern range, *X. muelleri* is found along the East African coastal belt from extreme southern Kenya through Tanzania into northwestern border areas of the Republic of South Africa, Botswana and the Caprivi region of Nambia [16,18]. In the western range, *X. muelleri* is widely distributed in a zone encompassing Burkina Faso, Cameroon, Côte d'Ivoire, northern Gabon, Nigeria, eastward to northeastern Democratic Republic of Congo [16,31]. It has been proposed that the western population represents a separate species with only the eastern population retaining the current scientific name *X. muelleri* [26]. For convenience, the western population is referred to as *X. muelleri* West in this article but this is not a recognized species name. The aim of the present study was to use peptidomic analysis (reversed-phase HLPC coupled with MALDI-TOF mass spectrometry) of norepinephrine-stimulated skin secretions from frogs from both ranges to determine whether the two populations should be regarded as conspecific. The study is part of a program of investigation with the dual purpose of gaining insight into the evolutionary history of the Xenopodinae and identifying antimicrobial peptides with the potential for development into therapeutically valuable anti-infective agents. The investigation complements previous work that has led to the characterization of antimicrobial and other peptides in skin secretions from *X. amieti* [5], *X. andrei* [28], *X. borealis* [27], and *X. clivii* [8].

There is no universally accepted nomenclature for designating antimicrobial peptides from frogs of the Pipidae family and so the peptides described in this study are classified according to the terminology used previously for peptides from the Xenopodinae [17]. The magainin, peptide glycine-leucine amide (PGLa), caerulein-precursor fragment (CPF), and xenopsin-precursor fragment (XPF) peptide families are recognized. The species origin is denoted by M for *X. muelleri* and MW for *X. muelleri* West. Paralogs are differentiated by numerals e.g. magainin-M1 and magainin-M2.

2. Experimental

2.1. Collection of skin secretions

All experiments with live animals were approved by the Animal Research Ethics committee of U.A.E. University (Protocol No. A21-09) and were carried out by authorized investigators. Adult *X. muelleri* from the eastern range (n = 2; male 43 g, female 43 g) were

collected in South Malawi. Adult individuals from the western range (n = 2; male 32 g, female 32 g) were collected in Nigeria, near Jos Plateau. Animals from both groups were supplied by Xenopus Express Inc. (Brooksville, FL). Each animal was injected via the dorsal lymph sac with norepinephrine hydrochloride (40 nmol/g body weight) and placed in a solution (100 ml) of distilled water for 15 min. The frog was removed and the collection solution was acidified by addition of trifluoroacetic acid (TFA) (1 ml) and immediately frozen for shipment to U.A.E. University. The solutions containing the secretions from each group were pooled and separately passed at a flow rate of 2 ml/min through 6 Sep-Pak C-18 cartridges (Waters Associates, Milford, MA) connected in series. Bound material was eluted with acetonitrile/water/TFA (70.0:29.9:0.1, v/v/v) and freeze-dried. The material was redissolved in 0.1% (v/v) TFA/water (2 ml).

2.2. Peptide purification

The pooled skin secretions from each group, after partial purification on Sep-Pak cartridges, were injected onto a (1.0 cm x 25 cm) Vydac 218TP510 (C-18) reversed-phase HPLC column (Grace, Deerfield, IL) equilibrated with 0.1% (v/v) TFA/water at a flow rate of 2.0 ml/min. 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 nm and 280 nm, and fractions (1 min) were collected. Purification of the peptides was monitored by incubating lyophilized aliquots of chromatographic effluent (100 µl) in Mueller-Hinton broth (50 µl) with an inoculum (50 µl of 10^6 colony forming units/ml) from a log-phase culture of reference strains of *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25726) in 96-well microtiter cell-culture plates for 18 h at 37^{o}C in a humidified atmosphere of air. After incubation, the absorbance at 630 nm of each well was determined using a microtiter plate reader. Fractions associated with antimicrobial activity were successively chromatographed on a (1.0 cm x 25 cm) Vydac 214TP510 (C-4) column and a (1.0 cm x 25 cm) Vydac 208TP510 (C-8) 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.

2.3. Structural characterization

The primary structures of the peptides were determined by automated Edman degradation using an Applied Biosystems model 494 Procise sequenator (Foster City, CA). MALDI-TOF mass spectrometry was carried out using a Voyager DE-PRO instrument (Applied Biosystems) that was operated in reflector mode with delayed extraction and the accelerating voltage in the ion source was 20 kV. The instrument was calibrated with peptides of known molecular mass in the 2 - 4 kDa range. The accuracy of mass

determinations was ± 0.02%. Amino acid composition analyses were performed by the University of Nebraska Medical Center Protein Structure Core Facility (Omaha, NE).

2.4. Peptide synthesis

Tigerinin-M1 (WCPPMIPLCSRF.NH_2) was supplied in crude form by China Peptides Co., Shanghai, China and was purified to near homogeneity (> 98% purity) by reversed-phase HPLC on a (2.2 cm x 25 cm) Vydac 218TP1022 (C-18) column. The concentration of acetonitrile in the eluting solvent was raised from 21% to 56% over 60 min and the flow rate was 6 ml/min. The structure of the peptide was confirmed by electrospray mass spectrometry.

2.5. Antimicrobial and hemolytic activities

Reference strains of microorganisms were purchased from the American Type Culture Collection (Rockville, MD). Minimum inhibitory concentrations (MIC) of the purified peptides against reference strains of *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25726), and *Candida albicans* (ATCC 90028) were measured in the concentration range of 3 - 200 μM by standard microdilution methods [1,2] and were taken as the lowest concentration of peptide where no visible growth was observed. The values were confirmed by measurement of absorbance at 630 nm. In order to monitor the validity and reproducibility of the assays, incubations were carried out in parallel with increasing concentrations of antibiotics (ampicillin for *S. aureus* and *E. coli*; and amphotericin for *C. albicans*) as previously described [27].

Hemolytic activity against human erythrocytes taken from a healthy donor was measured as previously described [27]. The LC_{50} value was taken as the mean concentration of peptide producing 50% hemolysis in two incubations.

2.6. Secondary structure prediction

Prediction of secondary structure and determination of helicity per residue for the peptides were performed using the AGADIR program [29]. AGADIR is a prediction algorithm based on the helix/coil transition theory which predicts the helical behaviour of monomeric peptides. Calculations were performed at pH 7 and 278 K. A minimum percentage of 1% helicity/residue was considered to predict the presence of a helix. Calculated mean hydrophobicities of the peptides were based upon the hydrophobicity scales for amino acids of Kyte and Doolittle [23].

2.7. Enzymatic assay

The ability of tigerinin-M1 (100 µM) to inhibit the activity of porcine pancreatic elastase (Calbiochem, San Diego, CA) was determined using succinyl-Ala-Ala-Pro-Abu-p-nitroanilide [Abu = L-α-aminobutyric acid] (Calbiochem) as substrate as described [24].

3. Results

3.1. Purification of the peptides from X. muelleri

The pooled skin secretions from *X. muelleri*, after partial purification on Sep-Pak C-18 cartridges, were chromatographed on a Vydac C-18 semipreparative reversed-phase HPLC column (Fig. 1). The prominent peaks designated 1 - 6 were collected and subjected to further purification. Under the conditions of assay, peak 2 was associated with the ability to inhibit the growth of *E. coli* only whereas peaks 1 and 4 - 6 inhibited the growth of both *E. coli* and *S. aureus*.

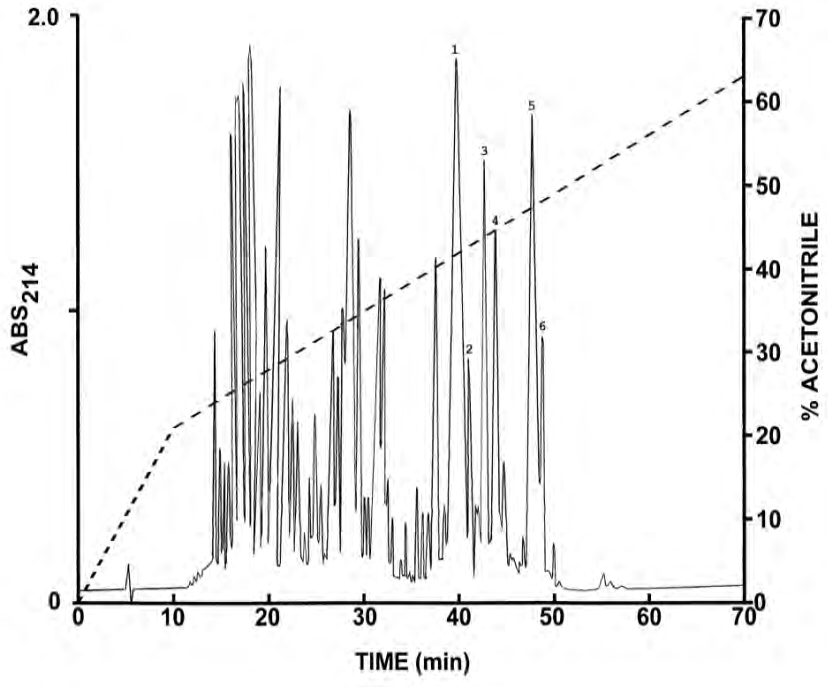

Fig. 1. Reversed-phase HPLC on a semipreparative Vydac C-18 column of skin secretions from *X. muelleri* after partial purification on Sep-Pak cartridges. The peaks designated 1 - 2 and 4 - 6 displayed antimicrobial activity and were purified further. Peak 3 contained tigerinin-M1. The dashed line shows the concentration of acetonitrile in the eluting solvent.

Subsequent structural analysis demonstrated that peak 1 contained magainin-M1, peak 2: magainin-M2, peak 3: tigerinin-M1, peak 4: XPF-M1, peak 5: CPF-M1, and peak 6: CPF-M2. The peptides were purified to near homogeneity, as assessed by a symmetrical peak shape and mass spectrometry, by further chromatography on semipreparative Vydac C-4 and Vydac C-8 columns as previously described. The approximate final yields of purified peptides (nmol) were magainin-M1 182, magainin-M2 20, tigerinin-M1 55, XPF-M1 50, CPF-M1 115, CPF-M2 40.

3.2. Purification of the peptides from X. muelleri West

The pooled skin secretions from *X. muelleri* West, after partial purification on Sep-Pak C-18 cartridges, were chromatographed on a Vydac C-18 semipreparative reversed-phase HPLC column using the same experimental conditions as for the *X. muelleri* skin secretions (Fig. 2).

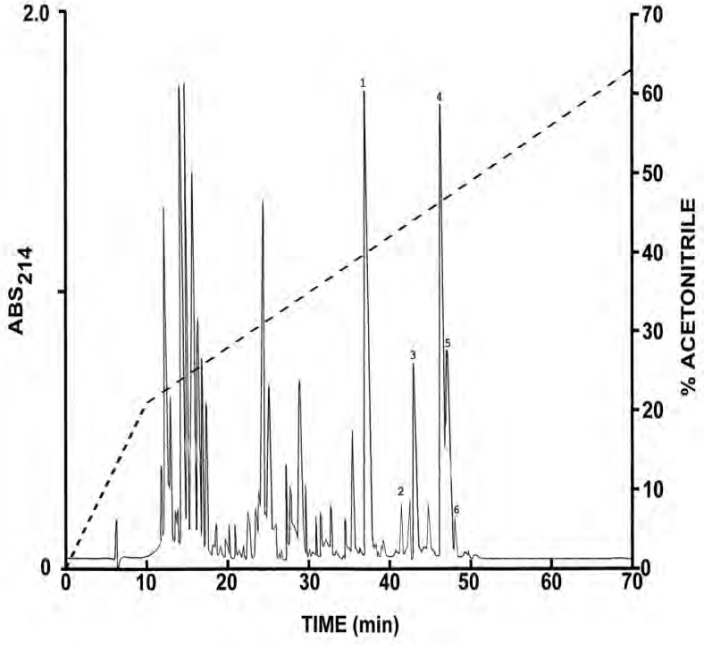

Fig. 2. Reversed-phase HPLC on a semipreparative Vydac C-18 column of skin secretions from *X. muelleri* West after partial purification on Sep-Pak cartridges. The peaks designated 1 - 6 displayed antimicrobial activity and were purified further. The dashed line shows the concentration of acetonitrile in the eluting solvent.

The prominent peaks designated 1 - 6 were collected and subjected to further purification. Under the conditions of assay, peaks 1 and 2 were associated with the ability to inhibit the growth of *E. coli* only whereas peaks 3 - 6 inhibited the growth of both *E. coli* and *S. aureus*. Subsequent structural analysis demonstrated that peak 1 contained magainin-MW1, peak 2: XPF-MW1, peak 3: PGLa-MW1 and PGLa-MW2, peak 4: CPF-MW1, peak 5: CPF-MW2, and peak 6: CPF-MW3. The peptides were purified to near homogeneity, as assessed by a symmetrical peak shape and mass spectrometry, by further chromatography on semipreparative Vydac C-4 and Vydac C-8 columns. The methodology is illustrated by the partial separation of PGLa-MW1 and PGLa-MW2 on a Vydac C-4 column (Fig. 3A), followed by purification to near homogeneity of PGLa-MW1 by further chromatograpy on a Vydac C-8 column (Fig. 3B).

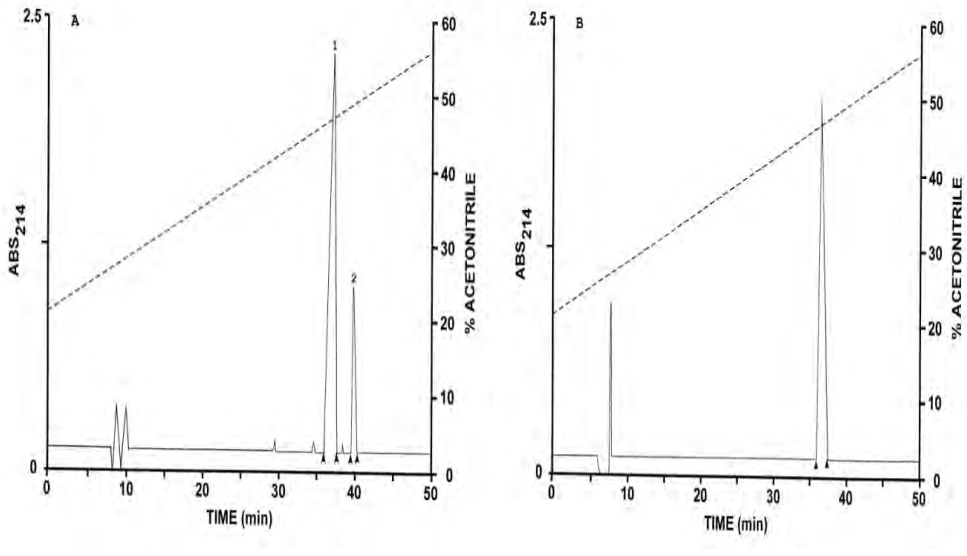

Fig. 3. (A) Separation of PGLa-MW1 (peak 1) and PGLa-MW2 (peak 2) on a semipreparative Vydac C-4 column and (B) purification to near homogeneity of PGLa-MW1 on a semipreparative Vydac C-8 columns. The arrowheads show where peak collection began and ended and the dashed line shows the concentration of acetonitrile in the eluting solvent.

The approximate final yields of purified peptides (nmol) were magainin-MW1 420, XPF-MW1 50, PGLa-MW1 230, PGLa-MW2 50, CPF-MW1 220, CPF-MW2 140 and CPF-MW38.

3.3. Structural characterization

The primary structures of the antimicrobial peptides isolated from *X. muelleri* and *X. muelleri* West skin secretions were established by automated Edman degradation and their complete primary structures are shown in Fig. 4.

Xenopus muelleri

		$[M_r+H]$obs	$[M_r+H]$calc
Magainin-M1	GIGKFLHSAGKFGKAFIGEIMKS	2423.1	2423.3
Magainin-M2	GFKQFVHSLGKFGKAFVGEMIKPK	2680.9	2680.5
Tigerinin-M1	WCPPMIPLCSRF.NH_2	1446.6	1446.6
XPF-M1	GWASKIGQTLGKMAKVGLKDLIQA	2513.8	2513.4
CPF-M1	GLGSLLGKAFKFGLKTVGKMMAGAPREQ	2892.6	2892.4
CPF-M2	GLGSLLGKAFKFGLKTVGKMMAGAPREE	2893.6	2893.6

Xenopus muelleri West

		$[M_r+H]$obs	$[M_r+H]$calc
Magainin-MW1	GIGKFLHSAGKFGKAFLGEVMKS	2409.1	2409.3
XPF-MW1	GWASKIGQTLGKLAKVGLKEFAQS	2518.6	2517.4
PGLa-MW1	GMASKAGSVLGKITKIALGAL.NH_2	1985.4	1985.2
PGLa-MW2	GMASKAGAIAGKIAKTAIKIAL.NH_2	2083.4	2083.3
CPF-MW1	GLGSLLGKAFKFGLKTVGKMMGGAPREQ	2878.6	2878.6
CPF-MW2	GLGSLLGKAFKFGLKTVGKMMGGAPREE	2879.8	2879.6
CPF-MW3	GLGSLLGKAFKFGLKTVGKMMGGAPR	2621.3	2621.5

Fig. 4. Amino acid sequences, observed molecular masses (M_r obs), and calculated molecular masses (M_r calc) of the antimicrobial peptides isolated from skin secretions of *X. muelleri* and *X. muelleri* West.

As there was some ambiguity with regard to identification of the amino acids at the very hydrophobic C-terminus of PGLa-MW2, the proposed sequence was confirmed by amino acid composition analysis [Found: Thr 1.2 (1), Ser 1.2 (1), Gly 3.5 (4), Ala 7.0 (7), Ile 3.5 (4), Leu 1.1 (1), Lys 3.9 (4) residues/mol peptide]. Figures in parentheses show the number of residues predicted from the proposed sequences. MALDI-TOF mass spectrometry was used to confirm the proposed structures and to demonstrate that tigerinin-M1, PGLa-MW1 and PGLa-MW2, are C-terminally α-amidated and tigerinin-M1 exists in the cyclic (disulphide-bridged) form. It is probable that CPF-MW3 is a product of the post-translational processing of CPF-MW1 and/or CPF-MW2 arising from proteolytic cleavage at a single arginine residue rather than the product of a separate gene.

3.4. Antimicrobial and hemolytic activities

The abilities of the most abundant peptides (> 50 nmol) in the *X. muelleri* and *X. muelleri* West skin secretions to inhibit the growth of reference strains of the Gram-positive bacterium *S. aureus*, the Gram-negative bacterium *E. coli*, and the opportunistic yeast pathogen *C. albicans* are compared in Table 1. CPF-MW1 (LC_{50} = 70 μM), CPF-MW2 (LC_{50} = 105 μM), and magainin-M1 (LC_{50} = 180 μM) showed moderately high hemolytic activity against human erythrocytes (Table 1) whereas the hemolytic activities of magainin-MW1, tigerinin-M1 and PGLa-MW1 were very low (LC_{50} > 200 μM). Tigerinin-M1 did not display antimicrobial activity and did not inhibit the activity of elastase at a concentration of 100 μM.

Table 1. Minimum inhibitory concentrations (μM) against microorganisms and hemolytic activity against human erythrocytes of the endogenous peptides isolated from skin secretions of *X. muelleri* and *X. muelleri* West.

	E. coli	***S. aureus***	***C. albicans***	**LC_{50}**
Magainin-M1	12.5	50	25	180
CPF-M1	12.5	6	25	ND
Tigerinin-M1	>200	>200	ND	>500
Magainin-MW1	50	>200	100	>200
PGLa-MW1	12.5	100	50	>200
CPF-MW1	3	6	25	70
CPF-MW2	6	6	ND	105

ND: not determined

4. Discussion

This study has described the purification of five peptides with varying degree of antimicrobial activity in norepinephrine-stimulated skin secretions from the tetraploid frog *X. muelleri* and seven antimicrobial peptides in skin secretions of *X. muelleri* West. Determination of the primary structures of the peptides reveals that they represent orthologs of previously described dermal peptides magainin, PGLa, CPF, and XPF isolated from the tetraploid frogs *X. laevis* [17], *X. borealis* [27], and *X. clivii* [8] and from the octoploid frogs *X. amieti* [5] and *X. andrei* [28]. A comparison of the primary structures of these peptides from frogs of the *X. muelleri* species group (Fig. 5) strongly suggests that *X. muelleri* and *X. muelleri* West are not conspecific thereby providing support for the proposal that the latter should eventually be described as a separate species. No orthologous peptide from the two populations of *X. muelleri* has the same amino acid sequence. Additionally, the *X. muelleri* secretions, like those from *X. clivii* [8], did not contain a PGLa peptide whereas the *X. muelleri* West secretions contained two members of this family. The data suggest that *X. muelleri* West is more closely related to *X. borealis* than to either *X. muelleri* or *X. clivii*. In particular, magainin-MW1 from *X. muelleri* West is identical to magainin-B2 from *X. borealis* and XPF-MW1 shows only two amino acid substitutions compared with the ortholog from *X. borealis* but five substitutions compared with the ortholog from *X. muelleri* (Fig. 5). This conclusion is supported by cladistic analysis based upon the nucleotide sequences of mitochondrial DNA that places X. *muelleri* West (described in the article as "X. new tetraploid") as sister group to *X. borealis* rather than to *X. muelleri* [14].

A disulphide-bridged peptide containing the RFamide motif at its C-terminus was isolated from the *X. muelleri* skin secretions. The peptide lacks antimicrobial or hemolytic activity but shows limited structural similarity to peptides of the tigerinin peptide family (Fig. 6) and so is provisionally designated tigerinin-M1. The presence of such peptide in skin secretions of species belonging to the Xenopodidae has not been reported previously. Tigerinin peptides have previously been identified only in *Hoplobatrachus tigerinus* [32], *Hoplobatrachus rugulosus* (unpublished data), and *Fejervarya cancrivora* [38] belonging to the subfamily Dicroglossinae of the family Dicroglossidae. Despite their low cationicity and their inability to adopt a α-helical conformation, the tigerinins from *Hoplobatrachus* and *Fejervarya* have been reported to display broad-spectrum antimicrobial activity [32,38]. However, under the conditions of assay used in this study, the peptide from *H. rugulosus* was not active against either *E. coli* or *S. aureus* (unpublished data). The biological role of tigerinin-M1, if any, and its evolutionary relationship to other vertebrate peptides containing the RFamide motif [34] are unknown. Skin secretions of several species of frogs have been shown to contain protease inhibitors [33]. The region of the cysteine-rich inhibitor of elastase, guamerin, first isolated from the leech *Hirudo nipponia* [20]

contains a Met(P1)-Ile(P1´) scissile bond. A Met-Ile bond is also found in tigerinin-M1 but the peptide did not inhibit porcine pancreatic elastase at a concentration of 100 μM.

Magainin-related peptides

X. muelleri West-MW1	GIGKFLHSAGKFGKAFLGEVMKS	
X. muelleri-M1	GIGKFLHSAGKFGKAFIGEIMKS	(2)
X. muelleri-M2	GFKQFVHSLGKFGKAFVGEMIKPK	(10)
X. borealis-B1	G**KFLHSAGKFGKAFLGEVMIG	(4)
X. borealis-B2	GIGKFLHSAGKFGKAFLGEVMKS	(0)
X. clivii-C1	GVGKFLHSAKKFGQALASEIMKS	(7)
X. clivii-C2	GVGKFLHSAKKFGQALVSEIMKS	(7)

Proxenopsin-derived peptides

X. muelleri West-MW1	GWASKIGQTLGKLAKVGLKEFAQS	
X. muelleri-M1	GWASKIGQTLGKMAKVGLKDLIQA	(5)
X. borealis-B2	GWASKIGQTLGKMAKVGLKEFVQS	(2)
X. clivii-C1	GWASKIGQALGKVAKVGLQQFIQPK	(6)

PGLa-related peptides

X. muelleri West-MW1	GMASKAGSVLGKITKIAL*GAL[a]	
X. borealis-B2	GMASKAGSIVGKIAKIAL*GAL[a]	(3)
X. muelleri West-MW2	GMASKAGAIAGKIAKTAIKLAL[a]	
X. borealis-B1	GMASKAGTIAGKIAKTAIKLAL[a]	(1)

Procaerulein-derived peptides

X. muelleri West-MW1	GLGSLLGKAFKFGLKTVGKMMGGAPREQ	
X. muelleri-M1	GLGSLLGKAFKFGLKTVGKMMAGAPREQ	(1)
X. borealis-B1	GLGSLLGKAFKIGLKTVGKMMGGAPREQ	(1)
X. clivii-C2	GLGSLLGKALKFGLKAAGKFMGGEP*QQ	(7)
X. muelleri West-MW2	GLGSLLGKAFKFGLKTVGKMMGGAPREE	
X. muelleri-M2	GLGSLLGKAFKFGLKTVGKMMAGAPREE	(1)
X. muelleri West-MW3	GLGSLLGKAFKFGLKTVGKMMGGAPR	
X. borealis-B2	GLGSLLGKAFKIGLKTVGKMMGGAPR	(1)

Fig. 5. A comparison of the primary structures of the orthologous peptides from *X. muelleri* West, *X. muelleri*, *X. borealis*, and *X. clivii*. Structural differences are emphasized by the shading and the values in parentheses show the number of amino acids that differ from the *X. muelleri* West sequence. (*) Denotes a residue deletion and ([a]) indicates that the peptide is C-terminally α-amidated.

Tigerinin-related peptides

X. muelleri	WCPPMIPL**CSRF[a]
H. tigerinus 1	FC*TMIPIPRCY[a]
H. tigerinus 2	RVC*FAIPLPICH[a]
H. tigerinus 3	RVC*YAIPLPICY[a]
H. tigerinus 4	RVC*YAIPLPIC[a]
F. cancrivora	RVC*SAIPLPICH
F. cancrivora	RVC*MAIPLPLCH
H. rugulosus	RVC*MAIPLPLCH[a]

Fig. 6. A comparison of the primary structures of the tigerinin-like peptide from *X. muelleri* with the tigerinins from *Hoplobatrachus tigerinus*, *Hoplobatrachus rugulosus*, and *Fejervarya cancrivora*. Structural similarity is emphasized by the shading. (*) Denotes a residue deletion and ([a]) indicates that the peptide is C-terminally α-amidated.

The physicochemical properties (mean hydrophobicity, cationicity, and degree of α-helicity) of the peptides isolated in this study are compared in Table 2. The antimicrobial activities of frog skin peptides against microorganisms and their cytotoxic activities against mammalian cells are determined by complex interactions among cationicity, hydrophobicity, α-helicity and amphipathicity [4,30].

Consistent with previous observations with peptides from related species [5,8,27,28], the CPF peptides show the greatest antimicrobial potency (MIC in the range 3 - 25 μM) against the Gram-negative *E. coli*, the Gram-positive *S. aureus*, and the opportunistic yeast pathogen *C. albicans*. This activity correlates with a high propensity to adopt an extended α-helical conformation in a membrane-mimetic solvent (Table 2). The 2-fold decrease in potency against *E. coli* of CPF-MW2 compared with CPF-MW1 may be explained by the decrease in cationicity associated with the amino acid substitution $Gln^{28} \rightarrow$ Glu. Several structure-activity studies involving both naturally occurring antimicrobial peptides and synthetic model peptides (reviewed in [4]) have shown a correlation between increasing cationicity and increased antimicrobial potency until a certain optimal molecular charge is reached. However, the therapeutic potential of the CPF peptides as anti-infective agents, particularly for systemic use, is limited by their hemolytic activity against human erythrocytes (LC_{50} in the range 70 – 105 μM).

Table 2. Physicochemical properties of the antimicrobial peptides isolated from skin secretions of *Xenopus muelleri and Xenopus muelleri* West

Peptide	Mean hydrophobicity	Charge at pH 7	α-helical domain
Magainin-M1	+5.3	+3	Non-helical
Magainin-M2	-2.1	+4	Non-helical
Tigerinin-M1	+7.0	+2	Non-helical
XPF-M1	+1.8	+3	10-16
CPF-M1	+1.6	+4	2-11, 14-22
CPF-M2	+1.6	+3	2-11, 14-22
Magainin-MW1	+4.3	+3	Non-helical
XPF-MW1	-2.6	+3	10-18
PGLa-MW1	+18.1	+4	12-19
PGLa-MW2	+18.0	+5	8-22
CPF-MW1	-0.6	+4	2-12, 14-17
CPF-MW2	-0.6	+3	2-11, 14-17
CPF-MW3	+6.4	+5	2-11, 14-17

Mean hydrophobicities (H) of the peptides are calculated using the hydrophobicity scales for amino acid residues of Kyte and Doolittle [23]. Predictions of helical domains are made using the AGADIR program [29].

It is more difficult to account for the greater antimicrobial potency and hemolytic activity of magainin-M1 compared with magainin-MW1. Both peptides bear a charge +3 at pH 7 but the substitutions $Leu^{17} \rightarrow$ Ile and $Val^{20} \rightarrow$ Ile in the *X. muelleri* West peptide result in an increase in mean hydrophobicity. Studies with synthetic analogs of magainin-2 from *X. laevis* have shown that increasing overall hydrophobicity enhanced activity against Gram-positive bacteria while, at the same time, increasing hemolytic activity [10,36]. Although the potency of magainin-MW1 is relatively low, it is known that the magainin peptides act synergistically with PGLa so that the peptides in combination show more potent growth inhibitory activity against *E. coli* than either peptide tested alone [37].

Consequently, in the *X. muelleri* West secretions, magainin-MW1 may act to potentiate the activities of PGLa-MW1 and PGLa-MW2 as well as the CPF peptides.

This study has given further support to the assertion that the amino acid sequences of antimicrobial peptides in skin secretions have value in inferring evolutionary relationships among frogs. The skin peptide profiles of Australian hylid frogs from the genera *Crinia*, *Litoria*, and *Uperoleia* have been used, together with morphological and cognate methods, to differentiate between sub-species and even different population clusters of the same species [19]. Previous investigations with ranid frogs have used the same methodology described in this article to demonstrate that *Rana tagoi tagoi* and *Rana tagoi okiensis* are probably not conspecific [6] and that the relict leopard frog *Lithobates onca* and the lowland leopard frog *Lithobates yavapaiensis*, although closely related phylogenetically, are separate species [7]. On the other hand, the presence of identical peptides in skin secretions from specimens of the Chiricahua leopard frog *Lithobates chiricahuensis* occupying regions in southern Arizona and from morphologically distinct specimens occupying the Mogollon Rim of central Arizona suggest that the two populations probably do not represent separate species [9].

Acknowledgments

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Chapter 6

The hymenochirins: a family of antimicrobial peptides from the Congo dwarf clawed frog *Hymenochirus boettgeri* (Pipidae)

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ABSTRACT

Skin secretions of frogs from the subfamily Xenopodinae (*Xenopus* + *Silurana*) within the family Pipidae are a rich source of antimicrobial peptides with therapeutic potential but species from the sister taxon *Hymenochirus* in the subfamily Pipinae (*Hymenochirus* + *Pseudhymenochirus* + *Pipa*) have not been investigated. Peptidomic analysis of norepinephrine-stimulated skin secretions from two distinct populations of the Congo dwarf clawed frog *Hymenochirus boettgeri* (Tornier, 1896) has led to identification of five structurally-related peptides with broad-spectrum antimicrobial activity. Hymenochirin-1B (IKLSPETKDNLKKVLKGAIKGAIAVAKMV.NH_2) is C-terminally α-amidated whereas hymenochirins-2B-5B have the general structure XKIPX_2VKDTLKKV AKGX_2SX_2AGAX_3.COOH. Hymenochirin-3B (IKIPAVVKDTLKKVAKGVLSAVA GALTQ) was the most abundant peptide in the secretions. The hymenochirins show very low structural similarity with the antimicrobial peptides isolated from skin secretions of *Silurana tropicalis* and *Xenopus laevis* consistent with the proposed ancient divergence of the Pipinae and Xenopodinae. Synthetic replicates of hymenochirin-1B - 4B inhibit the growth of *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Staphylococcus aureus* (MIC in the range 10 - 40 μM) and *Candida albicans* (MIC = 80 μM). The peptides display relatively weak hemolytic activity against human erythrocytes (LC_{50} in the range 160 to >300 μM).

1. Introduction

The clawed frogs comprise 33 well-characterized species distributed in five genera *Hymenochirus*, *Pipa*, *Pseudhymenochirus*, *Silurana*, and *Xenopus* within the family Pipidae [17,18]. All are found in Africa south of the Sahara except for members of the genus *Pipa* which are found in South America. The skins of frogs from the subfamily Xenopodinae (*Xenopus + Silurana)* have proved to be a rich source of host-defense peptides with antimicrobial and anti-inflammatory activities (reviewed in [10]). *Xenopus laevis* was the first amphibian species in which cutaneous peptides, the magainins with growth inhibitory activity against bacteria and fungi were unambiguously identified [39]. Subsequent analysis of *X. laevis* skin secretions has led to the isolation and characterization of several other peptides that show therapeutic potential as anti-infective agents. These include peptide glycine-leucine-amide (PGLa) and multiple structurally-related peptides termed caerulein-precursor fragment (CPF) and xenopsin-precursor fragment (XPF) that are derived from the post-translational processing of the biosynthetic precursors of caerulein and xenopsin respectively [21,35]. Peptides that belong to the PGLa family (peptide XT-5), the CPF family (peptides XT-1, XT-6, and XT-7), and the XPF family (peptides XT-2, XT-3, and XT-4) have been isolated from skin secretions of *Silurana tropicalis* but peptides orthologous to the magainins were not identified [1]. More recently, antimicrobial peptides belonging to the magainin, PGLa, CPF, and XPF families have been isolated from norepinephrine-stimulated skin secretions from *Xenopus amieti* [8], *Xenopus andrei* [29], *Xenopus borealis* [28], *Xenopus clivii* [9], *Xenopus lenduensis* [22], *Xenopus muelleri* [30] and an incompletely characterized species from West Africa provisionally designated *X. muelleri* West [30], *Xenopus petersii* [22], and *Xenopus pygmaeus* [22].

The genus *Hymenochirus* Boulenger, 1896 comprises four species (*H. boettgeri, H. boulengeri, H. curtipes, and H. feae*) that are found in equatorial Africa [18]. *H. boettgeri* ranges widely through the equatorial forest zone of central Africa from southeastern Nigeria and Cameroon south through Gabon to the Mayombe region of western Democratic Republic of Congo and the species has been introduced into southeastern Florida, USA non-indigenously [38]. The species is aquatic, generally found in still, shaded water in lowland rainforest and in pools by slow-flowing rivers [36]. *H. boettgeri* is unusual in that it relies solely on inertial suction to capture prey [4]. It is listed as a Species of Least Concern by the International Union for Conservation of Nature (IUCN) Red List of Threatened Species in view of its wide distribution. However, it is a popular species for the pet trade and numbers have also declined because of habitat modification from deforestation [37]. Chyridiomycosis has been reported in a population of *H. boettgeri* that were probably captive bred in Florida [33].

Up until this time, no species belonging to the genus *Hymenochirus* has been investigated for the presence of cutaneous host-defense peptides. The present study

describes the isolation and the structural and biological characterization of peptides with antimicrobial activity from norepinephrine-stimulated skin secretions of two distinct populations of the Congo dwarf clawed frog *H. boettgeri* (Tornier, 1896) that originated in the Cameroon and the Democratic Republic of the Congo.

2. Experimental

2.1. Collection of skin secretions

All experiments with live animals were approved by the Animal Research Ethics Committee of U.A.E. University (Protocol No. A21-09) and were carried out by authorized investigators. Two populations of the Congo dwarf clawed frog *H. boettgeri* were sampled. Adult animals from group 1 (n = 4; weights 3.0-5.0 g; 2 female) were kept at the Steinhart Aquarium, California Academy of Science, San Francisco, CA and were originally collected at a site in the Cameroon. Sub-adult animals from group 2 (n = 4; weights 0.69-0.74 g; sex not determined) were purchased at a pet store in St Louis, MO, USA and were originally collected at a site in the Democratic Republic of Congo.

Each animal was injected via the dorsal lymph sac with norepinephrine hydrochloride (40 nmol/g body weight) and placed in a solution (40 ml) of distilled water for 15 min. The frog was removed and the collection solution was acidified by addition of trifluoroacetic acid (TFA) (1 ml) and immediately frozen for shipment to U.A.E. University. The solutions containing the secretions from both groups were separately pooled and passed at a flow rate of 2 ml min^{-1} through 4 Sep-Pak C-18 cartridges (Waters Associates, Milford, MA, USA) connected in series. Bound material was eluted with acetonitrile/water/TFA (70.0:29.9:0.1, v/v/v) and freeze-dried. The material was redissolved in 0.1% (v/v) TFA/water (2 ml).

2.2. Peptide purification

The skin secretions from each group of *H. boettgeri*, after partial purification on Sep-Pak cartridges, were separately injected onto a preparative (2.2 cm x 25 cm) Vydac 218TP1022 (C-18) reversed-phase HPLC column (Grace, Deerfield, IL, USA) equilibrated with 0.1% (v/v) TFA/water at a flow rate of 6.0 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 nm and 280 nm, and fractions (1 min) were collected. Purification and biological activities of the peptides were monitored by incubating lyophilized aliquots of chromatographic effluent (100 µl) in Mueller-Hinton broth (50 µl) with an inoculum (50 µl of 10^6 colony forming units/ml) from a log-phase culture of reference strains of *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli*

(ATCC 25726) 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. Fractions associated with antimicrobial activity were successively chromatographed on a (1.0 cm x 25 cm) Vydac 214TP510 (C-4) column and a (1.0 cm x 25 cm) Vydac 208TP510 (C-8) 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}.

2.3. Structural characterization

The primary structures of the peptides were determined by automated Edman degradation using a model 494 Procise sequenator (Applied Biosystems, Foster City, CA, USA). MALDI-TOF mass spectrometry was carried out using a Voyager DE-PRO instrument (Applied Biosystems) that was operated in reflector mode with delayed extraction and the accelerating voltage in the ion source was 20 kV. The instrument was calibrated with peptides of known molecular mass in the 2 - 4 kDa range. The accuracy of mass determinations was ± 0.02%.

2.4. Peptide synthesis

Hymenochirins-1B - 5B were supplied in crude form by China Peptides Ltd. (Shanghai, China) and GL Biochem Ltd. (Shanghai, China) and were purified to near homogeneity (> 98% purity) by reversed-phase HPLC on a (2.2 cm x 25 cm) Vydac 218TP1022 (C-18) column. The concentration of acetonitrile in the eluting solvent was raised from 28% to 56% over 60 min and the flow rate was 6 ml min^{-1}. The identity and purity of the peptides was confirmed by electrospray mass spectrometry. Hymenochirin-5B was obtained in low yield and was observed to form a viscous gel in concentrated solution.

2.5. Antimicrobial and hemolytic activities

Reference strains of microorganisms were purchased from the American Type Culture Collection (Rockville, MD). Minimum inhibitory concentrations (MIC) of the synthetic hymenochirins against *S. aureus* (ATCC 25923), *E. coli* (ATCC 25726), *Klebsiella pneumoniae* (ATCC 700603), *Pseudomonas aeruginosa* (ATCC 27853), and *Candida albicans* (ATCC 90028) were measured in the concentration range of 2.5 - 160 μM by standard double dilution methods [5,6] and were taken as the lowest concentration of peptide where no visible growth was observed. The values were confirmed by measurement of absorbance at 630 nm. A minimum of three independent incubations were performed. In order to monitor the validity and reproducibility of the assays, incubations

were carried out in parallel with increasing concentrations of antibiotics (ampicillin for *S. aureus* and *E. coli*, ciprofloxacin for *K. pneumoniae* and *P. aeruginosa*, and amphotericin for *C. albicans*) as previously described [28-30].

Hemolytic activity against human erythrocytes taken from a healthy donor was measured as previously described [28-30]. Peptides were tested in the concentration range 37.5 - 300 μM. The LC_{50} value was taken as the mean concentration of peptide producing 50% hemolysis in three independent incubations.

2.6. Secondary structure prediction

Prediction of secondary structure and determination of % helicity per residue for the peptides were performed using the AGADIR program [31]. AGADIR is a prediction algorithm based on the helix/coil transition theory which predicts the helical behaviour of monomeric peptides. Calculations were performed at pH 7 and 278K. A minimum percentage of 1% helicity/residue was considered to predict the presence of a helix. Calculated mean hydrophobicities of the peptides were based upon the hydrophobicity scales for amino acids of Kyte and Doolittle [23].

3. Results

3.1. *Purification of the peptides*

The pooled skin secretions from the group 1 population of *H. boettgeri* (Cameroon), after partial purification on Sep-Pak C-18 cartridges, were chromatographed on a Vydac C-18 preparative reversed-phase HPLC column (Fig. 1). Under the conditions of assay, the prominent peaks designated 1 - 5 were associated with the ability to inhibit the growth of both *E. coli* and *S. aureus*. Subsequent structural analysis demonstrated that these peaks contained hymenochirins 1- 5. The peptides were purified to near homogeneity, as assessed by a symmetrical peak shape and mass spectrometry, by further chromatography on semipreparative Vydac C-4 and Vydac C-8 columns. The methodology is illustrated by purification of hymenochirin-3B on C-4 (Fig. 2A) and C-8 (Fig. 2B) columns. The approximate final yields of purified peptides (nmol) were hymenochirin-1B (40), hymenochirin-2B (55), hymenochirin-3B (425), hymenochirin-4B (90) and hymenochirin-5B (65).

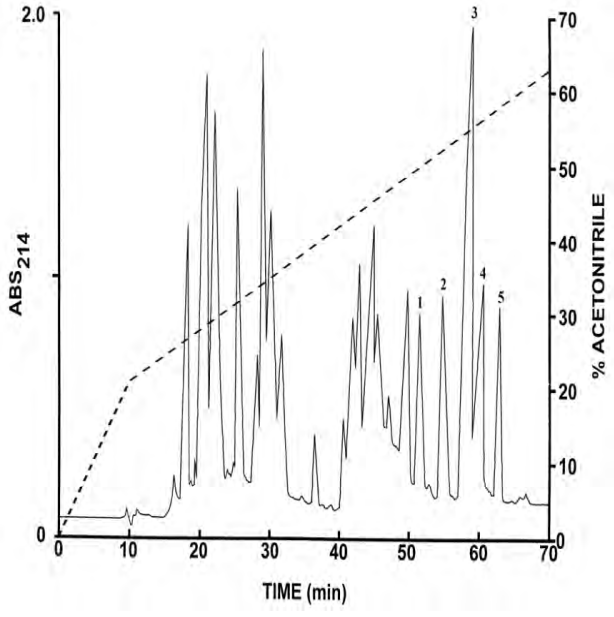

Fig. 1. Reversed-phase HPLC on a preparative Vydac C-18 column of skin secretions from *H. boettgeri* from the Cameroon (Group 1) after partial purification on Sep-Pak cartridges. Peaks 1-5 inhibited growth of *E. coli* and *S. aureus* and were purified further. The dashed line shows the concentration of acetonitrile in the eluting solvent.

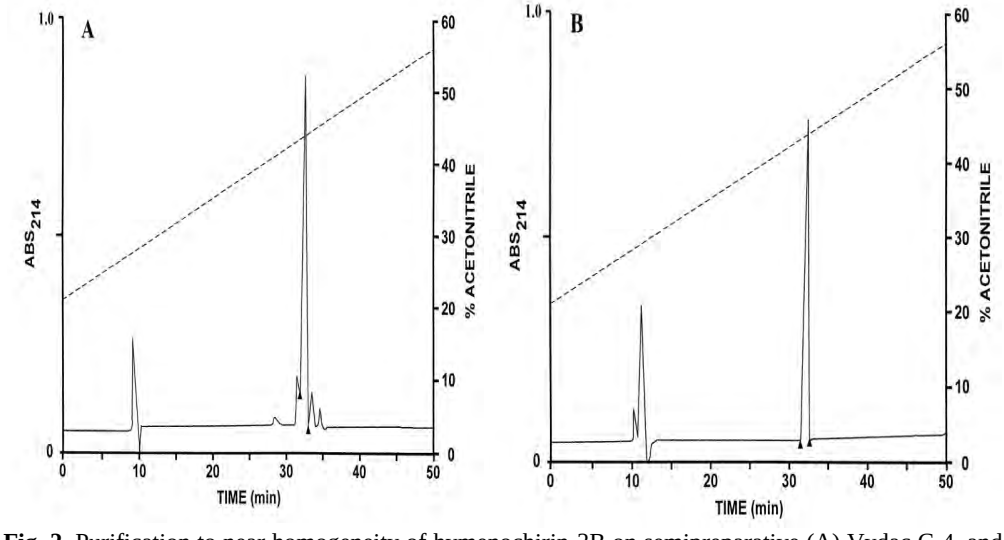

Fig. 2. Purification to near homogeneity of hymenochirin-3B on semipreparative (A) Vydac C-4, and (B) Vydac C-8 columns. The arrowheads show where peak collection began and ended. The dashed line shows the concentration of acetonitrile in the eluting solvent.

The pooled skin secretions from the group 2 population of *H. boettgeri* (Congo), after partial purification on Sep-Pak C-18 cartridges, were chromatographed on a Vydac C-18 preparative reversed-phase HPLC column under the same condition used for secretions from the group 1 population (Fig. 3). The prominent peaks designated 1 - 5 were collected and analysed by MALDI-TOF mass spectrometry.

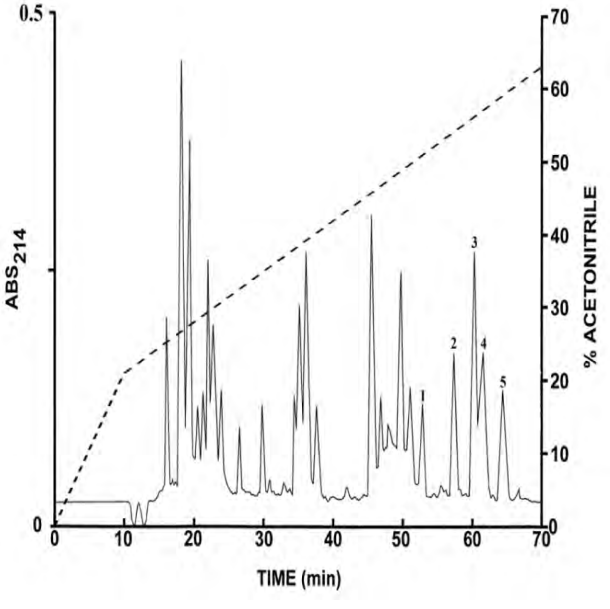

Fig. 3. Reversed-phase HPLC on a preparative Vydac C-18 column of skin secretions from *H. boettgeri* from the Democratic Republic of the Congo (Group 2) after partial purification on Sep-Pak cartridges. Peaks 1-5 were analysed by MALDI-TOF mass spectrometry and were purified further. The dashed line shows the concentration of acetonitrile in the eluting solvent.

3.2. Structural characterization

The primary structures of the antimicrobial peptides isolated from the group 1 population (Cameroon) of *H. boettgeri* were established by automated Edman degradation and their amino acid sequences are shown in Fig. 4.

		Cameroon	Congo	
		Mr_{obs}	Mr_{obs}	Mr_{calc}
Hymenochirin-1B	IKLSPETKDNLKKVLKGAIKGAIAVAKMV.NH_2	3061.8	3062.4	3061.8
Hymenochirin-2B	LKIPGFVKDTLKKVAKGIFSAVAGAMTPS	2973.5	2973.7	2973.7
Hymenochirin-3B	IKIPAVVKDTLKKVAKGVLSAVAGALTQ	2817.6	2817.9	2817.7
Hymenochirin-4B	IKIPAFVKDTLKKVAKGVISAVAGALTQ	2865.9	2865.5	2865.7
Hymenochirin-5B	IKIPPIVKDTLKKVAKGVLSTIAGALST	2861.0	2860.5	2860.8

Fig. 4. Amino acid sequences, observed molecular masses (M_r obs), and calculated molecular masses (M_r calc) of the antimicrobial peptides isolated from skin secretions of *H. boettgeri*.

As there was some ambiguity with regard to identification of the amino acids at the hydrophobic C-terminus of hymenochirin-1B, the proposed sequence was confirmed by amino acid composition analysis [Found: Asx 2.1 (2), Clx (1.0), Thr 1.1 (1), Ser 1.1 (1), Gly 2.1 (2), Ala 4.0 (4), Val 2.9 (3), Met 1.1 (1), Ile 2.7 (3), Leu 3.0 (3), Lys 6.7 (7), Pro 1.0 (1) residues/mol peptide]. Figures in parentheses show the number of residues predicted from the proposed sequences. The molecular masses of the peptides determined by MALDI-TOF mass spectrometry are consistent with their proposed structures and demonstrate that only hymenochirin-1B is C-terminally α-amidated. The amino acid sequences of hymenochirins from the group 2 population (Congo) were not determined but the close similarities in molecular masses (Fig. 4) and retention times on reversed-phase HPLC (Figs. 1 and 3) indicate that the peptides from both populations are probably identical.

3.3. Antimicrobial and hemolytic activities

The abilities of the synthetic hymenochirins to inhibit the growth of reference strains of the Gram-positive bacterium *S. aureus*, the Gram-negative bacteria *E. coli*, *K. pneumoniae*, and *P. aeruginosa*, and the opportunistic yeast pathogen *C. albicans* are compared in Table 1. The MIC values for hymenochirin-5B may be artefactually high because of the restricted solubility of the peptide in Mueller-Hinton broth and its tendency to form a gel in concentrated solution. All peptides displayed relatively low hemolytic activities against human erythrocytes (LC_{50} in the range 160 to > 300 μM).

Table 1. Minimum inhibitory concentrations against microorganisms and hemolytic activity against human erythrocytes (LC_{50}) of the peptides isolated from skin secretions of *H. boettgeri.*

Peptide	*E. coli*	*P. aeruginosa*	*K. pneumoniae*	*S. aureus*	*C. albicans*	LC_{50}
Hymenochirin-1B	20	40	20	10	80	225
Hymenochirin-2B	20	20	20	40	80	>300
Hymenochirin-3B	20	40	40	40	80	>300
Hymenochirin-4B	20	20	20	20	80	160
Hymenochirin-5B	40	ND	ND	80	160	ND

Data are expressed as μM and are derived from a minimum of three independent incubations. ND: not determined.

4. Discussion

The study has described the purification and characterization of five structurally related antimicrobial peptides from skin secretions of the frog *H. boettgeri* (Pipidae) belonging to two populations from different geographical locations. Skin secretions from a frog from the genus *Hymenochirus* have not been investigated previously. The peptides have been termed the hymenochirins and given the suffix B to denote the species. Although the amino acid sequences of the peptides from the population originating in the Democratic Republic of Congo were not determined, the close similarities in molecular masses and chromatographic properties indicate that they are probably identical to the peptides from frogs originating in the Cameroon. The work complements previous studies that have led to the isolation of antimicrobial peptides from several species from the genera *Silurana* and *Xenopus* within the family Pipidae (reviewed in [10,22]). Antimicrobial peptides were not identified in skin secretions from a representative of the genus *Pipa,* notably *P. pipa* [7], and the single species (*P. merlini*) belonging to the genus *Pseudhymenochirus* has not yet been investigated.

Pipidae is sister-group to the Rhinophrynidae (represented by a single species, the Mexican burrowing toad *Rhinophrynus dorsalis*) [17,18] but phylogenetic relationships among the clades within the family are not entirely clear. A study based upon morphological characters concluded that *Silurana* was more closely related to the genera *Hymenochirus*, *Pipa*, and *Pseudhymenochirus* than to *Xenopus* [3]. However, subsequent analyses based upon the comparison of the nucleotide sequences of the DNA from ribosomal 18S and 28S genes strongly supported the sister-group relationship between

Silurana and *Xenopus* rather than the relationship suggested by morphology [14]. Subsequent comparisons of the nucleotide sequences of mitochondrial DNA [15,16] provide support for this conclusion and suggest that the divergence of the Xenopodinae (*Silurana* + *Xenopus*) from the Pipinae (*Hymenochirus* + *Pipa* + *Pseudhymenochirus*) predates the divergence of the Pipinae subfamily which occurred at the time of separation of Africa and South America (105 - 119 million years ago, MYA) [27]. The origin of the Pipidae is at least Late Jurassic (150 MYA) and breakup of Gondwana led to the establishment of *Pipa* in South America and *Hymenochirus* + *Pseudhymenochirus* in Africa [2]. As shown in Fig. 5, the hymenochirins show only very low structural similarity to the magainins from *X. laevis* [39] and the xenopsin-precursor fragment peptides, the caerulein-precursor fragment peptides, and the PGLa-related peptides that were isolated from skin secretions of *S. tropicalis* [1] and *X. laevis* [21]. Amino acid sequence comparisons of orthologous antimicrobial peptides from *S. tropicalis* and a range of *Xenopus* species indicate an appreciably greater degree of sequence similarity (reviewed in [10]). The data are thus consistent with the proposed ancient divergence of *Hymenochirus* from the *Silurana* + *Xenopus* clade.

Magainin

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Hymemochirin-1B          IKLSPETKDNLKKVLKGAIKGAIAVAKMV[a]
Hymenochirin-2B          LKIPGFVKDTLKKVAKGIFSAVAGAMTPS
Hymenochirin-3B          IKIPAVVKDTLKKVAKGVLSAVAGALTQ
Hymenochirin-4B          IKIPAFVKDTLKKVAKGVISAVAGALTQ
Hymenochirin-5B          IKIPPIVKDTLKKVAKGVLSTIAGALST
X. laevis-1               GIGKFLHSAGKFGKAFVGEIMKS
X. laevis-2               GIGKFLHSAKKFGKAFVGEIMNS
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XPF

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Hymemochirin-1B          IKLSPETKDNLKKVLKGAIKGAIAVAKMV[a]
Hymenochirin-2B          LKIPGFVKDTLKKVAKGIFSAVAGAMTPS
Hymenochirin-3B          IKIPAVVKDTLKKVAKGVLSAVAGALTQ
Hymenochirin-4B          IKIPAFVKDTLKKVAKGVISAVAGALTQ
Hymenochirin-5B          IKIPPIVKDTLKKVAKGVLSTIAGALST
S. tropicalis XT-2        GVWSTVLGGLKKFAKGGLEAIVNPK
S. tropicalis XT-3        GLASTLGSFLGKFAKGGAQAFLQPK
X. laevis-1               GWASKIGQTLGKIAKVGLQGLMQPK
X. laevis-2               GWASKIGQTLGKIAKVGLKELIQPK
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PGLa

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Hymemochirin-1B          IKLSPETKDNLKKVLKGAIKGAIAVAKMV[a]
Hymenochirin-2B          LKIPGFVKDTLKKVAKGIFSAVAGAMTPS
Hymenochirin-3B          IKIPAVVKDTLKKVAKGVLSAVAGALTQ
Hymenochirin-4B          IKIPAFVKDTLKKVAKGVISAVAGALTQ
Hymenochirin-5B          IKIPPIVKDTLKKVAKGVLSTIAGALST
S. tropicalis XT-5        GMATKAGTALGKVAKAVIGAAL[a]
X. laevis                 GMASKAGAIAGKIAK*VALKAL[a]
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CPF

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Hymemochirin-1B          IKLSPETKDNLKKVLKGAIKGAIAVAKMV[a]
Hymenochirin-2B          LKIPGFVKDTLKKVAKGIFSAVAGAMTPS
Hymenochirin-3B          IKIPAVVKDTLKKVAKGVLSAVAGALTQ
Hymenochirin-4B          IKIPAFVKDTLKKVAKGVISAVAGALTQ
Hymenochirin-5B          IKIPPIVKDTLKKVAKGVLSTIAGALST
S. tropicalis XT-1       GFLGPLLKLAAKGVAKVIPHLI*PSRQQ
X. laevis CPF-1          GLASFLGKAL*KAGLKIGAHLLGGAPQQ
X. laevis CPF-2          GFASFLGKAL*KAALKIGANMLGGTPQQ
X. laevis CPF-3          GFGSFLGKAL*KAALKIGANALGGSPQQ
X. laevis CPF-4          GLASLLGKAL*KAGLKIGTHFLGGAPQQ
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Fig. 5. A comparison of the primary structures of peptides belonging to the magainin, XPF, PGLa, and CPF families from *Hymenochirus boettgeri, Silurana tropicalis, and Xenopus laevis.* [a] indicates that the peptide is C-terminally α-amidated. Shading is used to indicate conserved amino acid residues. Gaps, denoted by *, are inserted into some sequences to maximize structural similarity.

The physicochemical properties (grand average of hydrophobicity, cationicity, and degree of α-helicity) of the peptides isolated in this study are compared in Table 2. Retention time on reversed-phase HPLC has been shown to provide a measure of the effective hydrophobicity of a α-helical peptide [36]. However, the observed retention times of the hymenochirins do not correlate exactly with calculated hydrophobicity as hymenochirin-4B and -5B are eluted from the column with anomalously high retention times which may be related to their tendency to self-aggregate.

Table 2. Physicochemical properties of the antimicrobial peptides isolated from skin secretions of *H. boettgeri.*

Peptide	GRAVY	Charge at pH 7	α-helicity
Hymenochirin-1B	0.169	+6	5-27
Hymenochirin-2B	0.466	+4	8-16
Hymenochirin-3B	0.689	+4	9-16
Hymenochirin-4B	0.664	+4	7-16
Hymenochirin-5B	0.596	+4	9-16

Grand average of hydropathicity (GRAVY) of the peptides are calculated using the hydrophobicity scales for amino acid residues of Kyte and Doolittle [23]. Predictions of helical domains are made using the AGADIR program [31].

Analysis of the secondary structure of the peptides using the AGADIR program [31] indicates that hymenochirins -2B, -3B, -4B, and - 5B have a relatively weak propensity to adopt a α-helical conformation in the conserved central region of the molecule (maximum percentage helicity/residue = 1.5) whereas hymenochrin-1B is predicted to form a more stable (maximum percentage helicity/residue = 20) and extended α-helical domain from residues 5 to 27. A Schiffer-Edmundsen wheel representation [34] of N-terminal region of hymenochirin-1B illustrates the amphipathic nature of the α-helical conformation with the polar Lys^{2}, Glu^{6}, Lys^{8}, Asp^{9}, Lys^{12}, Lys^{13}, and Lys^{16} segregating on one face of the helix and the hydrophobic Leu^{3}, Leu^{11}, Val^{14}, and Leu^{15} residues on the opposite face (Fig. 6).

Hymenochirin-1B differs from the other structurally similar hymenochirins by its greater cationicity (charge of +6 at pH 7 compared with +4 for hymenochirins -2B, -3B, -4B, and -5B) and by its strong propensity to form a stable and extended α-helical conformation (Table 2). The antimicrobial activities of peptides against microorganisms and their cytotoxic activities against mammalian cells are determined by complex interactions among cationicity, hydrophobicity, α-helicity and amphipathicity [11,12]. Several structure-activity studies involving both naturally occurring antimicrobial peptides and synthetic model peptides (reviewed in [12]) have shown a correlation between increasing cationicity and increased antimicrobial potency until a certain optimal molecular charge is reached.

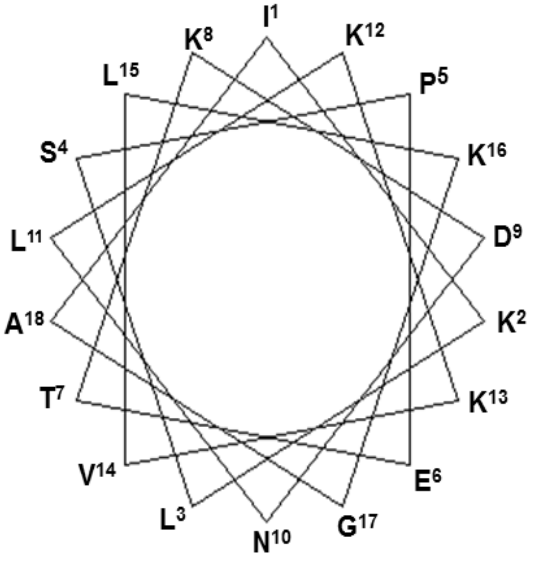

Fig. 6. Schiffer-Edmundson wheel representation of the (1-18) region of hymenochirin-1B demonstrating the amphipathic nature of the predicted α-helical conformation.

The increased growth inhibitory potency of hymenochirin-1B against the Gram-positive bacterium *S. aureus* is consistent with the results of these studies. The effects of increased cationicity on potency of hymenochirin-1B against Gram-negative bacteria are less pronounced (Table 2). It has been shown using model peptides that a stabilized amphipathic α-helical conformation is an absolute requirement for cytotoxic activity against Gram-positive bacteria whereas the structural requirements for activity against Gram-negative bacteria are less stringent [20]. Studies with analogs of the amphipathic model α-helical peptide KLALKLALKALKAAKLA.NH_2 have shown a positive correlation between hemolytic activity and degree of α-helicity [11]. The greater hemolytic activity of hymenochirin-1B compared with hymenochirin-2B and -3B is consistent with its increased propensity to form a stable α-helix. Hemolytic activity of an amphipathic α-helical peptide also increases with hydrophobicity [13,35]. The increased hemolytic activity of hymenochirin-4B correlates with its greater effective hydrophobicity as determined by retention time on reversed-phase HPLC. There was insufficient material to determine the LC_{50} value of hymenochirin-5B against human erythrocytes.

The emergence in all regions of the world of strains of pathogenic bacteria and fungi with resistance to commonly used antibiotics constitutes a serious threat to public health [32]. Although effective new types of antibiotics against multidrug-resistant Gram-positive

bacteria such as methicillin-resistant *S. aureus* (MRSA) have been introduced or are in clinical trials, the situation regarding new treatment options for infections produced by multidrug-resistant Gram-negative pathogens such as *E. coli*, *P. aeruginosa* and *K. pneumoniae* is less encouraging [26]. Infections caused by these carbapenemase-producing bacteria that are resistant to all beta-lactam antibiotics are particularly difficult to treat [19]. Colistin, the cyclic antibiotic peptide polymyxin E derived from *Bacillus polymyxa*, is used as a "last resort" therapy in the management of infections due to multidrug-resistant Gram-negative bacteria [25]. However, not only high nephrotoxicity limits its use but resistance to colistin has already been described and increasing use of this antibiotic is likely to lead to the emergence of more resistant strains [24]. This situation has necessitated a search for novel types of antimicrobial agents, with appropriate toxicological and pharmacokinetic properties, to which pan-resistant pathogenic microorganisms have not been exposed. Hymenochirins-2B and 3B, although displaying only moderate potency against Gram-negative bacteria (MIC in the range 20 - 40 μM), have the advantage over many naturally occurring antimicrobial peptides of having very low cytotoxicity against human erythrocytes (LC_{50} > 300 μM). Further structure-activity studies are warranted to develop analogs of these peptides with increased activity against drug-resistant pathogenic microorganisms that retain low hemolytic activity.

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Chapter 7

Hybridization between the tetraploid African clawed frogs *Xenopus laevis* and *Xenopus muelleri* (Pipidae) increases the multiplicity of antimicrobial peptides in the skin secretions of female offspring

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ABSTRACT

Peptidomic analysis was used to compare the distribution of host-defense peptides in norepinephrine-stimulated skin secretions from laboratory-generated female F1 hybrids of the common clawed frog *Xenopus laevis* (Daudin, 1802) and Mueller's clawed frog *Xenopus muelleri* (Peters, 1844) with the corresponding distribution in skin secretions from the parent species. A total of 18 peptides were identified in secretions from the hybrid frogs. Eleven peptides (magainin-1, magainin-2, CPF-1, CPF-3, CPF-4, CPF-5, CPF-6, CPF-7, XPF-1, XPF-2, and PGLa) were identified in secretions of both the hybrids and *X. laevis*. Four peptides (magainin-M1, XPF-M1, CPF-M1, and tigerinin-M1) were previously found in skin secretions of *X. muelleri* but magainin-M2 and CPF-M2 from *X. muelleri* were not detected. Three previously undescribed peptides (magainin-LM1, PGLa-LM1, and CPF-LM1) were purified from the secretions of the hybrid frogs that were not detected in secretions from either *X. laevis* or *X. muelleri*. Magainin-LM1 differs from magainin-2 from *X. laevis* by a single amino acid substitution ($Gly^{13} \rightarrow$ Ala) but PGLa-LM1 and CPF-LM1 differ appreciably in structure from orthologs in the parent species. CPF-LM1 shows potent, broad-spectrum antimicrobial activity and is hemolytic. The data indicate that hybridization increases the multiplicity of skin host-defense peptides in skin secretions. As the female F1 hybrids are fertile, hybridization may represent an adaptive strategy among *Xenopus* species to increase protection against pathogenic microorganisms in the environment.

1. Introduction

The clawed frogs of the genus *Xenopus* within the family Pipidae currently comprise 19 well characterized species although several unnamed species have been reported (Kobel et al., 1996; Frost, 2011). All are found in Africa south of the Sahara. The genus has a complex evolutionary history involving both bifurcating and reticulating modes of speciation (Kobel, 1996; Evans et al., 2004; Evans, 2008). Allopolyploidization events, in which two species hybridize and the descendant inherits the complete genome of both ancestors, have given rise to tetraploid, octoploid, and dodecaploid species with no extant *Xenopus* species retaining the diploid status that is thought to be related to the ancestral state existing prior to one or more whole genome duplications (Kobel and Du Pasquier, 1991). At this time, the ten tetraploid *Xenopus* species have been divided into three species groups on the basis of similarities in morphology, advertisement calls, and/or nucleotide sequences of mitochondrial genes: the *laevis* group includes *X. laevis, X. gilli, X. largeni, X. petersii, and X. victorianus*; the *muelleri* group includes *X. muelleri, X. borealis*, and *X. clivii*; and the *fraseri* group includes *X. fraseri* and *X. pygmaeus* (Kobel et al., 1996; Evans et al., 2004). It has been proposed that the seven extant octoploid species arose from three distinct allopolyploidization events (Evans, 2008; Evans et al., 2011). Thus, *X. lenduensis* and *X. vestitus* share a common tetraploid ancestor; *X. amieti*, *X. andrei*, and *X. boumbaensis* form a second group; and *X. itombwensis* and *X. wittei* constitute a third group. Further allopolyploidizations within the second group have given rise to the dodecaploid species *X. longipes* and *X. ruwenzoriensis*.

The skins of frogs from the genus *Xenopus* have proved to be a rich source of host-defense peptides with antimicrobial and anti-inflammatory activities [reviewed in (Conlon et al., 2012)]. The magainins were isolated from *X. laevis* skin more than 25 years ago, (Giovannini et al., 1987; Zasloff, 1987) and analysis of skin secretions from this species led to the isolation and characterization of several other peptides that show therapeutic potential as anti-infective agents (Zhang and Falla, 2010). These include peptide glycine-leucine-amide (PGLa) and multiple structurally-related peptides that are derived from the post-translational processing of the biosynthetic precursors of caerulein [termed caerulein-precursor fragment (CPF)] and xenopsin [termed xenopsin-precursor fragment (XPF)] (Gibson et al., 1986; Soravia et al., 1988). More recently, antimicrobial peptides belonging to the magainin, PGLa, CPF, and XPF families have been isolated from norepinephrine-stimulated skin secretions from *X. amieti* (Conlon et al., 2010), *X. andrei* (Mechkarska et al., 2011b) , *X. borealis* (Mechkarska et al., 2010), *X. clivii* (Conlon et al., 2011), *X. lenduensis* (King et al., 2012), *X. muelleri* and an incompletely characterized species from West Africa provisionally designated *X. muelleri* West (Mechkarska et al., 2011a), *X. petersii* (King et al., 2012), and *X. pygmaeus* (King et al., 2012).

There have been relatively few reports of natural hybridization between *Xenopus* species in the wild. Extensive interbreeding has been described only for *X. laevis* and the seriously endangered *X. gilli* (Picker, 1985). Hybrids between *X. borealis* and *X. victorianus* in western Kenya have also been described (Yager, 1996). Areas of sympatry between *X. laevis* and *X. muelleri* are few which mitigates against interbreeding but the two species were found in the same bodies of water in Mpumalanga province of South Africa and natural hybrids were characterized both biochemically and morphologically and from their distinctive advertisement calls (Fischer et al., 2000). In contrast, many *Xenopus* species can be hybridized in the laboratory and gametic incompatibilities are generally found to be absent (Kobel et al., 1996). In such interspecies crosses, the males are consistently sterile and the females are fully or partially fertile. As *Xenopus* females are heterogametic and males are homogametic, it has been pointed out that this genus represents an exception to Haldane's rule that the heterogametic sex typically suffers the greater dysfunctional effects of hybridization (Malone et al., 2007).

The generation of *X. laevis* x *X. muelleri* hybrids in the laboratory has been described and microarray analysis has been used to study gene expression in the testes of hybrid males (Malone et al., 2007) and in the ovary of hybrid females (Malone and Michalak, 2008). These animals have been made available to the investigators and the aim of the present study was to use peptidomic analysis (reversed-phase HLPC coupled with electrospray mass spectrometry) to compare the host-defense peptides in norepinephrine-stimulated skin secretions from female *X. laevis* x *X. muelleri* F1 hybrids with the corresponding peptides from the parent species. The peptides described in this study are classified according to the terminology used previously for peptides from the *X. laevis* (Gibson et al., 1986, Zasloff, 1987). The magainin, PGLa, CPF, and XPF peptide families are recognized. The term tigerinin refers to the family of cyclic peptides first identified in the Asian frog *Hoplobatrachus tigerinus* (Sai et al., 2001). Orthologs from *X. muelleri* are designated M and the novel peptides identified in the hybrids are denoted by LM. Paralogs are differentiated by numerals e.g. CPF-1 and CPF-2.

2. Materials and methods

2.1. Collection of skin secretions

All experiments with live animals were approved by the Animal Research Ethics committee of U.A.E. University (Protocol No. A21-09) and were carried out by authorized investigators. *X. laevis* x *X. muelleri* F1 hybrids were produced as previously described (Malone et al., 2007). The female parents were *X. laevis* originating from the Cape region of South Africa and the male parents were *X. muelleri* originating from the Nkambeni area of Swaziland. Female hybrids (n = 3; 5 -6 years old; weights 43 – 48 g) were injected via

the dorsal lymph sac with norepinephrine hydrochloride (40 nmol/g body weight) and placed in a solution (100 mL) of collecting buffer (50 mM sodium chloride-25 mM sodium acetate, pH 7.0) for 15 min. The frogs were removed and the collection solution was acidified by addition of concentrated hydrochloric acid (1 mL) and immediately frozen for shipment to U.A.E. University. The solutions containing the secretions from each group were pooled and separately passed at a flow rate of 2 mL/min through 6 Sep-Pak C-18 cartridges (Waters Associates, Milford, MA, USA) connected in series. Bound material was eluted with acetonitrile/water/trifluoroacetic acid (TFA) (70.0:29.9:0.1, v/v/v) and freeze-dried. The material was redissolved in 0.1% (v/v) TFA/water (2 mL).

A single adult female specimen of *X. laevis* (weight 263 g) was supplied by Xenopus Express Inc. (Brooksville, FL, USA) and housed in the vivarium at U.A.E. University. Skin secretions were obtained and partially purified on Sep-Pak cartridges using the same protocol used for the hybrids. The collection of skin secretions from *X. muelleri* has been described previously (Mechkarska et al., 2011a).

2.2. Peptide purification

The pooled skin secretions from the hybrid frogs, after partial purification on Sep-Pak cartridges, were injected onto a (2.2 cm x 25 cm) Vydac 218TP1022 (C-18) reversed-phase HPLC column (Grace, Deerfield, IL, USA) equilibrated with 0.1% (v/v) TFA/water at a flow rate of 6.0 mL/min. 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 nm and 280 nm, and fractions (1 min) were collected. Purification of the peptides was monitored by incubating lyophilized aliquots of chromatographic effluent (100 μL) in Mueller-Hinton broth (50 μL) with an inoculum (50 μL of 10^6 colony forming units/mL) from a log-phase culture of reference strains of *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25726) in 96-well microtiter cell-culture plates for 18 h at 37^{o}C in a humidified atmosphere of air. After incubation, the absorbance at 630 nm of each well was determined using a microtiter plate reader. Fractions associated with antimicrobial activity were successively chromatographed on a (1.0 cm x 25 cm) Vydac 214TP510 (C-4) column and a (1.0 cm x 25 cm) Vydac 208TP510 (C-8) 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.

Skin secretions from *X. laevis* (20% of the total amount collected) were subjected to the same chromatographic procedures used for purification of the peptides from the hybrid frogs. The purification of antimicrobial peptides from *X. muelleri* skin secretions has been described previously (Mechkarska et al., 2011a).

2.3. Structural characterization

Electrospray-ionization mass spectrometry was carried out using an Agilent 6310 Series ion trap mass spectrometer as previously described (Zahid et al., 2011). The polarity of the mass spectrometer was set to positive and the scan range was from 200 to 2200 m/z with maximum accumulation time of 300 ms. The capillary voltage was set to -3500V, the skimmer voltage was 36.3 V and the trap drive was 74.2 V. The flow of drying gas was set to 10 l/min, the nebulizer gas pressure was set to 70 psi, and the drying temperature was 350 °C. The accuracy of mass determinations was ± 0.05%. The primary structures of the novel peptides were determined by automated Edman degradation using a model 492 Procise sequenator (Applied Biosystems, Foster City, CA, USA).

2.4. Peptide synthesis

PGLa-LM1 and CPF-LM1 were supplied in crude form by GL Biochem. Ltd. (Shanghai, China) and were purified to near homogeneity (> 98% purity) by reversed-phase HPLC on a (2.2 cm x 25 cm) Vydac 218TP1022 (C-18) column. The concentration of acetonitrile in the eluting solvent was raised from 21% to 56% over 60 min and the flow rate was 6 mL/min. The structures of the peptides were confirmed by electrospray mass spectrometry.

2.5. Antimicrobial and hemolytic activities

Reference strains of microorganisms were purchased from the American Type Culture Collection (Rockville, MD, USA). Minimum inhibitory concentrations (MIC) of synthetic PGLa-LM1 and CPF-LM1 against reference strains of *S. aureus* (ATCC 25923), *E. coli* (ATCC 25726), and *Candida albicans* (ATCC 90028) were measured in the concentration range of 3 - 200 μM by standard microdilution methods (Clinical Laboratory Standards Institute, 2008a; 2008b) and were taken as the lowest concentration of peptide where no visible growth was observed. The values were confirmed by measurement of absorbance at 630 nm. In order to monitor the validity and reproducibility of the assays, incubations were carried out in parallel with increasing concentrations of antibiotics (ampicillin for *S. aureus*

and *E. coli*; and amphotericin for *C. albicans*) as previously described (Mechkarska et al., 2011a).

Hemolytic activity against human erythrocytes taken from a healthy donor was measured as previously described (Mechkarska et al., 2011a). The LC_{50} value was taken as the mean concentration of peptide producing 50% hemolysis in three independent incubations.

3. Results

3.1. *Purification of the peptides from X. laevis* x *X. muelleri hybrids*

The pooled skin secretions from the hybrid frogs, after partial purification on Sep-Pak C-18 cartridges, were chromatographed on a Vydac C-18 preparative reversed-phase HPLC column (Fig. 1).

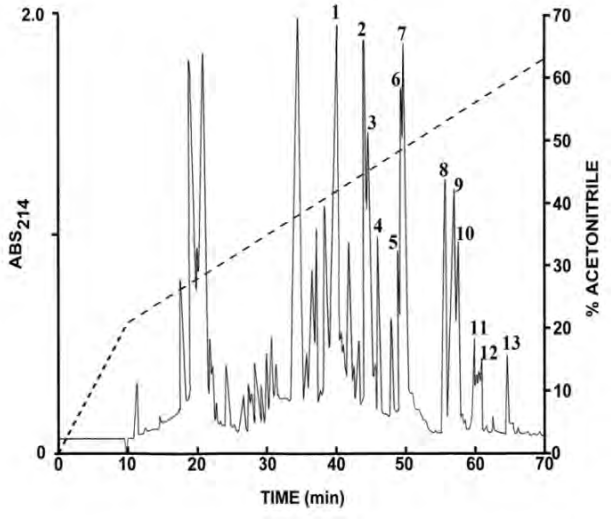

Fig. 1. Reversed-phase HPLC on a semipreparative Vydac C-18 column of skin secretions from F1 *X. laevis* x *X. muelleri* hybrids after partial purification on Sep-Pak cartridges. The peaks designated 1 - 13 displayed varying degrees of antimicrobial activity against *E. coli* and *S. aureus* and were purified further. The dashed line shows the concentration of acetonitrile in the eluting solvent.

The prominent peaks designated 1 - 13 were collected and the masses of the major components present in the peaks are shown in Table 1. Subsequent structural analysis revealed that peak 1 contained magainin-2, peak 2 magainin-1, peak 3 magainin-M1 + PGLa-LM1, peak 4 PGLa, peak 5 magainin-LM1, peak 6 XPF-1 + XPF-M1, peak 7 tigerinin-M1 + XPF-2, peak 8 CPF-M1, peak 9 CPF-4 + CPF-5, peak 10 CPF-3 + CPF-6, peak 11 CPF-1, peak 12 CPF-LM1, peak 13 CPF-7. Under the conditions of assay, material from peaks 1 - 11 inhibited to varying degrees the growth of *E. coli* and material from peaks 2, 3, and 8-10 inhibited growth of *S. aureus*. The peptides were purified to near homogeneity, as assessed by a symmetrical peak shape and mass spectrometry, by further chromatography on semipreparative Vydac C-4 and Vydac C-8 columns.

3.2. *Purification of the peptides from X. laevis*

The pooled skin secretions from *X. laevis*, after partial purification on Sep-Pak C-18 cartridges, were chromatographed on a Vydac C-18 preparative reversed-phase HPLC column using the same experimental conditions as for the skin secretions from the hybrid frogs (Fig. 2).

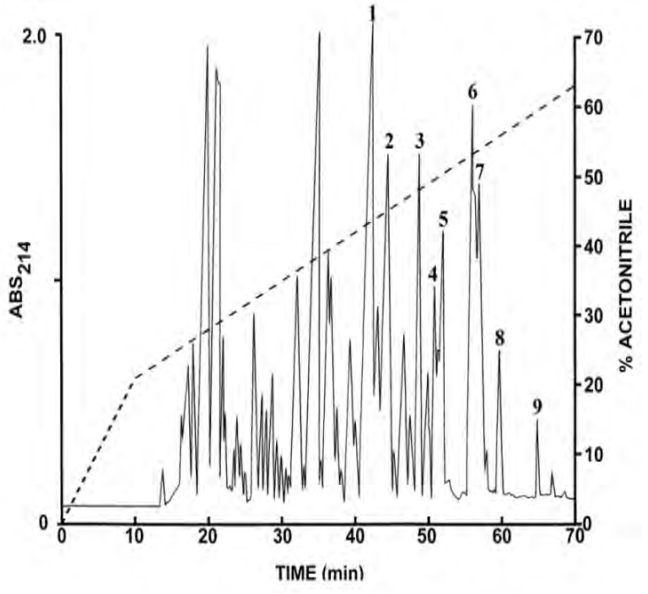

Fig. 2. Reversed-phase HPLC on a semipreparative Vydac C-18 column of skin secretions from *X. laevis* after partial purification on Sep-Pak cartridges. The peaks designated 1 - 9 displayed varying degrees of antimicrobial activity against *E. coli* and *S. aureus* and were purified further. The dashed line shows the concentration of acetonitrile in the eluting solvent.

The prominent peaks designated 1 - 9 were collected and subjected to further purification. Under the conditions of assay, material from all peaks inhibited the growth of *E. coli* at least partially and peaks 2, 3, and 6-9 inhibited the growth of *S. aureus*. The masses of the major components present in the peaks are shown in Table 1. Subsequent structural analysis demonstrated that peak 1 contained magainin-2, peak 2 magainin-1, peak 3 PGLa, peak 4 XPF-1, peak 5 XPF-2, peak 6 CPF-4 + CPF-5, peak 7 CPF-3 + CPF-6, peak 8 CPF-1, peak 9 CPF-7. The peptides were purified to near homogeneity, as assessed by a symmetrical peak shape and mass spectrometry, by further chromatography on semipreparative Vydac C-4 and Vydac C-8 columns.

Purification of antimicrobial peptides (magainin-M1, magainin-M2, XPF-M1, CPF-M1, CPF-M2, and tigerinin-M1) from *X. muelleri* has been described previously (Mechkarska et al., 2011a).

3.3. Structural characterization

The molecular masses of the purified peptides from *X. laevis*, *X. muelleri*, and the *X. laevis* x *X. muelleri* F1 hybrids are shown in Table 1.

Table 1. Molecular masses of the host-defense peptides isolated from skin secretions of *Xenopus laevis*, *Xenopus muelleri*, and *X. laevis* x *X. muelleri* F1 hybrids.

Peptide	*X. laevis*	*X. muelleri*	F1 hybrid
Magainin-1	2410.3		2410.3
Magainin-2	2467.3		2467.4
Magainin-M1		2424.9	2424.4
Magainin-M2		2682.3	
Magainin-LM1			2480.4
PGLa	1968.8		1969.0
PGLa-LM1			1948.0
CPF-1	2618.6		2617.5
CPF-3	2602.4		2602.6
CPF-4	2647.3		2647.4
CPF-5	2632.3		2632.3
CPF-6	2586.8		2586.5
CPF-7	2660.2		2659.6
CPF-M1		2894.5	2894.1
CPF-M2		2895.5	
CPF-LM1			2791.0
XPF-1	2610.6		2610.3
XPF-2	2664.6		2664.8
XPF-M1		2515.1	2514.5
Tigerinin-M1		1446.6	1446.7

The primary structures of the peptides from the F1 hybrids are shown in Fig. 3. This list includes both peptides that have been previously identified and characterized in *X. laevis* and *X. muelleri* and newly discovered peptides present in skin secretions of the hybrid frogs that are not present in skin secretions of the parent species. The data indicate that magainin-1, magainin-2, CPF-1, CPF-3, CPF-4, CPF-5, CPF-6, CPF-7, XPF-1, XPF-2,

and PGLa are present in skin secretions of both the hybrids and *X. laevis*. Magainin-M1, XPF-M1, CPF-M1, and tigerinin-M1 are present in skin secretions of both the hybrids and *X. muelleri*. Magainin-M2 and CPF-M2, present in skin secretions of *X. muelleri*, were not detected in secretions from the hybrids. Magainin-LM1, PGLa-LM1, and CPF-LM1 are present in the secretions of the hybrid frogs but were not detected in secretions from either *X. laevis* or *X. muelleri*.

		$[M_r+H]$obs	$[M_r+H]$calc
Magainin-1	GIGKFLHSAGKFGKAFVGEIMKS	2410.3	2410.9
Magainin-2	GIGKFLHSAKKFGKAFVGEIMNS	2467.4	2467.9
Magainin-M1	GIGKFLHSAGKFGKAFIGEIMKS	2424.4	2424.9
Magainin-LM1	GIGKFLHSAKKFAKAFVGEIMNS	2480.4	2480.3
PGLa	GMASKAGAIAGKIAKVALKAL.NH_2	1969.0	1969.5
PGLa-LM1	GMASKAGSVAGKIAKFALGAL.NH_2	1948.0	1948.4
CPF-1	GLASFLGKALKAGLKIGAHLLGGAPQQ	2617.5	2618.2
CPF-3	GFGSFLGKALKAALKIGANALGGSPQQ	2602.6	2603.1
CPF-4	GLASLLGKALKAGLKIGTHFLGGAPQQ	2647.4	2648.2
CPF-5	GFGSFLGKALKTALKIGANALGGSPQQ	2632.3	2633.1
CPF-6	GFASFLGKALKAALKIGANMLGGAPQQ	2659.6	2659.4
CPF-7	GFGSFLGKALKAALKIGANALGGAPQQ	2586.5	2585.5
CPF-M1	GLGSLLGKAFKFGLKTVGKMMAGAPREQ	2894.1	2894.5
CPF-LM1	GFGSFLGSLFKTGLKIIPKLLPSIQQ	2791.0	2789.6
XPF-1	GWASKIGQTLGKIAKVGLQGLMQPK	2610.3	2611.2
XPF-2	GWASKIGQTLGKIAKVGLKELIQPK	2664.8	2665.3
XPF-M1	GWASKIGQTLGKMAKVGLKDLIQA	2514.5	2515.0
Tigerinin-M1	WCPPMIPLCSRF.NH_2	1446.7	1446.7

Fig. 3. Amino acid sequences, observed average molecular masses (M_r obs), and calculated average molecular masses (M_r calc) of the antimicrobial peptides isolated from skin secretions of the *X. laevis* x *X. muelleri* hybrids.

3.4. Antimicrobial activity

The MIC values for synthetic PGLa-LM1 and CPF-LM1 against reference strains of the Gram-positive bacterium *S. aureus*, the Gram-negative bacterium *E. coli*, and the opportunistic yeast pathogen *C. albicans*, together with their hemolytic activities against human erythrocytes, are shown in Table 2.

Table 2. Minimum inhibitory concentrations (μM) against microorganisms and hemolytic activity against human erythrocytes (μM) of synthetic replicates of PGLa-LM1 and CPF-LM1 from *X. laevis* x *X. muelleri* F1 hybrids.

	E. coli	***S. aureus***	***C. albicans***	**HC_{50}**
PGLa-LM1	80	80	160	>500
CPF-LM1	20	5	40	30

4. Discussion

This study has described the purification of 18 peptides with varying degree of antimicrobial activity in norepinephrine-stimulated skin secretions from female *X. laevis* x *X. muelleri* F1 hybrids. Characterization of the peptides by mass spectrometry and Edman degradation reveals that they represent orthologs of the previously described host-defense peptides magainin, PGLa, CPF, and XPF, first identified in *X. laevis* (Gibson et al., 1986; Zasloff, 1987) and tigerinin, first identified in the Asian frog *H. tigerinus* (Sai et al., 2001) and subsequently in *X. muelleri* (Mechkarska et al., 2011a). The data indicate that the hybrid is expressing genes derived from both parent species so that the multiplicity of the antimicrobial peptides in the skin secretions of the hybrid is greater than in either parent species. As shown in Table 1, all the antimicrobial peptides identified in the *X. laevis* secretions are also present in the secretions of the hybrid. Four out of six peptides identified in the skin secretions of *X. muelleri* were found in the secretions of the hybrid but two peptides (magainin-M2 and CPF-M2) were not detected. In addition, three previously undescribed peptides (magainin-LM1, PGLa-LM1, and CPF-LM1) were purified from the secretions of the hybrid frogs that were not detected in secretions from either *X. laevis* or *X. muelleri* (Fig. 4).

Magainin

Hybrid-LM1	GIGKFLHSAKKFAKAFVGEIMNS
X. laevis-1	GIGKFLHSAGKFGKAFVGEIMKS
X. laevis-2	GIGKFLHSAKKFGKAFVGEIMNS
X. muelleri-M1	GIGKFLHSAGKFGKAFIGEIMKS
X. muelleri-M2	GFKQFVHSLGKFGKAFVGEMIKPK

PGLa

Hybrid-LM1	GMASKAGSVAGKIAKFALGAL.NH_2
X. laevis	GMASKAGAIAGKIAKVALKAL.NH_2

CPF

Hybrid-LM1	GFGSFLGSLFKTGLKI*IPKLL*PSIQQ
X. laevis-1	GLASFLGKALKAGLKI*GAHLLGGAPQQ
X. laevis-2	GFASFLGKALKAALKI*GANMLGGTPQQ
X. laevis-3	GFGSFLGKALKAALKI*GANALGGSPQQ
X. laevis-4	GLASL LGKALKAGLKI*GTHFLGGAPQQ
X. laevis-5	GFGSFLGKALKTALKI*GANALGGSPQQ
X. laevis-6	GFASFLGKALKAALKI*GANMLGGAPQQ
X. laevis-7	GFGSFLGKALKAALKI*GANALGGAPQQ
X. muelleri-M1	GLGSLLGKAFKFGLKTVGKMMAGAPREQ
X. muelleri-M2	GLGSLLGKAFKFGLKTVGKMMAGAPREE

Fig. 4. A comparison of the primary structures of the novel peptides magainin-LM1, PGLa-LM1, and CPF-LM1 identified in skin secretions of the hybrid with orthologous peptides from *X. laevis* and *X. muelleri*. The shaded residues indicate amino acid differences. Residue deletions (denoted by *) have been introduced into some sequences to maximize structural similarity.

The data complement, and are consistent with, the results of an earlier study which investigated the distribution of host-defense peptides in the skin secretions of F1 hybrid treefrogs produced in captivity from female *Litoria splendida* and male *Litoria caerulea* (Pukala et al., 2006). The skin secretions contained peptides common to only one parent together with peptides synthesized by both parental species and four peptides (caerins 1.10, 2.6, 2.7, and 5.1) found only in secretions of the hybrid but not in either parental species. The authors speculated that the appearance of these novel peptides is a result of "some form of altered regulation of expression in the hybrid genetic background". Firm support for this assertion is provided by a comprehensive analysis using microarray technology of gene expression in the ovary of fertile F1 female hybrids produced by crossing maternal *X. laevis* with paternal *X. muelleri* (Malone and Michalak, 2008). The hybrids showed a differential pattern of gene expression with 839 of genes upregulated in the hybrids relative to *X. laevis* and 777 genes upregulated in *X. leavis* relative to the hybrids. Similarly, a total of 2930 genes were upregulated in the hybrids relative to *X. muelleri* and 4349 genes upregulated in *X. muelleri* relative to hybrids. In a related study of sterile F1 hybrid males of *X. laevis* and *X. muelleri*, a pattern of differential gene expression was also observed and it was found that approximately 1.2% of genes inherited from *X. laevis* were misexpressed in the hybrids whereas approximately 35% genes inherited from *X. muelleri* were misexpressed (Malone et al., 2007). It was suggested, therefore, that the *X. muelleri* component of the hybrid genome was preferentially silenced.

Three novel peptides (magainin-LM1, PGLa-LM1 and CPF-LM1) were identified in the secretions of the hybrids. Their origins are a matter for speculation. As shown in Fig. 4, magainin-LM1 shows only one amino acid substitution ($Gly^{13} \rightarrow Ala$) compared with magainin-2 from *X. laevis*. Magainin-1 and magainin-2 are encoded by the same gene in *X. laevis* (Zasloff, 1987) but the deduced precursor sequence contains five copies of the magainin-2 sequence and one copy of the magainin-1 sequence (Terry et al., 1988). Both peptides are present in secretions of the hybrid and *X. laevis* with magainin-2 in higher concentration as expected (Figs. 1 and 2). It is probable, therefore, that magainin-LM1 arises from the expression of a duplicate magainin gene derived from *X. laevis* that has been subject to mutation in the domain encoding magainin-2. Magainin-M1, but not magainin-M2, was detected in secretions of the hybrid. Magainin-M2 is present in much lower concentration (approximately 9-fold) than magainin-M1 in *X. muelleri* secretions (Mechkarska et al., 2011a) so that it is possible that its presence in the secretions of the hybrid was missed.

PGLa was not detected in skin secretions of *X. muelleri* (Mechkarska et al., 2011a). However, if the gene is present but expressed at a very low level, PGLa-LM1in the hybrid may have arisen from upregulation of the PGLa-M1 gene inherited from *X. muelleri*. However, PGLa was detected along with PGLa-LM1 in the hybrid secretions so that the alternative explanation that PGLa-LM1 results from the expression of a mutated duplicated

PGLa gene inherited from *X. laevis* is another possibility. The origin of CPF-LM1 is unclear. The peptide shows a greater structural similarity to the CPF peptides identified in the *X. laevis* secretions compared with CPF-M1 and CPF-M2 from *X. muelleri* (Fig. 4). However, the degree of sequence identity is low (12 amino acid substitutions and one amino acid deletion compared with CPF-5, the peptide from *X. laevis* with the closest resemblance). All six CPF peptides present in the *X. laevis* secretions (CPF-1, -3, -4, -5, -6, and -7) are found in the secretions of the hybrids suggesting that CPF-LM1 may have arisen from the expression of a mutated duplicate CPF gene inherited from *X. laevis.* The amino acid sequence of CPF-2 is predicted from the nucleotide sequence of a cDNA (Wakabayashi et al., 1985; Richter et al., 1986) but the peptide was not detected in secretions from either *X. laevis* or the hybrids. The proposed origin of the host-defense peptides in the hybrid secretions is represented schematically in Fig. 5.

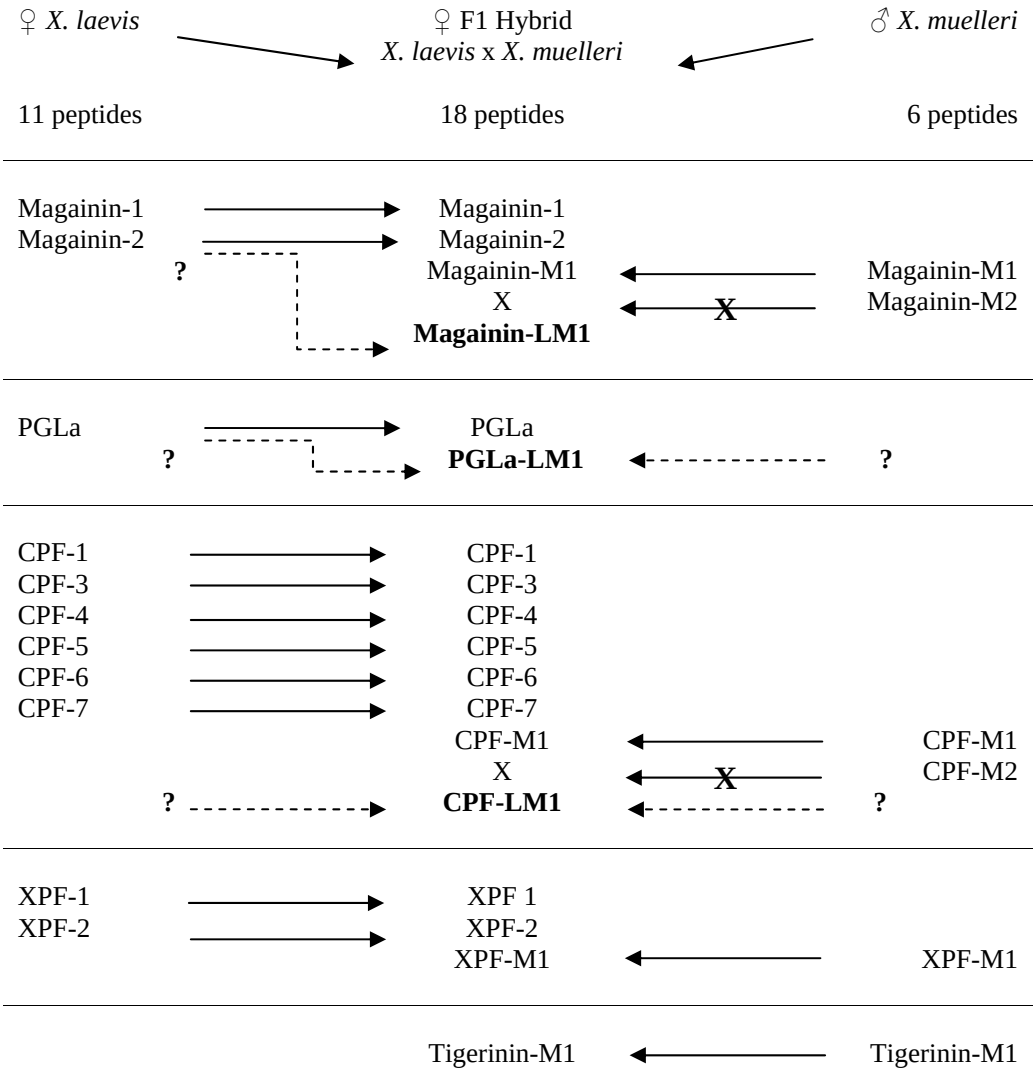

Fig. 5. Proposed origin of the host-defense peptides present in skin secretions of female F1 *X. laevis* x *X. muelleri* hybrids. Magainin-M2 and CPF-M2 from *X. muelleri* were not detected in the hybrids as indicated by X. Three novel peptides (magainin-LM1, PGLa-LM1, and CPF-LM1), indicated by bold type, are found in the hybrid skin secretions only. The dashed line indicates that it is unclear whether the gene encoding the peptide is inherited from *X. laevis* or *X. muelleri*.

It should be pointed out that the *X. muelleri* male parents of the hybrids originated from the Nkambeni area of Swaziland whereas the *X. muelleri* individuals studied by Mechkarska et al. (2011a) were from South Malawi. It is possible that the two populations are synthesizing different molecular variants so that the three novel peptides identified in the secretions of the hybrids could be derived from the male parent. This is unlikely, however, as it has been shown that skin secretion from populations of *X. laevis* from widely separated regions of Africa contain antimicrobial peptides with the same primary structures (M. Mechkarska and J.M. Conlon, unpublished data).

The study permits a comparison of the effects on expression of antimicrobial peptide genes of inter-species hybridization in the laboratory with ancient allopolyploidization events in the lineage. Previous investigations analysing the multiplicity of antimicrobial peptides in the skin secretions of tetraploid and octoploid *Xenopus* species (Mechkarska et al., 2011b; King et al., 2012) have shown that the numbers of paralogs from the octoploid frogs were not significantly greater than the corresponding numbers from the tetraploid frogs. It was concluded, therefore, that nonfunctionalization (gene silencing) (Lynch and Katju, 2004) has been the most common fate of duplicated antimicrobial peptide genes following the putative allopolyploidization events in the *Xenopus* lineage (Conlon et al., 2012). In contrast, the full complement of antimicrobial peptide genes from *X. laevis* are expressed in the F1 hybrids as well as four out of six antimicrobial peptide genes from *X. muelleri.* These hybrids, like the parent species, are tetraploid (2n = 36) (Koroma et al., 2011). Additionally, hybridization has resulted in expression of three novel genes encoding peptides not detected in skin secretions of the parent species. One such peptide, CPF-LM1 shows potent, broad spectrum antimicrobial activity. One may speculate, therefore, that the hybrid, with a greater complement of host-defense peptides, is better protected against invasion by pathogenic microorganisms in the environment than the parent species but firm evidence to support this claim is required.

During the process of interspecies hybridization, even without polyploidization, epigenetic changes that control gene expression often take place in addition to genome reconfigurations. There is an increasing body of evidence coming from studies ranging across biological systems from yeast and plants to humans that demonstrate that epigenetic changes induced by hybridization or environmental stress may be inherited by future generations and therefore may contribute to adaptation to novel environments [reviewed in (Whitelaw and Whitelaw, 2006)]. All of the inheritable epigenetic effects, such as DNA methylation, histone modification, microRNA, small interfering RNA and spatial location of DNA, have been shown to trigger changes in gene expression and ultimately phenotype in the next generation, without variation in genotype (Finnegan, 2002; Jablonka and Raz, 2009). Cytosine methylation is one of the most important epigenetic mechanisms operating in plant and vertebrate genomes and analysis of methylated fragments of *X. laevis* x *X. muelleri* F1 hybrids (Koroma et al., 2011) revealed four distinct methylation patterns

clusters with the two parental species *X. laevis* and *X. muelleri* separate from hybrid males and females. The hybrids were characterized by a significantly higher proportion (70.6%) of methylated fragments compared with the parental species (64.5%), and this difference is consistent with previously observed disruptions of hybrid transcriptomes. Hybrid-specific methylated fragments (20.9% of the markers analyzed) were identified indicative of novel methylation states due to interspecies hybridization. It remains to be determined whether the novel peptides identified in the hybrids in this study arise from stably inherited epigenetic effects. Since the female hybrids are fertile, it is important to ascertain whether the offspring from the mating of the hybrids with either of the parental species will inherit the increased complement of host-defense peptides.

Acknowledgments

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Chapter 8

General discussion

Milena Mechkarska

Part of the discussion is published in the review: Host-defense peptides in skin secretions of African clawed frogs (Xenopodinae, Pipidae). J.M. Conlon, M. Mechkarska, J.D. King. Gen Comp Endocrinol (2012a) 176:513-518.

The aim of the work described in this thesis is the isolation of AMPs with therapeutic potential against multidrug-resistant pathogens from skin secretions of frogs belonging to the Pipidae family. At the beginning of 2010 only three species belonging to the subfamily Xenopodinae had been analyzed for the presence of AMPs - the diploid frog *S. tropicalis* (Ali et al., 2001), the tetraploid frog *X. laevis* (Zasloff, 1987; Giovannini et al., 1987) and the octoploid *X. amieti* (Conlon et al., 2010a). During the course of this research more than 50 AMPs were purified from the skin secretions of an additional seven members of the family Pipidae: *X. borealis* (Chapter **2**), *X. clivii* (Chapter **3**), *X. andrei* in parallel with "*S.* new tetraploid 1" (Chapter **4**), *X. muelleri* together with the incompletely characterized *X. muelleri* West (Chapter **5**), and *H. boettgeri* (Chapter **6**). In addition, an attempt was made to understand the mode of inheritance of AMP genes by studying the AMP profile of F1 hybrid frogs *X. laevis* x *X. muelleri* and comparing it to those of the parents (Chapter **7**).

The importance of the AMPs isolated from Pipidae frogs is discussed from the following viewpoints:

- Development of novel therapeutically active compounds against multidrug-resistant pathogens.
- Clarification of taxonomic relationships between frog species and study of their evolutionary history.
- Analysis of the effect of polyploidization and interspecies hybridization on the fate and mode of inheritance of duplicated AMP genes.

Therapeutic potential of AMPs from frogs of the Pipidae family

In common with the vast majority of frog skin AMPs, all the newly identified AMPs from skin secretions of frogs of the Pipidae family (Chapters **2**-**6**) belong to the class of cationic α-helical peptides (Chapter **1**).

Seven peptides have been identified that show therapeutic potential for development into anti-infective agents: CPF-B1, CPF-C1, CPF-MW1 and CPF-SP1 (from Xenopodinae) and the hymenochirins-1B, -2B and -3B (from Pipinae). Their primary structures are shown in Table 1 and their antimicrobial and hemolytic activities are compared in Table 2.

The CPF peptides are potent against the clinically relevant microorganisms *E. coli*, *S. aureus*, *C. albicans*, *K. pneumonia* and *P. aeruginosa* with minimum inhibitory concentration (MIC) $\leq$ 25 μM but are only moderately cytotoxic against human erythrocytes. In addition, the CPF peptides are very active against MRSA and MDRAB with MIC $\leq$ 12.5 μM (Chapters **1**, **2** and **4**). Although the hymenochirins display only moderate antimicrobial and antifungal activity, they have the advantage of very low hemolytic activity - mean concentration of peptide producing 50% hemolysis (HC_{50}) $\geq$ 225 μM.

Table 1. Naturally-occuring AMPs from skin secretions of Pipidae frogs with potential for development into potent non-toxic, anti-infective agents for use against drug resistant microorganisms.

Frog species	AMP	Primary structure
"*S.* new tetraploid 1"	CPF-SP1	GFLGPLLKLGLKGVAKVLPHLIPSRQQ
X. borealis	CPF-B1	GLGSLLGKAFKIGLKTVGKMMGGAPREQ
X. clivii	CPF-C1	GFGSLLGKALRLGANVL.NH_2
X. muelleri West	CPF-MW1	GLGSLLGKAFKFGLKTVGKMMGGAPREQ
H. boettgeri	Hymenochirin-1B	IKLSPETKDNLKKVLKGAIKGAIAVAKMV.NH_2
	Hymenochirin-2B	LKIPGFVKDTLKKVAKGIFSAVAGAMTPS
	Hymenochirin-3B	IKIPAVVKDTLKKVAKGVLSAVAGALTQ

Table 2. MIC against microorganisms and hemolytic activity against human erythrocytes (HC_{50}) of the peptides isolated from skin secretions of Pipidae frogs.

	E. coli	***S.aureus***	***C. albicans***	***K. pneumoniae***	***P. aeruginosa***	**HC_{50}**
CPF-SP1	25	6	25	25	6	>100
CPF-B1	5	5	25	-	-	>200
CPF-C1	6	6	-	25	25	140
CPF-MW1	3	6	25	-	-	70
Hymenochirin-1B	20	10	80	20	40	225
Hymenochirin-2B	20	40	80	20	20	>300
Hymenochirin-3B	20	40	80	40	40	>300

Data are expressed in μM.

Because the potency of the AMPs is determined by four different parameters that are strongly interrelated (Chapter **1**) (Powers and Hancock, 2003), it is difficult to predict the antimicrobial activity, selectivity, and mode of action of a given peptide from its amino acid sequence or physicochemical properties. Comparison of the primary sequences of the CPFs and the hymenochirins reveals that there is no conserved amino acid sequence associated with antimicrobial activity (Table 1). In addition, there is no obvious common

set of physicochemical parameters which determine the activity of a given peptide (Table 3).

Table 3. Physicochemical properties of the CPFs and the hymenochirins, isolated from skin secretions of Pipidae frogs.

Peptide	Hydrophobicity	Charge at pH 7	pI	Alpha-helical domain
CPF-SP1	+10.5	+4	11.73	5-11
CPF-B1	-1.1	+4	10.98	2-12, 14-17
CPF-C1	+15.3	+2	11.49	5-14
CPF-MW1	-0.6	+4	10.98	2-12, 14-17
Hymenochirin-1B	+4.9	+6	10.65	5-27
Hymenochirin-2B	+13.5	+4	10.71	8-16
Hymenochirin-3B	+19.5	+4	10.71	9-16

There is no simple correlation between peptide charge and membrane activity. Thus, the activity of CPF-C1 with a +2 charge is similar to that exerted by peptides with a charge of +4. Similarly, the activity of hymenochirin-1B with a charge of +6 is similar to that observed for hymenochirin-2B and -3B with a charge of +4. Another strong driving force for the binding of peptides to membranes is the formation of α-helical structures (Wieprecht et al., 1999). This feature is essential for the activity against neutral (eukaryotic) membranes, but seems less important for the action towards negatively charged (prokaryotic) ones. In terms of effect on microorganisms, helicity is an absolute requirement for the antimicrobial activity towards Gram-positive bacteria, while the situation regarding the Gram-negative bacteria is less stringent (Dathe et al., 1997; Giangaspero et al., 2001). Analysis of the secondary structure of the peptides included in Table 3 using the AGADIR program (Munoz and Serrano, 1994) indicate that all can adopt an α-helical conformation over at least part of their structures. A Schiffer-Edmundson wheel representation (Schiffer and Edmundson, 1967) of the structures illustrates the amphipathic character of the helices (Fig. 1).

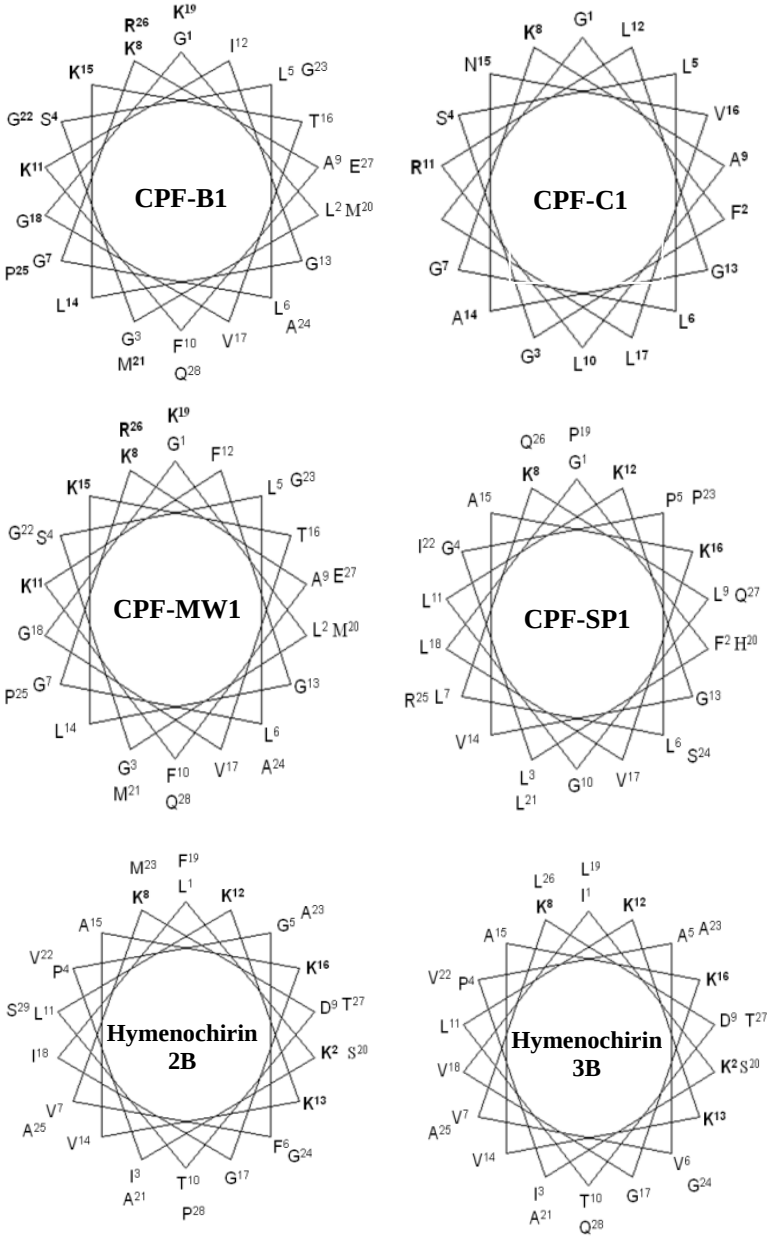

Fig. 1. Schiffer-Edmundson wheel representation of the most active CPFs and hymenochirins demonstrating the amphipathic nature of the predicted α-helical conformation. The polar Lys, Glu, Asp and Arg segregate on one face of the helix with the hydrophobic Leu, Ile, Pro and Val residues on the opposite face. The basic residues in the peptides, essential for potent antimicrobial activity, are shown in bold type.

The wheel representation of the amphipathic structure of hymenochirin-1B is shown in Chapter **6**. Therefore, it is difficult to explain why the hymenochirins have reduced activity towards *S. aureus* or are less hemolytic when compared to the CPF peptides.

Hydrophobicity correlates with the higher affinity of peptides for zwitterionic lipids (Wieprecht et al., 1997a) but plays a secondary role in their interaction with negatively charged membranes (Kiyota et al., 1996; Wieprecht et al., 1997b; Kwon et al., 1998). An increase in hydrophobicity is often followed by an increase of the hemolytic potency (Javadpour et al., 1996; Skerlavaj et al., 1996; Dathe et al., 1997). The findings presented in this thesis are not fully consistent with this rule. For example, the hymenochirins are the most hydrophobic but are the least hemolytic of the peptides in Tables 2 and 3.

Possible clinical applications of α-helical AMPs isolated from skin secretions of different frog species have been recently reviewed (Conlon and Sonnevend, 2011). The following peptides, by exerting activity against multidrug-resistant Gram-positive and Gram-negative bacteria, as well as the opportunistic yeast pathogen *C. albicans*, show therapeutic potential as anti-infective agents: alyteserin-1c (from *Alytes obstetricans*); ascaphin-8 (*Aschapus truei*); brevinin-1BYa (*Rana boylii*); brevinin-2PRa (*Rana pirica*); brevinin-2-related peptide (B2RP) (*Lithobates septentrionalis*); B2RP-ERa (*Hylarana erythraea*); kassinatuerin-1 (*Kassina senegalensis*); pseudin-2 (*Pseudis paradoxa*); and temporin-DRa (*Rana draytonii*).

Further support for the CPF peptides as candidates for development into therapeutic agents comes from other work carried out in the laboratory on AMPs from *X. amieti* (Conlon et al., 2010a), *X. pygmaeus*, *X. petersii* and *X. lenduensis* (King et al., 2012), and *S. epitropicalis* (Conlon et al., 2012b). Thus, CPF-AM1 shows broad spectrum bactericidal activity with MIC values ≤ 25 μM against reference strains of *E. coli* and *S. aureus* and against multiple clinical isolates of antibiotic-resistant *A. baumannii*, including strains that are resistant to colistin (Conlon et al., 2012c), and *Stenotrophomonas maltophilia* (unpublished data). Two peptides - CPF-SE2 and CPF-SE3 - from *S. epitropicalis* show potent growth-inhibitory activity (MIC = 2.5 - 5μM) against a range of clinical isolates of MRSA but are hemolytic (Conlon et al., 2012b). Similarly, a synthetic replicate of CPF-PG1 from *X. pygmaeus* has relatively high potency against the Gram-positive bacterium *S. aureus* (MIC = 6 μM) and shows moderate hemolytic activity (HC_{50} = 145 μM) (King et al., 2012). More members of the CPF family are described in this thesis: CPF-B2, CPF-B3 and CPF-B4 (from *X. borealis*), CPF-AN1 (from *X. andrei*) and CPF-MW3 (from *X. muelleri* West). However, their antimicrobial potential was not investigated as these peptides were isolated from skin secretions in very low yields.

The use of peptide-based anti-infective agents is limited by their toxicities to host cells such as erythrocytes. If the therapeutic index (ratio of MIC against microorganisms to HC_{50} for hemolysis of human erythrocytes) of the promising peptides is found to be too low for

systemic use, alternative routes of application may still be feasible. For example, skin infections in otherwise healthy young individuals are a common clinical manifestation of community-acquired MRSA infections (Skov et al., 2012). MRSA is a serious problem in infections of the superficial epidermis, such as impetigo in infants and children (Geria and Schwartz, 2010) and in colonization of surface lesions such as the foot ulcers of diabetic patients (Bowling et al., 2009). Consequently, the peptides may find application as topical treatment options in combating skin infections and in therapeutic regimes to promote wound healing.

AMPs have attracted interest as therapeutic agents for treatment of acne vulgaris (Melo et al., 2006; Popovic et al., 2012). Acne vulgaris is a disease affecting the pilosebaceous unit (PSU), with both bacterial and inflammatory components (Bhambri et al., 2009; Grange et al., 2010). A major role in the development of an acne lesion is proliferation by *Propionibacterium acnes* within the PSU (Bojar and Holland, 2004; Kurokawa et al., 2009). The inflammatory immune response of the host to *P. acnes* is important in the pathogenesis of acne and contributes to the complications during the course of the disease (Mouser et al., 2003; Sugisaki et al., 2009). Frog skin peptides show potential for development into a topical agent because they possess the desired dual action: directly inhibit growth of *P. acnes* and suppress the inflammatory immune response (Popovic et al., 2012).

Natural resistance to AMPs has been demonstrated *in vitro* (Banemann et al., 1998; Bishop and Finlay, 2006). Development of induced resistance will also occur (Groisman et al., 1992; Nikaido, 1996; Otto, 2006; Llobet et al., 2008) but at rates that are orders of magnitude lower than those observed for conventional antibiotics (Ge et al., 1999; Perron et al., 2006). However, sub-toxic AMP concentrations which do not damage the cell membranes can stimulate bacterial populations to switch to a metabolically dormant state associated with biofilm formation (Berditsch et al., 2012). This mechanism can lead to bacterial persistence responsible for chronic diseases (Lewis, 2010; Berditsch et al., 2012). Moreover, Habets and Brockhurst (2012) reported that evolved resistance to pexiganan generated experimentally provides *S. aureus* with cross-resistance to human-neutrophil-defensin-1 which plays a key role in the innate immune response to infections.

Taxonomy and evolutionary history of Pipidae frogs

Back in the 19th and early 20th centuries, the recognition of taxa, such as genera, species and subspecies and establishing the phylogeny of animal species was based entirely upon morphological characteristics and the fossil record (Gould, 1977; Hennig, 1979). In the 1950s the definition of taxa was facilitated by including behavioural characteristics such as advertisement calls and other attributes. Following the development of biochemical and

molecular biology techniques, the study of animal evolution has increasingly relied upon comparison of molecular features (Guttman, 1973; Graur and Li, 2000; Carroll et al., 2001).

There is an increasing body of evidence that AMPs present in frog skin secretions possess the necessary features to be considered as a reliable marker for anuran taxonomy and for study of evolutionary history of the different frog families. AMPs are genetically encoded and evolve rapidly via positive selection, explaining the extraordinary diversity of the peptide families (Duda et al., 2002; Tennessen, 2005). On the basis of limited similarities in their amino acid sequences, frog skin AMPs may be grouped together in families that share a common evolutionary origin (Goraya et al., 2000; Duda et al., 2002). Skin secretions from a single species frequently contain several members of a particular peptide family (Tennessen and Blouin, 2007). Since there is virtually no overlap in the sequences of individual peptides from one species to another (Conlon et al., 2004), the dermal peptides of frogs are valuable in taxonomic evaluations of closely related genera and species (Conlon et al., 2004; Apponyi et al., 2004; Zheng et al., 2005).

On the basis of morphological characters alone, it was concluded that *Silurana* is more closely related to the genera *Hymenochirus*, *Pipa*, and *Pseudhymenochirus* than to *Xenopus* (Cannatella and Trueb, 1988). However, subsequent analyses based upon comparisons of the nucleotide sequence of mitochondrial genes provided support for divergence of the Xenopodinae (*Silurana* + *Xenopus*) from the Pipinae (*Hymenochirus* + *Pipa* + *Pseudhymenochirus*) before the divergence of the Pipinae subfamily (de Sa and Hillis, 1990; Evans et al., 2004; Evans, 2008). Collectively, the amino acid sequences of AMPs isolated from Xenopodinae and Pipinae provide support for the latter view. The AMPs isolated to-date from skin secretions of species belonging to Xenopodinae (Chapters **2-5**; Conlon et al., 2010a; Conlon et al, 2012b; King et al., 2012) are grouped in the same four peptide families - magainin, PGLa, CPF, and XPF - initially identified in skin secretions from *X. laevis* (Figs. 2-4). This finding is not unexpected as the genomic DNA sequences of *S. tropicalis* and *X. laevis* are 90% identical within coding regions and roughly half of the duplicated genes are maintained in *X. laevis* after the putative tetraploidization took place (Yanai et al., 2011). The hymenochirins isolated from skin secretions of *H. boettgeri* show very low structural similarity to the peptides from Xenopodinae consistent with an earlier divergence of the Pipinae from the Xenopodinae (Chapter **6**).

Magainin-related peptides

S. tropicalis ST1	GLKEVAHSAKKFAKGFISGLTGS
S. epitropicalis SE1	GLKEVLHSTKKFAKGFITGLTGQ
X. laevis 1	GIGKFLHSAGKFGKAFVGEIMKS
X. laevis 2	GIGKFLHSAKKFGKAFVGEIMNS
X. petersii P1	GIGKFLHSAGKFGKAFVGEIMKS
X. petersii P2	GIGQFLHSAKKFGKAFVGEIMKS
X. borealis B1	G**KFLHSAGKFGKAFLGEVMIG
X. borealis B2	GIGKFLHSAGKFGKAFLGEVMKS
X. muelleri M1	GIGKFLHSAGKFGKAFIGEIMKS
X. muelleri M2	GFKQFVHSLGKFGKAFVGEMIKPK
X. muelleri West MW1	GIGKFLHSAGKFGKAFLGEVMKS
X. laevis x *X. muelleri* LM1	GIGKFLHSAKKFAKAFVGEIMNS
X. clivii C1	GVGKFLHSAKKFGQALASEIMKS
X. clivii C2	GVGKFLHSAKKFGQALVSEIMKS
X. pygmaeus PG1	GVGKFLHAAGKFGKALMGEMMKS
X. pygmaeus PG2	GVSQFLHSASKFGKALMGEIMKS
X. lenduensis L1	GIGKFLHSAKKFGKAFVGEVMKS
X. lenduensis L2	GISQFLHSAKKFGKAFAGEIMKS
X. amieti AM1	GIKEFAHSLGKFGKAFVGGILNQ
X. amieti AM2	GVSKILHSAGKFGKAFLGEIMKS
X. andrei AN1	GIKEFAHSLGKFGKAFVGGILNQ
X. andrei AN2	GVSKILHSAGKFGKAFLGEIMKS

PGLa-related peptides

S. tropicalis ST1	GMATKAGTALGKVAKAVIGAAL[a]
"S. new tetraploid 1" SP1	GMATKAGTALGKVAKAVIGAAL[a]
S. epitropicalis SE1	GMATKAGTALGKVAKAVIGAAL[a]
S. epitropicalis SE2	GMATAAGTTLGKLAKFVI*GAV[a]
X. laevis 1	GMASKAGAIAGKIAKVALK*AL[a]
X. petersii P1	GMASTAGSIAGKIAKVALK*AL[a]
X. borealis B1	GMASKAGTIAGKIAKTAIKLAL[a]
X. borealis B2	GMASKAGSIVGKIAKIAL*GAL[a]
X. muelleri West MW1	GMASKAGSVLGKITKIAL*GAL[a]
X. muelleri West MW2	GMASKAGAIAGKIAKTAIKLAL[a]
X. laevis x *X. muelleri* LM1	GMASKAGSVAGKIAKFAL*GAL[a]
X. pygmaeus PG1	GMASKAGTIVGKIAKVAL*NAL[a]
X. lenduensis L1	GMASTAGSIFGKLAKTAL*GAL[a]
X. lenduensis L2	GMASTAGSVLGKLAKVAIKAAL[a]
X. lenduensis L3	GMASTAGSVLGKLAKVAL*GAL[a]
X. lenduensis L4	GMASTVGSIFGKLAKTAL*GAL[a]
X. amieti AM1	GMASKAGSVLGKVAKVALKAAL[a]
X. amieti AM2	GMASTAGSVLGKLAKAVAIGAL[a]
X. andrei AN1	GMASKAGSVLGKVAKVALKAAL[a]
X. andrei AN2	GMASKAGSVLGKLAKVAI*GAL[a]

Fig. 2. A comparison of the primary structures of magainin and PGLa-related peptides from species belonging to the taxon Xenopodinae. Amino acid residue deletions denoted by * have been introduced in to some sequences to maximize structural similarity. [a] denotes C-terminal α-amidation. Conservation of amino acid residues is emphasized by the shading.

CPF-related peptides

S. tropicalis ST1	GFLGPLLKLAAKGVAKVIPHLI**PSRQQ
"S. new tetraploid 1" SP1	GFLGPLLKLGLKGVAKVLPHLI**PSRQQ
"S. new tetraploid 1" SP2	GFLGPLLKLGLKGAAKLLPQLL**PSRQQ
S. epitropicalis SE1	GFLGPLLKLGLKGVAKVIPHLI**PSRQQ
S. epitropicalis SE2	GFLGPLLKLGLKGAAKLLPQLL**PSRQQ
X. laevis 1	GLASFLGKALKAGL*KIGAHLLGGAP*QQ
X. laevis 2	GFASFLGKALKAAL*KIGANMLGGTP*QQ
X. laevis 3	GFGSFLGKALKAAL*KIGANALGGSP*QQ
X. laevis 4	GLASLLGKALKAGL*KIGTHFLGGAP*QQ
X. laevis 5	GFGSFLGKALKTAL*KIGANALGGSP*QQ
X. laevis 6	GFASFLGKALKAAL*KIGANMLGGAP*QQ
X. laevis 7	GFGSFLGKALKAAL*KIGANALGGAP*QQ
X. petersii P1	GFGSFLGKALKAAL*KIGANALGGAP*QQ
X. petersii P2	GLASFLGKALKAGL*KIGSHLLGGAP*QQ
X. petersii P3	GFGSFLGKALKAAL*KIGANVLGGAP*QQ
X. petersii P4	GFGSFLGKALKAAL*KIGANVLGGAP*EQ
X. petersii P5	GFGSFLGKALKAAL*KIGADVLGGAP*QQ
X. borealis B1	GLGSLLGKAFKIGL*KTVGKMMGGAPREQ
X. borealis B2	GLGSLLGSLFKFIP*K****LL**PSIQQ
X. muelleri M1	GLGSLLGKAFKFGL*KTVGKMMAGAPREQ
X. muelleri M2	GLGSLLGKAFKFGL*KTVGKMMAGAPREE
X. muelleri West MW1	GLGSLLGKAFKFGL*KTVGKMMGGAPREQ
X. muelleri West MW2	GLGSLLGKAFKFGL*KTVGKMMGGAPREE
X. laevis x X. muelleri LM1	GFGSFLGSLFKTGL*KIIPKLL**PSIQQ
X. clivii C2	GLGSLLGKALKFGL*KAAGKFMGGEP*QQ
X. pygmaeus PG2	GFGSFLGKALKAGL*KLGANLLGGAP*QQ
X. pygmaeus PG3	GFGSLLGKALKAGL*KLGANLLGGAP*QQ
S. tropicalis ST2	GFLGSLLKTGLKV****GSNLL[a]
S. tropicalis ST3	GLLGPLLKIAAKV****GSNLL[a]
"S. new tetraploid 1" SP3	GFLGSLLKTGLKV****GSNLL[a]
S. epitropicalis SE3	GFLGSLLKTGLKV****GSNLL[a]
X. clivii C1	GFGSLLGK*ALRL****GANVL[a]
X. lenduensis L1	GIGSLLAK*AAKL****GANLL[a]
X. lenduensis L2	GIGSALAK*AAKL****VAGIV$_a$
X. lenduensis L3	GLGTFLGN*ALKTGLKIGANLL$_a$
X. amieti AM1	GLGSVLGK*ALKI****GANLL[a]
X. amieti AM2	GIGSALAK*AAKL****VAGIV[a]
X. amieti AM4	GLGSLVGN*ALRI****GAKLL[a]
X. andrei AN1	GFASVLGK*ALKL****GANLL[a]

Fig. 3. A comparison of the primary structures of CPF-related peptides from species belonging to the taxon Xenopodinae. Amino acid residue deletions denoted by * have been introduced in to some sequences to maximize structural similarity. [a] denotes C-terminal α-amidation. Conservation of amino acid residues is emphasized by the shading.

XPF-related peptides

S. tropicalis ST1	GLASTLGSFLGKFAKGGAQAFLQPK
"S. new tetraploid 1" SP1	GFWSSALEGLKKFAKGGLEALTNPK
"S. new tetraploid 1" SP2	GLASTIGSLLGKFAKGGAQAFLQPK
S. tropicalis ST2	GVWSTVLGGLKKFAKGGLEAIVNPK
S. tropicalis ST3	GVFLDA***LKKFAKGGMNAVLNPK
S. epitropicalis SE2	GLASTIGSLLGKFAKGGAQAFLQPK
S. epitropicalis SE3	GFWTTAAEGLKKFAKAGLASILNPK
S. epitropicalis SE4	GVWTTILGGLKKFAKGGLEALTNPK
S. epitropicalis SE1	GLFLDT***LKKFAKAGMEAVINPK
X. laevis 1	GWASKIGQTLGKIAKVGLQGLMQPK
X. laevis 2	GWASKIGQTLGKIAKVGLKELIQPK
X. borealis B1	GFKQFVHSM*GKFGKAFVGEIINPK
X. borealis B2	GWASKIGTQLGKMAKVGLKEFVQS*
X. muelleri M1	GWASKIGQTLGKMAKVGLKDLIQA*
X. muelleri West MW1	GWASKIGQTLGKLAKVGLKEFAQS*
X. clivii C1	GWASKIGQALGKVAKVGLQQFIQPK
X. amieti AM1	GWASKIAQTLGKMAKVGLQELIQPK
X. andrei AN1	GWASKIGQTLGKMAKVGLQELIQPK
X. andrei AN2	GWVSKIGQTLGKMAKVGLQELIQPK

Fig. 4. A comparison of the primary structures of XPF-related peptides from species belonging to the taxon Xenopodinae. Amino acid residue deletions denoted by * have been introduced in to some sequences to maximize structural similarity. [a] denotes C-terminal α-amidation. Conservation of amino acid residues is emphasized by the shading.

Evolutionary pressure to conserve the primary structures of the antimicrobial peptides from Xenopodinae has not been uniform (Figs. 2-4). Peptides belonging to the PGLa family are most strongly conserved (7 out of 22 amino acids invariant) followed by the magainin family (4 out of 23 amino acids invariant). The amino acid sequences of the XPF family peptides have been poorly conserved (only 3 out of 25 amino acids invariant). A comparison of the primary structures of the CPF-peptides suggests that they divide naturally into two groups with and without a C-terminally α-amidated amino acid residue (Fig. 3). Even after deletions have been introduced into some sequences in order to maximize structural similarity, only the 3 amino acids are invariant in the first group and only the N-terminal glycine residue is invariant in the group of the amidated CPFs. Nevertheless, in both the XPF and CPF peptides, amino acid substitutions are generally conservative and secondary structure predictions indicate that the amphipathic α-helical character of all the peptides has been maintained (Chapters **2-5**; King et al., 2012; Conlon et al., 2012a).

During the last 4 years, the genus *Xenopus* has been expanded to include two new entries with a status of fully recognized octoploid species – *X. itombwensis* (Evans et al., 2008) and *X. lenduensis* (Evans et al., 2011). At the same time, there are several incompletely characterized members of the Xenopodinae for which further confirmation is needed in order to gain recognized species status (Evans et al., 2004; Evans et al., 2005; Evans, 2007). Two such members have been addressed in this thesis with the aim of providing evidence to confirm their putative species designation.

The first example is the tetraploid *Silurana* analysed in Chapter **4**. There are several studies involving this frog first described as "*Xenopus species* nova VII" (Graf and Fishberg, 1986; Tymowska, 1991) and subsequently as "*Silurana* new tetraploid 1" (Evans et al., 2004). In the publication arising from the work in Chapter **4** (Mechkarska et al., 2011), it was referred as *S. paratropicalis* according to the terminology of Flajnik et al. (1993). However, as it is not recognized as a separate species, it was pointed out that the term *S. paratropicalis* is a *nomen nudum* and therefore invalid (Blackburn and Beier, 2011). Consequently, the name "*Silurana* new tetraploid 1" is used instead of *S. paratropicalis* in the Discussion (Chapter **8**).

In the genus *Silurana*, analysis of mitochondrial DNA suggests that tetraploidization occurred by allopolyploidization. Hence another diploid (2n=20) ancestral species is predicted to have existed in addition to the ancestor of the diploid species *S. tropicalis* (Evans et al., 2005; Evans, 2007). All AMP families - PGLa, XPF and CPF - found in *S. tropicalis* skin secretions (Ali et al., 2001) were detected in skin secretions from "*Silurana* new tetraploid 1" (Figs. 2-4). The magainins were not detected in either species and so it was speculated that the gene encoding the magainins arose in the *Xenopus* lineage after its divergence from *Silurana*. However, a peptide belonging to the magainin family

was recently purified from the tetraploid *S. epitropicalis* (Conlon et al., 2012b). A BLAST search of the *S. tropicalis* genome database (Hellsten et al., 2010) reveals the presence of a gene encoding a magainin-related peptide (referred to as magainin-ST1) that would bear, if expressed, three amino acid differences from the *S. epitropicalis* ortholog (magainin-SE1). However, the reported antimicrobial activity of magainin-SE1 is very weak (Conlon et al., 2012b) so that it is possible that the presence of magainin peptide(s) in *S. tropicalis* and in "*S.* new tetraploid 1" secretions may have been missed. An alternative explanation may be that the polyploidization event in the *Silurana* lineage gave rise to species with differential expression of the tetraploid genomes so that more AMP genes have been silenced in "*S.* new tetraploid 1" - including the gene for the magainin - than in *S. epitropicalis*.

The second example of an incompletely characterized species is the frog which is given the working name *X. muelleri* West (Measey et al., 2004) that is analysed in Chapter **5**. Two discontinuous (allopatric) populations *X. muelleri* occupy ranges in east and west Africa (Rodel, 2000; Harper et al., 2010; Frost, 2011). It has been proposed that the western population represents a separate species (Measey et al., 2011). In previous reports involving molecular genetics studies and analysis of the evolution of advertisement calls, this population is referred to as *X. new tetraploid* 1 (Evans et al., 2004; Tobias et al., 2011). Comparison of the AMP profiles of the two frog populations suggests that they are not conspecific because (a) no orthologous peptide has the same amino acid sequence, and (b) two PGLa members are detected in *X. muelleri* West secretions while PGLa is lacking in the *X. muelleri* secretions. On the basis of the AMP sequences, *X. muelleri* West is more closely related to *X. borealis* than to *X. muelleri*. Therefore, the data provide strong support for the separate species designation of *X. muelleri* West.

Previous investigations of ranid frogs have used the same methodology described in this thesis to demonstrate that *Rana tagoi tagoi* and *Rana tagoi okiensis* are probably not conspecific (Conlon et al., 2010b) and that the relict leopard frog *Lithobates onca* and the lowland leopard frog *Lithobates yavapaiensis*, although closely related phylogenetically, are separate species (Conlon et al., 2010c). On the other hand, the presence of identical peptides in skin secretions from specimens of the Chiricahua leopard frog *Lithobates chiricahuensis* occupying regions in southern Arizona and from morphologically distinct specimens occupying the Mogollon Rim of central Arizona suggest that the two populations probably do not represent separate species (Conlon et al., 2011). The skin peptide profiles of Australian hylid frogs from the genera *Crinia*, *Litoria*, and *Uperoleia* have been used, together with morphological and cognate methods, to differentiate between sub-species and even different population clusters of the same species (Jackway et al., 2011).

The eight octoploid species have complex taxonomic relationships and can be divided into two subgroups (Chapter **1**, Fig.11). According to this proposed evolutionary scenario,

X. amieti and *X. andrei* share a common octoploid ancestor that differs from the octoploid ancestor of the "*vestitus-wittei*" subgroup. More detailed analysis of the mitochondrial DNA and gene sequences of RAG1 and RAG2 of the newly identified octoploids *X. lenduensis* and *X. itombwensis* suggest that there have been at least three independent allooctopolyploidization events in *Xenopus* (Evans et al., 2008; Evans et al., 2011). The results from our work on AMPs in skin secretions confirm the particularly close phylogenetic relationship between *X. andrei* (Chapter **4**) and *X. amieti* (Conlon et al., 2010a). The observed differences in primary structures between AMPs from *X. lenduensis* (King et al., 2012) and orthologs from the sister-pair species *X. amieti* and *X. andrei* are consistent with the fact that *X. lenduensis* (which belongs to the "*vestitus-wittei*" subgroup) originated from a different octoploid ancestor. However, further analysis is necessary to clarify the relations between the different octoploid species.

Effect of polyploidization and hybridization in Xenopodinae

All extant vertebrates carry large numbers of duplicated genes suggesting evolutionary conservation of genes that arise through local, regional or global DNA duplication events (Lynch and Conery, 2000). The 2R hypothesis – influenced initially by studies on the Hox gene clusters - postulates that two rounds of whole genome duplications (WGD) occurred in relatively rapid succession, perhaps within 10 million years, sometime very early in vertebrate evolution but after divergence of amphioxus approximately 550-600 MYA (Escriva et al., 2002; Furlong and Holland, 2002; Lundin et al., 2003). A further round of WGD in the main branch of the vertebrate lineage that produced all the teleost fishes (approximately 320-350 MYA) (Robinson-Rechavi et al., 2001). In addition to the WGD postulated in the 2R hypothesis, there have been duplication events involving multiple duplications and translocations of individual genes, segmental duplications of large stretches of DNA and perhaps whole chromosomes (Hughes, 1999; Martin, 2001; Robinson-Rechavi et al., 2001).

Gene or genome duplications *per se* will not lead to an increase in the complexity of life forms but they are an important evolutionary strategy by which organisms acquire phenotypic diversity (Ohno, 1970). *A priori* three evolutionary fates for a duplicated gene are proposed (Lynch and Katju, 2004): (a) nonfunctionalization which involves deletion of the daughter gene or its degeneration into a psuedogene; (b) subfunctionalization in which the mother and daughter genes are retained and share the same biological role, and (c) neofunctionalization in which the mother and daughter genes are retained but the daughter gene evolves to adopt a new biological role. Usually, WGD events are followed by massive gene loss (Gu et al., 2002). However, the mechanisms that lead to retention, followed by adoption of new function, versus loss of duplicated genes have broader implications at both a genetic and an evolutionary level.

The duplication of the genome in the ancestor of the Xenopodinae is significantly more recent than the teleost-specific duplication and the ancient vertebrate specific 2R duplication. At the same time, it is older than the typical lifetime of duplicated genes in a diploid background (several million years) (Lynch and Conery, 2000). Only in the Pipidae family do polyploids comprise the majority of the species (Duellman and Trueb, 1994; Martino and Sinsch, 2002) and ploidy reaches one of its highest levels - dodecaploidy (Tymowska, 1991). *X. laevis* has retained ~50% of the duplicated gene loci when compared to its unduplicated relative *S. tropicalis*. Because the taxon is so well defined, the Xenopodinae constitutes an excellent system in which to study the evolutionary trajectory of duplicated genes following one or more polyploidization events.

The distribution of AMPs in skin secretions from all species from Xenopodinae studied to date are compared in Table 4 (Chapters **2-5**; Conlon et al., 2010a; King et al., 2012; Conlon et al., 2012b). The multiplicity of the peptides belonging to the PGLa, CPF, and XPF families is no greater in the tetraploids *S. epitropicalis* and "*S.* new tetraploid 1" than in the diploid *S. tropicalis*. Similarly, the multiplicity of the magainin, PGLa, CPF, and XPF families is not significantly greater in the octoploid *Xenopus* species than in the tetraploid *Xenopus* species. The data suggest, therefore, that nonfunctionalization is the most common fate of duplicated AMP genes following polyploidization events.

A similar high level of gene deletion and/or gene silencing in octoploid and dodecaploid *Xenopus* species is seen among major histocompatibility complex (MHC) genes (Sammut et al., 2002; Du Pasquier et al., 2009) and also in the case of genes encoding RAG1 and RAG2 proteins crucial to the process of somatic rearrangement of DNA in adaptive immunity. In the latter example, degeneration of RAG1/RAG2 paralogs occurs via incorporation of premature stop codons and frameshift mutations (Evans, 2007; Evans et al., 2005).

Table 4. Multiplicity of the AMPs present in skin secretions of diploid, tetraploid, and octoploid frogs of the Xenopodinae.

	Ploidy level (2n)	Magainin	PGLa	CPF	XPF	Total
S. tropicalis	20	0	1	3	3	7
"*S.* new tetraploid 1"	40	0	1	3	2	6
S. epitropicalis	40	1	2	3	4	10
X. laevis	36	2	1	6	2	11
X. petersii	36	2	1	5	0	8
X. pygmaeus	36	2	1	3	0	6
X. borealis	36	2	2	4	2	9
X. muelleri	36	2	0	2	1	5
X. mulleri West	36	1	2	3	1	7
X. clivii	36	2	0	2	1	5
X. amieti	72	2	2	4	1	9
X. andrei	72	2	2	1	2	7
X. lenduensis	72	2	4	3	0	9

Peptidomic analysis does not allow discrimination between deletion of the duplicated gene from the frog genome and silencing at the level of transcription or translation. Similarly, it is not claimed that the AMPs isolated from skin secretions in these studies necessarily represent the full complement of expressed AMP gene products as components present in very low abundance and/or with very weak growth-inhibitory activity may have been missed.

The Xenopodinae provide examples of subfunctionalization among the AMPs. As shown in Table 5, the two CPF peptides (CPF-AM1 and CPF-AM4) from *X. amieti* (Conlon et al., 2010a), the two CPF peptides (CPF-SP1 and CPF-SP3) from "*S.* new tetraploid 1" (Chapter **4**), and the two PGLa peptides (PGLa-B1 and PGLa-B2) from *X. borealis* (Chapter **2**) have moderately well conserved primary structures and show comparable potencies against the Gram-positive bacterium *S. aureus* and the

Gram-negative bacterium *E. coli*. They appear to be sharing the responsibility of protecting the organism against environmental pathogens.

Table 5. Examples of subfunctionalization and possible neofunctionalization among AMPs from Xenopodinae.

Peptide	**Amino acid sequence**	**MIC** ***E. coli***	**MIC** ***S. aureus***
CPF-AM1	GLGSVLG**K**AL**K**IGANLL.NH_2	12.5	6
CPF-AM4	GLGSLVGNAL**R**IGA**K**LL.NH_2	25	12.5
CPF-SP1	GFLGPLL**K**LGL**K**GVA**K**VLPHLIPS**R**QQ	25	6
CPF-SP3	GFLGSLL**K**TGL**K**VGSNLL.NH_2	50	25
PGLa-B1	GMAS**K**AGTIAG**K**IA**K**TAI**K**LAL.NH_2	12.5	25
PGLa-B2	GMAS**K**AGSIVG**K**IA**K**IALGAL.NH_2	25	50
PGLa-AM1	GMAS**K**AGSVLG**K**VA**K**VAL**K**AAL.NH_2	12.5	25
PGLa-AM2	GMASTAGSVLG**K**LA**K**AVAIGAL.NH_2	>200	>200

The basic residues in the peptides, essential for potent antimicrobial activity, are shown in bold type.

In order to appreciate the possibility of neofunctionalization among the AMP genes in subfamily Xenopodinae, it is necessary to discuss structure-activity relationships among the gene products. The potency of an AMP (Chapter **1**) is determined by complex interactions among four parameters: cationicity (net positive charge), hydrophobicity, stability of the α-helix, and degree of amphipathicity (Conlon et al., 2007a; Powers and Hancock, 2003). Increasing cationicity while maintaining amphipathicity promotes interaction between the peptide and the more negatively charged cell membrane of prokaryotes, and so generally increases antimicrobial potency (Conlon et al., 2007a). However, in the case of the PGLa peptides from *X. amieti*, the amino acid substitutions $Lys^{5} \rightarrow$ Thr and $Lys^{19} \rightarrow$ Ile have resulted in a selective decrease in cationicity which in turn has resulted in complete disappearance of antimicrobial activity (Table 5). This suggests the possibility of neofunctionalization with PGLa-AM2 having adopted a new biological role but the nature of this new function is currently unknown.

Interspecies hybridization and AMP multiplicity

Polyploidization in clawed frogs occurs mainly through allopolyploidization – genome duplication associated with hybridization between species (reviewed in Evans, 2008). Skin secretion AMPs profile from hybrid *X. laevis* x *X. muelleri* female frogs (Chapter **7**) was compared to that of *X. laevis* and *X. muelleri* (Chapter **5**) to study the mode of inheritance of AMPs. It is possible that the interspecies hybrids generated in the laboratory represent a model of ancient allopolyploidization events in the lineage (Chapter **1**) without increasing the total amount of genetic information as these hybrids, like the parent species, are tetraploid (2n = 36) (Koroma et al., 2011). It was confirmed that the full complement of AMPs from *X. laevis* as well as four out of six AMPs from *X. muelleri* were present in secretions of the hybrid (Chapter **7**). In addition, three novel AMPs, not detected in skin secretions of the parent species, were present in the hybrids and are presumed to have arisen as a result of hybridization. Therefore, comparing the total number of AMPs in the hybrids, with those from the tetraploid *Xenopus* species (Table 3) suggests the conclusion that interspecies hybridization leads to increased multiplicity of AMPs in the skin secretions.

The origin of the Pipidae dates back at least to the Mesozoic (Late Jurassic around 150 MYA) (Roelants and Bossuyt, 2005; Roelants et al., 2007; San Mauro, 2010; San Mauro et al., 2005). The extensive fossil record that spans about 90 million years (from the Cretaceous) (Trueb, et al., 2005) indicates that large scale population movements of Pipidae species have probably occurred in the Central African highlands during different periods of time. Sympatry observed amongst representatives of *Xenopus* may have been an important factor in the evolution of these frogs. Thus, *X. wittei* and *X. vestitus* are interpreted as

allopolyploids, the products of interspecies hybridization (Tinsley and Jackson, 1998) but conclusive evidence is lacking.

Many present day representatives of *Xenopus* and *Silurana* live in sympatry. However, there have been relatively few reports of naturally occurring hybrids in the genus *Xenopus* in the wild, mainly amongst members of the *laevis* and the *muelleri* group (Picker, 1985; Yager, 1996; Fischer et al., 2000). In contrast, many *Xenopus* species can be hybridized in the laboratory and gametic incompatibilities are generally found to be absent (Kobel et al., 1996). As *Xenopus* females are heterogametic and males are homogametic, it has been pointed out that this genus represents an exception to Haldane's rule that the heterogametic sex typically suffers the greater dysfunctional effects of hybridization (Malone et al., 2007). In interspecies crosses the males are consistently found to be sterile and the females are fully or partially fertile. The fertile female hybrids may play an important role in the evolution of the *Xenopus* frogs.

Future perspectives

More than 150 years have passed since Mark Twain penned the words: "I don't see no p'ints about that frog that's any better'n any other frog" in *The Celebrated Jumping Frog of Calaveras County* (1867). Now we actually do know why some frogs are better than others.

The new insights gained from the work on AMPs from skin secretions of polyploid frogs from Pipidae family suggest the following directions for future research:

Design of analogues of the CPFs and the hymenochirins with potential for development into therapeutic agents

Development of frog AMP-based anti-infectives as a platform for next-generation therapeutics will help address the growing threat of multidrug-resistant infections. Despite displaying potent activity against strains of antibiotic resistant bacteria and against certain pathogenic fungi, no anti-infective peptide based upon their structures has been adopted in clinical practice. Consequently, the therapeutic potential of frog skin AMPs has yet to be realized.

The CPF-B1, CPF-C1, CPF-MW1, CPF-SP1 peptides, as well as hymenochirin-1B, -2B and -3B that are highlighted in the Discussion (Table 1) exert broad spectrum antimicrobial activity and so represent good templates for targeted drug design. They will be the subject of extensive structure-activity relationships studies aimed at generating analogues with improved antimicrobial potency against MDR pathogens that are non-hemolytic. A successful strategy for structural modification of promising naturally occurring AMPs has been established in the laboratory (Conlon et al., 2007a). It involves

selective increase in cationicity concominant with decrease in helicity and hydrophobicity in the transformation of the naturally occurring AMPs into non-toxic therapeutic agents. Subsequently, the efficacy and pharmacokinetic properties of the best analogues of the naturally occurring peptides will be investigated both *in vitro* and using *in vivo* models of diseases caused by MDR pathogens.

A major obstacle to the development of effective peptide-based anti-infective drugs is their short half-lives in the circulation which severely limits their usefulness for systemic applications. Peptides lacking stable secondary structures are susceptible to proteolysis by a range of microbial proteases (Schmidtchen et al., 2002). Several approaches have been developed to increase the stability of peptide analogues towards proteolytic enzymes which involve coupling of the peptides to fatty acids and substitutions of L-amino acids by D-amino acids or unnatural amino acids (Chen et al., 2006; Conlon et al., 2007b; Tossi et al., 2012).

The conjugation of a fatty acid moiety at the N-terminus of peptide scaffolds has been demonstrated to improve significantly antimicrobial activity (Shalev et al., 2006; Jerala, 2007) and is known also to compensate for a loss of hydrophobicity within the peptidic chain based on amino acid residue substitutions (Malina and Shai, 2005). As only natural L-amino acid residues are targets of the proteases, substitutions by D-amino acids at susceptible sites would render the peptide partially or totally resistant to proteolysis without loss of antimicrobial activity (Chen et al., 2006; Conlon and Sonnevend, 2011; Conlon, et al., 2012d). D-stereoisomers, by decreasing α-helicity, also tend to be less hemolytic than their L-counterparts (Papo et al., 2002). An alternative strategy is to select unnatural amino acids, such as α-aminoisobutyric acid (Aib) (Conlon et al., 2007b) and ornithine (Strom et al., 2003). Aib is a non-chiral, α,α-dimethylated amino acid and its incorporation into a peptide introduces conformational constraints. As a result, Aib substitution leads to stabilization of an existing helical conformation and provides stability against proteases.

Another worthwhile avenue for future exploitation is the evaluation of CPF peptides and the hymenochirins, as well as their analogues, for antitumour and insulin-releasing potential. Since cancer and diabetes are conditions associated with increased susceptibility to infections due to a compromised immune system and/or impaired wound healing, the combined antimicrobial and antitumor or insulin-releasing activity of these peptides may prove particularly helpful in the development of new therapies to counter the increasing health burden of the diseases.

Cancer chemotherapy faces challenges posed by tumours becoming unresponsive to commonly used anti-cancer agents (Chen and Tiwari, 2011). There is an urgent need for new types of drugs with appropriate pharmacokinetic and toxicological profiles that exert preferential cytotoxicity towards tumour cells compared with non-neoplastic cells. Anti-cancer activity of AMPs has been studied in much less detail but it has been shown that

alyteserin-2a, isolated from skin secretions from *A. obstetricans* (Conlon et al., 2009a), represent a good template to design more potent analogues that selectively target tumour cells (Conlon et al., 2012e).

Similarly, many frog skin peptides have been shown to stimulate insulin-release from BRIN-BD11 clonal β-cells at low concentrations that are not toxic to the cells (Ojo et al., 2013). Consequently, these compounds show potential for treatment of patients with Type 2 diabetes The CPF peptides from skin secretions of *X. laevis* and *S. epitropicalis* show particularly high potency, producing a significant increase in the rate of insulin release at concentrations as low as 3×10^{-11} M (Srinivasan et al., 2013). CPF peptides from *X. borealis*, *X. clivii*, *X. muelleri* West and "*S.* new tetraploid 1", described in Chapters **2-5**, may show increased potency and therefore greater therapeutic potential.

Further studies to elucidate taxonomic relationships among frogs from the Pipidae

Based on advertisement calls and/or molecular characters, *X. fraseri* forms a monophyletic group with tetraploid *X. pygmaeus*, the octoploids *X. amieti*, *X. andrei* and *X. boumbaensis*, and the dodecaploid *X. ruwenzoriensis* (Kobel, 1996). However, more detailed comparative studies of the evolution of globin expression of the tetraploids in genus *Xenopus* suggest that *X. fraseri*, despite sharing the same chromosome number with the rest of the species, may have evolved as a result of an independent genome duplication (Burki and Fischberg, 1985). *X. fraseri* may occupy an anomalous position in the evolution of Xenopodinae being more closely related to *Silurana* than the other tetraploids. Both *X. fraseri* and *S. tropicalis* are morphologically similar, e.g. possess equal number of claws, and display similar electrophoretic patterns for their albumins (Burki and Fischberg, 1985). Two other tetraploids similar to *X. fraseri* have been identified and referred as *X.* cf. *fraseri* 1 and 2 (Evans et al., 2004). Characterization of their species status is still incomplete. The AMP secretory profile of *X. fraseri* will provide insight into the taxonomic relations of this species with the other members of the Xenopodinae.

The taxonomic relationship between the octoploids *X. vestitus* and *X. wittei* is complex. Based on molecular analysis of the mtDNA, at least three independent allooctoploidization events are suggested (Evans et al., 2011). According to the scenario, *X. vestitus* forms a sister group with *X. lenduensis* which shares common ancestry of half of their genome with *X. itombwensis* and *X. wittei*, and the other half with *X. amieti* and *X. andrei*. Comparison of the electrophoretic patterns of the albumins reveals that more globin genes have been silenced in *X. vestitus* than in *X. wittei* (Burki and Fischberg, 1985). By applying the same approach used in Chapters **2-5**, the full complement of AMPs in skin secretions of these species will be determined. Once their primary structures are known, they will be compared with those of the previously characterized orthologous peptides from *X. andrei*, *X. amieti*, and *X. lenduensis* (Chapter **4**; Conlon et al., 2010a; King et al., 2012). An expected

outcome of this research will be a better understanding of the evolutionary history of these frogs and may permit differentiation between the alternative hypotheses.

Relationships between different taxa in the subfamily Pipinae have been much less studied than those in the Xenopodinae. It is proposed to analyze the skin secretions from the single species *P. merlini* belonging to the genus *Pseudhymenochirus*. Since this genus is taxonomically closely related to *Hymenochirus* (Evans et al., 2004; Bewick et al., 2012), it is expected that the skin secretions of *P. merlini* will contain AMPs. A comparison of the structures of these putative peptides with the hymenochirins will provide insight into the taxonomic relationship between *Pseudhymenochirus* and *Hymenochirus*. Moreover, the only representative of genus *Pipa* studied to-date is *P. pipa* and, unexpectedly, AMPs are absent from skin secretions (Conlon et al., 2009b). In order to answer the question whether the lack of AMPs is species-specific or is common to all the members of the genus, other species will be analyzed. It is tempting to speculate that the AMP genes have evolved after the divergence of *Pipa* and the other genera in Pipidae family. An alternative explanation is nonfunctionalization of the AMP genes necessitated by the unusual reproductive behavior of *Pipa*. This includes female dorsal skin brooding and AMPs may be toxic to the eggs.

Investigations of the link between polyploidization and multiplicity of AMPs

The evolutionary scenario described in Chapter **1** outlines the importance of allopolyploidization for the speciation of Xenopodinae. Both *X. ruwenzoriensis* and *X. longipes* evolved independently and are considered to be products of such allopolyploidization events between different octoploid and tetraploid ancestors (Evans et al., 2004). Further investigations are required to address ambiguities regarding the origin of these species. Since *X. ruwenzoriensis* and *X. longipes* are dodecaploid (the highest level of ploidy observed in *Xenopus*), they represent a good model to extend the study on effect of gene dosage on AMPs multiplicity in skin secretions. In light of the findings presented in Chapter **4**, it is expected that the number of the AMPs will not be substantially different from the number in tetraploids and octoploids, as many AMP genes will have been silenced. However, the dodecaploids are considered as a model of interspecies hybridization between parents with different chromosome sets. Consequently, an alternative (although less likely) outcome is a possibility: an increased number of AMPs which include peptides common with both parents together with novel components.

Insight into the mode of inheritance of AMP genes in interspecies hybrids

Interspecies hybridization between female *X. laevis* and male *X. muelleri* increases the multiplicity of the AMPs and leads to expression of novel peptides in the skin secretions of

female F1 frogs (Chapter **7**). It is proposed to extend this research by studying other species hybrids such as female *X. laevis* x *X. borealis*. Both *X. borealis* and *X. muelleri* belong to the *muelleri* lineage and are thought to be evolutionary equidistant from *X. laevis* (Evans et al., 2004; Evans et al., 2005; Evans, 2007). Using both directions of interspecies cross, in which the mother is either *X. laevis* or *X. borealis*, would provide more information relating to: 1) multiplicity of the AMPs in skin secretions of hybrid frogs; 2) influence of the maternal and paternal genomes on the mode of inheritance of the AMP genes; and 3) origin of the novel peptides. An increase in AMP multiplicity is expected but the possibility that this finding might be species-specific is not excluded.

Dramatic genetic and epigenetic reconfigurations are known to occur in the hybrid *Xenopus* background such as: mis-expression and novel gene expression pattern, or changes in the cytosine methylation pattern and histone modifications (Chain et al., 2008; Malone and Michalak, 2008; Koroma, et al., 2011). As many AMP genes in a species are silenced after genome duplications take place (Chapter **4**), it seems reasonable to speculate that their expression will be re-activated due to the presence of transcription factors from the other parent present in the hybrid environment. This mechanism may explain the presence of novel peptides in the skin secretions. The hypothesis that a hybrid with a greater complement of AMPs, including novel active peptides, will be better protected against invasion by pathogenic microorganisms in the environment than the parent species also needs further investigation.

The issue of whether epigenetic changes will play a role in the inheritance of the full complement of AMPs including the novel peptides needs to be addressed. Since the female hybrids are fertile, analysis of the AMPs in the offspring from mating the *X. laevis* x *X. muelleri* and *X. laevis* x *X. borealis* hybrids with either of their parental species (F2 generation) will be undertaken. The main question to be answered is whether the novel complement of AMPs is stably transferred into the next generation.

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Summary

The emergence of pathogenic bacteria and fungi resistant to commonly used antibiotics poses a serious threat to public health and necessitates novel treatment approaches in order to control infections. Antimicrobial peptides (AMPs) are one of the central components of the system of innate immunity and due to their non-specific and highly destructive mechanism of killing, pathogens will develop resistance at lower rates than conventional antibiotics. Skin secretions of frogs from the family Pipidae are a rich source of AMPs which show potential for development into therapeutic agents.

Until recently, the only representatives of the Pipidae family frogs from which dermal AMPs had been identified were the diploid frog *Silurana tropicalis*, the tetraploid frog *Xenopus laevis* and the octoploid frog *Xenopus amieti*. Therefore, this program of research was undertaken with the aim to isolate, purify and characterize AMPs with therapeutic potential from skin secretions of other polyploid species of African clawed frogs of the Pipidae family. Emphasis is given to the application of the AMPs as markers to elucidate the taxonomic relationships and evolutionary history of the frogs. The study also investigates the effects which polyploidization and interspecies hybridization have had on the multiplicity of AMPs in frog skin secretions.

Chapter **2** and Chapter **3** present data from the peptidomic analysis of norepinephrine-stimulated skin secretions of two well-characterized and closely related tetraploid *Xenopus* species – *X. borealis* and *X. clivii*. Multiple peptides with varying degrees of antimicrobial activity were isolated. Structural characterization demonstrated that they were orthologous to magainins, peptide glycine-leucine-amide (PGLa), caerulein-precursor fragments (CPFs) and xenopsin-precursor fragments (XPFs), previously isolated from *S. tropicalis*, *X. laevis* and *X. amieti*. CPF-B1 and CPF-C1 were the most abundant AMPs in the skin secretions of *X. borealis* and *X. clivii* respectively. CPF-B1 (GLGSLLGKAFKIGLKTV GKMMGGAPREQ) was active against clinical isolates of the hospital-associated pathogens, methicillin-resistant *Staphylococcus aureus* (MRSA) and multidrug-resistant *Acinetobacter baumannii* (MDRAB) with MIC = 5 μM and showed low hemolytic activity against human erythrocytes (HC_{50} >200 μM). CPF-C1 (GFGSLLGKALRLGANVL.NH_2) also showed potent activity against a range of Gram-negative bacteria. CPF peptides, therefore, represent promising candidates for development into therapeutic agents for use against these emerging antibiotic-resistant pathogens.

The genera *Silurana* and *Xenopus* are united in the subfamily Xenopodinae and have a complex evolutionary history. Chapter **4** includes data from the peptidomic analysis of skin secretions from an incompletely characterized tetraploid species termed "*S.* new tetraploid 1" with chromosome number 2n=40 and the octoploid species *X. andrei* (2n=72). The species represent model systems in which to study of the fate of duplicated AMP genes following putative allopolyploidization events. Multiple peptides belonging to the PGLa, XPF, and CPF familes were identified. The primary structures of the AMPs from *X. andrei* indicate a

close phylogenetic relationship between this species and the previously studied *X. amieti*. Three CPF peptides from "*S.* new tetraploid 1" showed potent, broad-spectrum antimicrobial activity and are present in high abundance. In contrast, only a single CPF peptide was isolated in low yield from the *X. andrei* secretions. There is no increase in the multiplicity of the AMPs in skin secretions of "*S.* new tetraploid 1" and the octoploid *X. andrei* when compared to the diploid *S. tropicalis* (2n=20) and the tetraploid *X. laevis* (2n=36). It is concluded that nonfunctionalization (gene silencing) has been the most common fate of duplicated AMP genes following polyploidization in the *Silurana* and *Xenopus* lineages.

AMPs constitute a characteristic ''fingerprint'' of a particular frog species that may be used for an unequivocal taxonomic classification. Two populations of the tetraploid *X. muelleri*, occupying separate non-contiguous ranges in east and west Africa, are studied in Chapter **5**. Their taxonomic relationship is unclear and it has been proposed that the western population represents a separate species referred to as *X. muelleri* West while the eastern population retains the original name *X. muelleri*. A comparison of the primary sequences of AMPs in skin secretions reveals that no orthologous peptide from the two populations of *X. muelleri* has the same amino acid sequence. Additionally, the *X. muelleri* secretions, like those from *X. clivii*, did not contain a PGLa peptide whereas the *X. muelleri* West secretions contained two members of this family. The data indicate that *X. muelleri* West is more closely related to *X. borealis* than to *X. muelleri* and so provide strong support for the proposal that *X. muelleri* and *X. muelleri* West should be described as separate species.

In contrast to species in the subfamily Xenopodinae, frogs from the subfamily Pipinae have not been investigated as a source of AMPs. The AMP profile in skin secretions from *Hymenochirus boettgeri* as a representative of genus *Hymenochirus* (subfamily *Pipinae*) is described in Chapter **6**. A novel family of five structurally-related peptides, designated as hymenochirins, was identified. Hymenochirin-1B (IKLSPETKDNLKKVLKGAIKGAIAV AKMV.NH2) is C-terminally α-amidated whereas hymenochirins-2B - 5B have the general structure $XKIPX_2VKDTLKKVAKG\ X_2SX_2\ AGAX_3$.COOH. The most abundant peptide in the secretions was hymenochirin-3B (IKIPAVVKDTLKKVAKGVLSAVAGALTQ). Synthetic replicates of hymenochirin-1B - 4B possess broad-spectrum antimicrobial activity and relatively weak hemolytic activity and so represent potential candidates for development into therapeutically valuable agents against drug-resistant pathogens. The hymenochirins show very low structural similarity with the antimicrobial peptides isolated from skin secretions of *S. tropicalis* and *X. laevis* consistent with the proposed ancient divergence of the Pipinae and Xenopodinae.

The F1 hybrid frogs *X. laevis* x *X. muelleri* represent a model of interspecies hybridization in the Pipidae family that does not result in an increase in ploidy. They are

studied in Chapter **7** and the data obtained provide an insight into the mode of inheritance of AMPs. A total of 18 different AMPs were isolated from skin secretions of the female hybrids. In addition to the complement of AMPs from the parent species, three previously undescribed peptides (magainin-LM1, PGLa-LM1, and CPF-LM1) were purified from the secretions of the hybrid frogs that were not detected in secretions from either *X. laevis* or *X. muelleri*. Magainin-LM1 differs from magainin 2 from *X. laevis* by a single amino acid substitution ($Gly^{13} \rightarrow Ala$) but PGLa-LM1 and CPF-LM1 differ appreciably in structure from orthologs in the parent species. CPF-LM1 shows potent, broad-spectrum antimicrobial activity and is hemolytic. The data indicate that hybridization increases the multiplicity of host-defense peptides in skin secretions. As the female F1 hybrids are fertile, hybridization may represent an adaptive strategy among *Xenopus* species to increase protection against pathogenic microorganisms in the environment.

The thesis is completed by a general discussion in Chapter **8** of the results and conclusions in Chapters **2**-**7**. The potential of AMPs from skin secretions of frogs belonging to the Pipidae family is reviewed from three different aspects: promising candidates for development into therapeutic valuable anti-infective agents; reliable taxonomic and phylogenetic markers; and tools to study the fate of duplicated genes in *Xenopus* and *Silurana*. The interspecies *Xenopus* hybrids are proposed as a suitable model to perform future studies on the mode of inheritance of skin AMPs.

Samenvatting

De opkomst van pathogene bacteriën en schimmels, die resistent zijn tegen conventionele antibiotica vormen een serieus gevaar voor de volksgezondheid en vereist een nieuwe aanpak om infecties in bedwang te houden. Antimicrobiële peptiden (AMPs) vormen een van de centrale componenten in het aangeboren immuun system en door hun niet specifieke en hoogst vernietigende mechanismen, zullen pathogenen langzamer resistentie opbouwen dan bij gebruik van conventionele antibiotica. De huidsecreties van kikkers van de Pipidae familie vormen een rijke bron van AMPs die het ontwikkelen van therapeutische middelen mogelijk zou kunnen maken.

Tot voor kort waren alleen AMPs- geïdentificeerd van de kikkerhuid van de diploid kikker *Silurana tropicalis,* van de tetraploide kikker *Xenopus laevis* en van de octoploide kikker *Xenopus amieti*, die allen behoren tot de Pipidae familie. Om die reden werd dit onderzoeksprogramma uitgevoerd met het doel om AMPs met therapeutisch potentiaal te isoleren, te zuiveren en te karakteriseren ten opzichte van de huidsecreties van andere polyploide soorten van de Afrikaanse klauwkikker van de Pipidae familie. De nadruk ligt op het gebruik van AMPs als markers om de taxonomische relaties en de evolutionaire geschiedenis van de kikkers in kaart te brengen. Deze studie onderzoekt de effecten die polyploidizatie en interspecies hybridisatie gehad hebben op het multipliceren van AMPs in de huidsecretie van deze kikkers.

Hoofdstukken **2** en **3** presenteren de data van de peptidomische analyse van norepinephrine-gestimuleerde huidsecreties van twee goed gekarakteriseerde en nauw verwante tetraploide *Xenopus* soorten – *X. borealis* en *X. clivii*. Meerdere peptiden met verscheidene antimicrobiële activiteiten werden geïsoleerd. Structuurkarakterisatie toonde aan dat deze ortholoog waren tot magainins, peptide-leucine-amide (PGLa), caerulein-precursor fragmenten (CPFs) en xenopsin-precursor fragmenten (XPFs), die al eerder waren geïsoleerd van *S. tropicalis*, *X. laevis* en *X. amieti.* CPF-B1 en CPF-C1 waren de meest voorkomende AMPs in de huidsecreties van respectievelijk *X. borealis* en *X. clivii*. CPF-B1 (GLGSLLGKAFKIGLKTVGKMMGGAPREQ) was actief bij de klinische isolatie van de ziekenhuis-gerelateerde pathogene, methicillin-resistente *Staphylococcus aureus* (MRSA) en de meervoudig-resistente *Acinetobacter baumannii* (MDRAB) met een MIC = 5 µM en een lage hemolytische activiteit tegen menselijke erythrocyten (HC_{50} > 200 µM). CPF-C1 (GFGSLLGKALRLGANVL.NH_2) was ook sterk actief tegen verschillenden Gram-negatieve bacteriën. CPF peptiden representeren daarom een groep van veelbelovende kandidaten voor de ontwikkeling van therapeutische middelen tegen het verschijnen van antibiotica resistente pathogenen.

De genera *Silurana* en *Xenopus* zijn verenigd in de subfamilie Xenopodinae en hebben een complexe evolutionaire geschiedenis. Hoofdstuk **4** bevat data van de peptidomische analyse van huidsecretie van een onvolledig gekarakteriseerde tetraploide species genaamd "*S.* new tetraploid 1" met chromosoom nummer 2n=40 en de octoploid species *X. andrei*

(2n=72). Deze soorten vertegenwoordigen een model waarin het van gedupliceerde AMP genen bestudeerd kunnen worden, na een putatieve allopolyploidizatie gebeurtenis. Verscheidene peptiden behorende tot de PGLa, de XPF, en de CPF families werden geïdentificeerd. De primaire structuren van de AMPs van *X. andrei* wijzen op een nauwe phylogenetische verwantschap tussen deze species en de eerder bestudeerde *X. amieti*. Drie CPF peptiden van de "*S.* new tetraploid 1" vertoonden een sterke, breed-spectrum antimicrobiële activiteit en zijn in overmaat aanwezig. Daarentegen werd slechts een enkel CPF peptide en in lage kwantiteit geïsoleerd uit de secreties van de *X. andrei*. Er is geen toename in de multipliciteit van de AMPs in huidsecreties van de "*S.* new tetraploid 1" en de octoploid *X. andrei* ten opzichte van de diploid *S. tropicalis* (2n=20) en de tetraploid *X. laevis* (2n=36). De conclusie is dat de meest voorkomende uitkomst van gedupliceerde polyploidisatie in de *Silurana* en *Xenopus* geslacht is niet-functionalisatie (gene silencing).

AMPs vormen een karakteristieke "vingerafdruk" van een specifiek kikkersoort die gebruikt zou kunnen worden voor een eenduidige taxonomische classificatie. Twee populaties van de tetraploid *X. muelleri,* die apart voorkomen in twee afzonderlijke en niet aan elkaar verbonden gebieden in oost en west Afrika zijn bestudeerd in hoofdstuk **5.** Hun taxonomische relatie is niet duidelijk en er is voorgesteld dat de westerse populatie een aparte soorten vertegenwoordigt, genaamd *X. muelleri* West, terwijl de oostelijke populatie zijn oorspronkelijke naam *X. muelleri* behoudt. Een vergelijking van de primaire sequenties van de AMPs in de huidsecreties maakt duidelijk dat geen orthologe peptide van de twee *X. muelleri* populaties dezelfde serie aminozuren vertoont. Bovendien, bevatten de huidsecreties van *X. muelleri,* net als die van de *X. clivii*, niet een PGLa peptide terwijl de *X. muelleri* West secreties twee leden van deze familie bevatten. De resultaten tonen aan dat *X. muelleri* West meer gelieerd is aan *X. borealis* dan aan *X. muelleri*. Dit het voorstel ondersteunt dat *X. muelleri* en *X. muelleri* West het best als twee verschillende soorten kunen beschreven worden.

In tegenstelling tot de species in de subfamilie Xenopodinae, zijn de kikkers van de subfamilie Pipinae nog niet onderzocht als een potentiele bron van AMPs. Het AMP profiel in de huidsecreties van *Hymenochirus boettgeri* als vertegenwoordiger van de genus *Hymenochirus* (subfamily Pipinae) is beschreven in hoofdstuk **6**. Een nieuwe familie van vijf structuur-gerelateerde peptiden, hymenochirins genoemd, is geïdentificeerd. Hymenochirin-1B (IKLSPETKDNLKKVLKGAIKGAIAVAKMV.NH_2) is C-terminally α-amidated terwijl hymenochirins-2B - 5B de algemene structuur XKIPX_2VKDTLKKV AKGX_2SX_2 AGAX_3.COOH hebben. De meest voorkomende peptide in de secreties was hymenochirin-3B (IKIPAVVKDTLKKVAKGVLSAVAGALTQ). Synthetische kopieën van hymenochirin-1B - 4B bevatten breed-spectum antimicrobiële activiteit en een relatief zwakke hemolytische activiteit en vertegenwoordigen dus potentiele kandidaten voor de ontwikkeling van therapeutisch waardevolle middelen tegen medicijnen-resistente pathogene. De hymenochirins vertonen een zeer geringe structurele gelijkenis met de

antimicrobiële peptiden die geïsoleerd waren van huidsecreties van *S. tropicalis* en *X. laevis* wat weer consistent is met de voorgestelde oude afscheiding van de Pipinae en de Xenopodinae.

De F1 hybride kikkers *X. laevis* x *X. muelleri* vormen een interspecies hybridizatie model in de Pipidae familie die geen toename in ploidy initieert. Deze zijn bestudeerd in hoofdstuk **7** en de verkregen resultaten geven een inzicht in de overerving van AMPs. In totaal werden 18 AMPs geïsoleerd van de huidsecretie van de vrouwelijke hybriden. Naast het aantal AMPs, afkomstig van de ouderlijke soorten, werden drie niet eerder beschreven peptiden (magainin-LM1, PGLa-LM1, en CPF-LM1) gezuiverd uit de secreties van de hybride kikkers die niet ontdekt waren in secreties afkomstig van of *X. laevis* of van *X. muelleri*. Magainin-LM1 verschilt van magainin 2 van de *X. laevis* met een enkele aminozuur substitutie ($Gly^{13} \rightarrow Ala$), maar PGLa-LM1 en CPF-LM1 verschillen aanzienlijk in structuur vergeleken met hun orthologen in de ouderlijke speciën. CPF-LM1 vertoont een sterke breedspectrum antimicrobiële activiteit en is hemolytisch. De data suggereren dat hybridisatie de multipliciteit van gastheer-verdedigende peptiden in huidsecreties doet toenemen. Aangezien de vrouwelijke F1 hybriden vruchtbaar zijn, zou hybridisatie een aanpassingsstrategie voor de *Xenopus* soorten kunnen zijn om zich beter te verdedigen tegen pathogene micro-organismen in de hun omgeving.

Het proefschrift wordt in hoofdstuk **8** afgesloten met een algemene discussie over de resultaten en de conclusies uit hoofdstukken **2**-**7**. Het potentieel van AMPs afkomstig van huidsecreties van de Pipidae kikkerfamilie wordt beschreven vanuit drie verschillende aspecten: als potentiele kandidaten voor de ontwikkeling van therapeutisch interessante anti-infectie middelen; als betrouwbare taxonomische en phylogenetische markers; en als gereedschap om het lot van gedupliceerde genen te bestuderen in *Xenopus* en *Silurana*. De interspecies *Xenopus* hybride wordt voorgesteld als een geschikt model voor verdere studie in de soort van vererving van dermaal afkomstige AMPs.

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“Ancora imparo. [I am still learning.]”

-Michelangelo, at age 87 in 1562

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Dear Past, I am grateful for the lessons! Dear Future, I am ready!

Personalia

Curriculum vitae

List of publications

Curriculum Vitae

Milena Mechkarska was born on 9th of October 1969 in Sofia, Bulgaria. She graduated with a gold medal from the National School of Mathematics and Natural Sciences (NSMNS), class Biotechnology in Sofia in 1987 and immediately continued her education in Genetic and Cellular Engineering at Sofia University "St. Kliment Ohridski" (Sofia, Bulgaria). During the course of the study she participated in the research of the Laboratory of Virology under the supervision of the late Prof. Tatiana Varadinova and wrote a thesis entitled "Zinc-containing complexes as a novel therapy for herpes simplex virus type 1 infection".

After receiving her MSc degree in 1992 she specialized further in the field of Virology and Immunology at the Faculty of Biology, Sofia University (Sofia, Bulgaria), the Bulgarian Academy of Sciences (Sofia, Bulgaria) and the International Centre for Genetic Engineering and Biotechnology (New Delhi, India). She gained extensive experience in the field of host-pathogen interactions working on herpes simplex and hepatitis E viral models. She obtained a second MSc in Ecology and Environment from Sikkim Manipal Academy (New Delhi, India) with a thesis entitled "Interfaces between ecology and virology: the SARS model" with Prof. Norbert Nowotny (Department of Microbiology and Immunology, United Arab Emirates University) as a supervisor. Subsequently, she spent four years in the laboratory of Prof. Basel Al-Ramadi (Department of Microbiology and Immunology, United Arab Emirates University) on a project studying the differential activation of murine macrophages by cytokine-expressing *Salmonella* strains.

At the beginning of 2010 she joined the laboratory of Prof. J. Michael Conlon at the Department of Biochemistry (United Arab Emirates University). Her main research focus since then has been isolation and characterization of biologically active peptides from frog skin secretions with particular emphasis on antimicrobial peptides from skin secretions of polyploid frogs from the Pipidae family as the PhD topic. The PhD research, under the supervision of Prof. J. Michael Conlon and Prof. Jerry M. Wells, was completed in 2012 and is described in this thesis. Currently, she is focusing on finding answers to some of the questions proposed in this thesis for future research.

List of publications

Directly related to the PhD topic:

Mechkarska, M., Ahmed, E., Coquet, L., Leprince, J., Jouenne, T., Vaudry, H., King, J.D., Conlon., J.M., 2010. Antimicrobial peptides with therapeutic potential from frog skin secretions of the Marsabit clawed frog *Xenopus borealis* (Pipidae). Comp Biochem Physiol 4:467-472.

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