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ORIGINAL ARTICLE

Skin micro-organs from several frog species secrete a repertoire of powerful antimicrobials in culture

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This work is an attempt to take advantage of the rich biodiversity that exists in Colombia in order to start a systematic analysis of antimicrobial substances that have emerged through amphibian evolution. For this purpose we have developed a technique to grow intact frog skin derived micro-organs (SMOs) *in vitro* in the absence of serum. We show that in SMOs, the skin glands remain intact and continue to secrete into the medium substances with potent antibacterial activity, for several days in culture. Our strategy has been to create a bank of substances secreted by amphibian skin from different species. This bank contains at present around 50 species and is of particular importance as some of the species are in danger of disappearing. We show that some of the species tested displayed very strong antibacterial activity without being toxic to somatic cell lines, even at 10-fold higher concentration.
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INTRODUCTION

In spite of significant advances in molecular biology, more than 40% of compounds used by modern medicine are derived from nature. In certain areas, such as antimicrobials, anticancer, antihypertensive and anti-inflammatory drugs, the numbers are even higher and constitute about 75% of the total.[1,2] Frogs and toads have developed a successful strategy for surviving microbe-laden hostile environments, which rely heavily on the secretion of chemical cocktails from specialized skin glands.[3] These secretions not only produce large amounts of biologically active peptides that are similar to mammalian neuropeptides and hormones, but they also contain a rich arsenal of broad-spectrum, cytolytic antimicrobial peptides.[4,5] Their high degree of chemical complexity is evidenced by the fact that they contain proteins, peptides, biogenic amines, alkaloids and other as yet uncharacterized biochemicals. Interestingly, peptides have <5 kDa molecular mass are the predominant molecules in the secretions of many frogs,[6] Five thousand living anuran frog species may produce about 100 000 different antimicrobial peptides. Colombia has $\sim 10\%$ of the world's biodiversity with more than 700 amphibian species representing a 'natural treasure trove' for novel discovery.[7,8]

We have adapted the micro-organ (MO) technology to study the *in vitro* secretions of frog skin derived MOs (SMOs) in serum-free defined-media. The technology is based on the fact that preservation of the basic epithelial–mesenchymal interactions allows for highly complex *ex vivo* function of epidermal cells. The approach is based on the preparation of organ fragments that preserve the basic microenvironment encountered by epithelial cells *in vivo* but with geometry and dimensions that ensure appropriate diffusion of nutrients and gases to all cells in culture.[9,10] Such fragments have been termed MOs to distinguish them from other tissue fragments that do not encompass the true organ structure.[9,11,12]

In the present work we report the collection and initial characterization of activities of frogs collected from highly varied habitats of Colombia. In addition we have collected DNA and RNA samples from each species for our records and for further analysis. We have obtained both *in vivo* and *in vitro* secretions and we further show that MOs prepared from frog skin (SMOs) can be successfully cultured for several days *in vitro*. During that period the frog SMOs secrete into the serum-free medium a whole repertoire of substances with powerful broad-spectrum antibacterial activities. Activities obtained from *in vitro* secretions were found, in most cases and the same concentrations, to be higher per gram of tissue than the actual secretions *in vivo*. More importantly, these active secretions were found not to be toxic to somatic cells even at 10-fold higher concentrations. We also confirmed that frog SMOs transcribe housekeeping genes when cultured for several days in serum-free medium.

MATERIALS AND METHODS

Frogs specimens

We have created a bank of skin secretions from 50 species of frogs collected from different regions of Colombia including the Guajira, upper Magdalena Valley, Amazone region, Andes piedmont and at the base of the eastern

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mountain range. Some of the species examined include: *Hypsiboas lanciformis*, *Sphaenorhynchus lacteus*, *Hypsiboas boans*, *Dendrobates truncatus* and *Pipa pipa*. The frogs were kept alive in a purpose-built amphibian facility at 25±1 °C under a 12-h light/dark cycle and fed with fruit flies three times per week.[13] All procedures involving frogs adhered to resolution 008430 of the Ministry of Health for the use of animals in research and the guidelines of the ethical committee on animal research from the Andes University. All captures were performed under the collection license No. 2 of the 20th of January 2009, from the Ministerio de Medio Ambiente, Colombia.

In vivo frog skin secretions

In vivo skin secretions were obtained from dorso-lateral skin folds by first washing the live animals with sterile distilled water and then were softly dried. This washing was followed by a s.c. injection of 70 µl (10 µg ml^{-1}) of norepinephrine[3] per gram of animal weigh. Four minutes after injection, the secretions were collected by washing the frog with 1 ml cm^{-2} of skin of a solution containing 70% Dulbecco's modified Eagle's medium (DMEM), 30% H_2O, over a period of 10 min and the solution obtained was then aliquoted and stored frozen at −80 °C before analysis.

Frog skin MOs

Adult specimens were anesthetized and killed applying 100 mg MS222 (3-aminobenzoic acid ethyl ester methanesulfonate salt, Sigma), directly to the tongue. Skin were removed immediately after the animals were killed and washed twice for 30 min with 70% DMEM supplemented with penicillin (1000 IU ml^{-1}), streptomycin (1000 IU ml^{-1}) and fungizone (1 µg ml^{-1}). Skin fragments from dorsal and ventral regions were cut into 4 mm width and 30 mm length flaps, and then transverse sectioned under sterile conditions parallel to the shortest dimension every 300 mm using a Mc Ilwain Tissue Chopper (Stoelting Co., Wood Dale, IL, USA). The SMOs obtained (4 mm long ×0.3 mm wide) were washed five times for 10 min with 70% DMEM supplemented with penicillin (1000 IU ml^{-1})/streptomycin (1000 IU ml^{-1}) and fungizone (1 µg ml^{-1}), and then twice for 10 min in 70% DMEM containing no antibiotics, nor antimycotic agents. Finally, 40 SMOs (0.5 cm^2 of skin equivalent) were cultured per well in 500 µl of serum-free 70% DMEM (Sigma-Aldrich, St Louis, MO, USA). No exogenous growth factors were added to the medium. The cultures were kept at 26 °C in 5% CO_2, in 24-well plates.[9]

Frog skin secretions

The media in which the SMOs were maintained in culture, denoted as conditioned media (CM), contained the *in vitro* secretions derived from frog skin. The CM was collected every day and replaced by the same volume of fresh 70% DMEM. Medium collected at days 1, 2 and 3 were filtered (0.2 µm pore size) and labeled CM1, CM2 and CM3, respectively, aliquoted and stored at −80 °C until required. Total concentration of skin peptides in each CM ranged between 300 µg and 600 µg ml^{-1} of CM, and was determined using a nanodrop 2000 with the protocol of A205 custom method for protein and peptide quantification (Thermo Scientific, Austin, TX, USA). Also, we used bradykinin (RPPGFSPFR) (Sigma Chemical, St Louis, MO, USA) to establish a standard curve.[14]

Histology

Histology was done by preparing 8 mm thick paraffin sections. SMOs were fixed for 30 min in phosphate buffered solution containing 4% paraformaldehyde, rinsed, dehydrated and embedded in paraffin. Staining was done with hematoxylin/eosin according to routine histological methods.[15]

Antibacterial assay

The antibacterial activities of frog skin secretions were tested using growth inhibition assays against human pathogenic bacteria. Colonies of *Staphylococcus aureus*, *Escherichia coli*, *Salmonella sp.* and *Enterobacter cloacae* (all isolated from clinical cases) were selected from agar and incubated overnight at 37 °C on BHI media (Brain Heart Infusion). Following incubation, the bacterial suspension was adjusted to achieve an OD (OD_{595}) of 0.04 and used as inoculum for the growth inhibition assay, plated in 96-well microtiter plates plus increasing amounts (0, 5 and 25% of CM. A total volume of 100 µl was obtained by adding the required volume of 70% DMEM. The positive control wells received SMO culture media (serum-free 70% DMEM (Sigma-Aldrich) instead of CM and negative control wells received 2 µg ml^{-1} of ampicillin.[13] Each test was performed in duplicate for each experimental condition. Plates were incubated at 37 °C and the OD_{595} was read at three different time points: 6, 8 and 24 h.

Growth inhibition was calculated using the following formula ((positive control OD − sample OD)/(positive control OD)) × 100 and expressed as a percentage.[13]

Cell culture

The cell lines used for the cytotoxicity of the CM, were *Cricetulus griseus* ovary chinese hamster cells (CHO-K1 cell line ATCC CCL-61, ATCC, Manassas, VA, USA), *Canis familiaris* Madin-Darby canine kidney (MDCK cell line ATCC CCL-34) and *Cercopithecus aethiops* African monkey kidney cells (COS-7cell line ATCC CRL-1651), respectively. The CHO-K1 cell line was grown as a monolayer culture in RPMI 1640 medium and MDCK and COS-7 in DMEM. Both media were supplemented with 10% FBS, (100 IU ml^{-1})/streptomycin (100 IU ml^{-1}) and fungizone (1 µg ml^{-1}). The cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

Cytotoxicity assay

Cell toxicity was monitored by determining the effect of the CM dilution (10 and 50%) on cell viability. Each CM dilution was made by diluting the collected CM with culture medium and added it to confluent CHO-K1 cell monolayers (3×10^5 cells per ml) in flat-bottomed, 96-well, microtiter tray. The cells with the CM dilutions were incubated at 37 °C in humidified 5% CO_2 for 48 h.

The cytotoxicity was determined by colorimetric methods based on the reduction of tetrazolium salts by viable cells (MTT assay).[16]

Briefly, 10 µl of MTT solution (2 mg ml^{-1} phosphate buffered saline solution) were added to each well of the microtiter tray after 48 h of incubation. The tray was then incubated at 37 °C for 4 h more. To dissolve the formazan crystals, 70 µl of dimethylsulphoxide was added to each well. After shaking the tray for 10 min, whereby formazan crystals were completely dissolved, the absorbance of the wells was read in a computer-controlled microplate reader (Bio-Rad, Philadelphia, PA, USA) at 595 nm. The percentage of viable treated cells was calculated in relation to untreated controls (viability percentage = OD-treated cells/OD control cells × 100%). The untreated control was taken as 100%.

RT-PCR analysis

For each sample, total RNA was extracted from five equal-sized SMOs, with acid-guanidine and phenol as described,[17] and reverse-transcribed (Promega Corporation, Madison, WI, USA). RT-PCR was done by running parallel reactions for each set of primers. Dermaseptin B4 primers were designed based on the published sequence of *Phyllomedusa bicolor*.[18]

Primer sets were as follows:
β-actin 5′-CGGAACCGCTCATTGCC-3′
5′-ACCACAACTGTGCCCATCTA-3′
Dermaseptin B4 5′-GACCAGACATGGCTTTCCT-3′
5′-TTGCTCCCTTGATTTCCA-3′

PCR products obtained were gel-purified, and sequenced using an ABI Prism 310 automated sequencer (Applied Biosystems, Carlsbad, CA, USA). The sequence obtained was subjected to homology search using the BLAST tool available at the NCBI database.

Statistical analysis

Shapiro–Wilk normality tests were performed on all data and one-way analysis of variances were applied after the distributions of data were found to meet the assumptions for parametric tests. Student's *t*-tests were used to compare the significance of growth inhibition and Tukey tests were used to compare significance of cytotoxicity effects. All statistics were performed using the software Statistics version 9 (StatSoft, Tulsa, OK, USA).

RESULTS

Xenopus SMOs remain viable for several days in culture

As a first step we used *Xenopus laevis* as a source of SMOs. We found that *Xenopus* SMOs remain viable for at least 1 week *in vitro* when cultured in 70% DMEM in the absence of serum at 26 °C and 5% CO_2. Figure 1a shows one SMO stained with MTT (right) as compared with an unstained SMO on the left after 8 days in culture. Figures 1b and c show standard hematoxilin eosin 8 μm histology sections of *Xenopus* SMOs after 8 days in culture at different magnifications, to indicate that both the epidermis remains stratified and the glands retain their intact architecture.

Frog skin secretes antibacterial substances for several days when cultured as SMOs in serum-free medium *in vitro*

In order to determine whether frog-derived SMOs secrete into the medium antibacterial activity, CM obtained as described in the methods section was tested against four strains of bacteria: *S. aureus*, *Salmonella sp.*, *E. cloacae* and *E. coli*. For each assay the *in vivo* skin secretions from each species was also collected as described above and tested in parallel to the *in vitro* secretions. In some cases as the one shown in Figure 2, CM was more powerful than the *in vivo* secretion. In others (Figure 3), a more similar pattern of activity was observed from the *in vivo* secretions as compared with the *in vitro* secretions. SMO cultures have been prepared from all species collected and CM has been systematically tested against the four bacterial strains as described in the previous section. On the whole, antibacterial activity was found in all species tested. Some of the species that showed more powerful antibacterial activities were tested further for their effect on somatic cell lines.

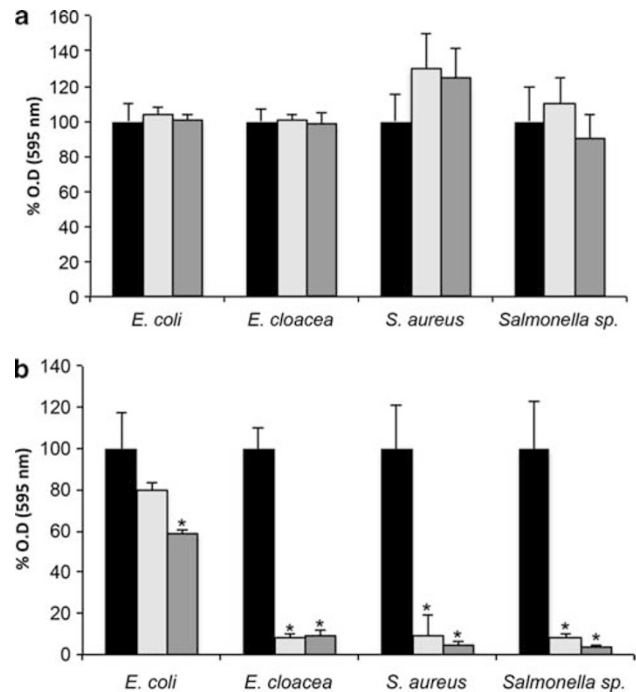

Figure 2 Comparison of antibacterial activity from *in vivo* (IV) (**a**) whole skin secretions and (**b**) SMOs *in vitro* secretion of *Sphaenorhynchus lacteus*. Bacterial growth control—no CM added—(solid bar), increasing amounts of CM 5% (light bar) and 25% (gray bar) of CM. The data are presented as the mean of two replicate samples from two independent experiments. $*P<0.05$.

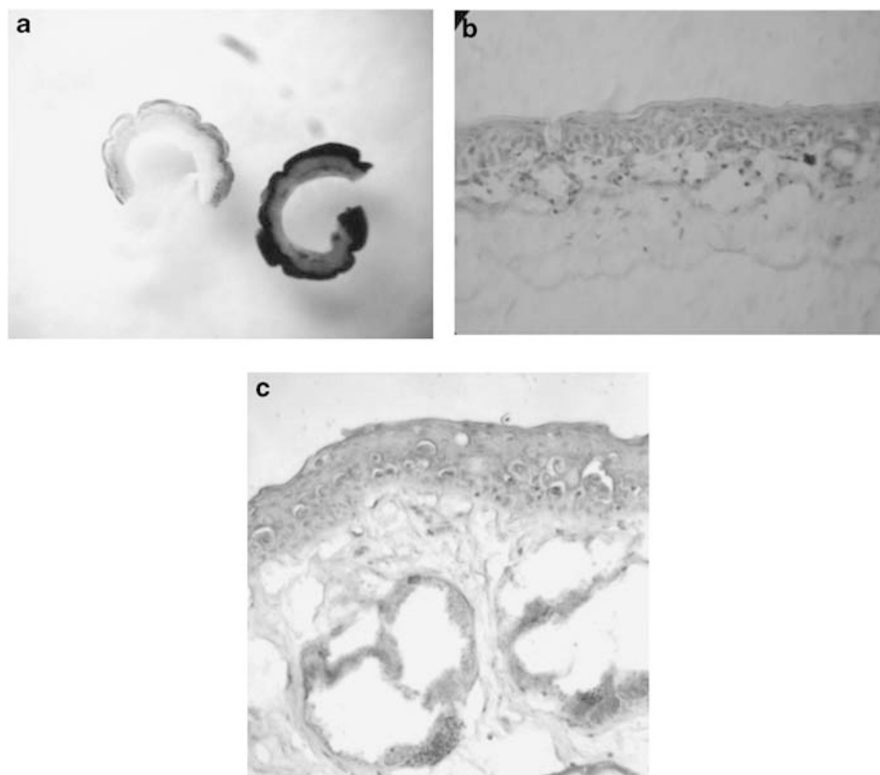

Figure 1 (**a**) MTT viability test of SMOs. (**b**) Light microscopic section of dorsal skin *Xenopus laevis* stained with haematoxilin-eosin. (**c**) Higher magnification illustrating an intact serous granular gland. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

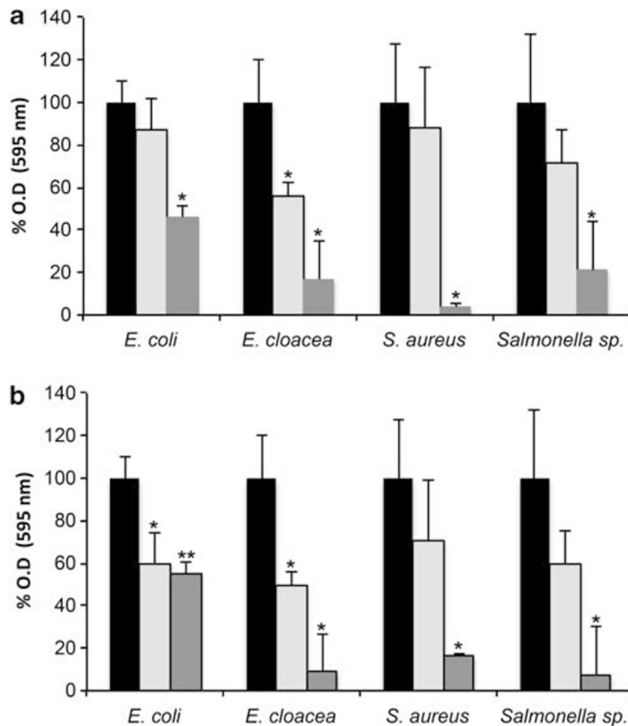

Figure 3 Comparison of antibacterial activity from *in vivo* (IV) (**a**) whole skin secretions and (**b**) SMOs *in vitro* secretion CM taken 1 day after culture of *Hypsiboas lanciformis*. Bacterial growth control—no CM added—(solid bar), increasing amounts of CM 5% (light bar) and 25% (gray bar) of CM. The data are presented as the mean of two replicate samples from two independent experiments. $^{*}P<0.05$, $^{**}P<0.001$.

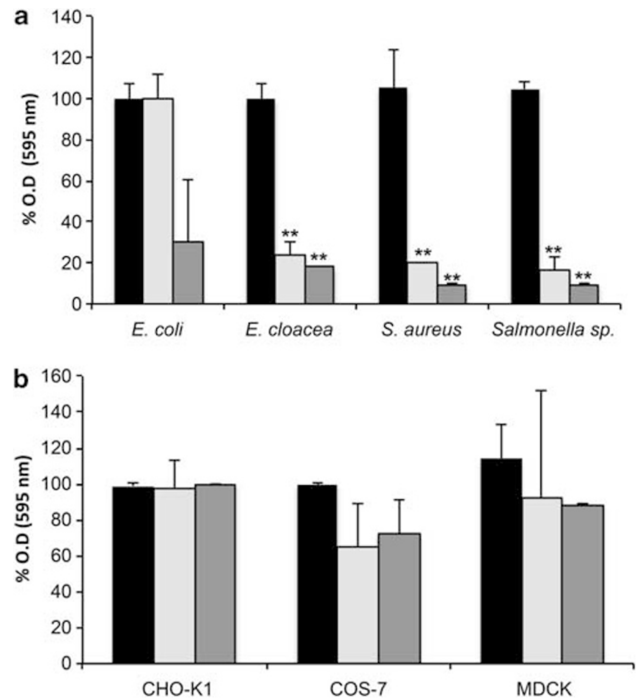

Figure 4 Secretion from SMOs of *Hypsiboas boans* (**a**) inhibited bacterial activity, CM obtained after 2 days, (**b**) with virtually no effect (CM2) on cell viability. Bacterial growth control—no CM added—(solid bar), increasing amounts of CM 5% (light bar and 25% (gray bar) of CM in antibacterial assays, 10% (light bar) and 50% (gray bar) in cytotoxic assays. The data are presented as the mean of two replicate samples from two independent experiments. $^{**}P<0.001$.

Secretions from SMOs in culture are not toxic to somatic cell lines even at 10-fold higher concentrations

As mentioned earlier, the major obstacle to the use of peptide-based anti-infective agents as useful drugs is their toxicities, particularly if they are to be administered systemically.[19] We were therefore interested to determine the toxicity of in culture secretions of frog SMOs. To that extent CM from the same species were tested in parallel against three different vertebrate somatic cell lines: CHO-K1, COS-7 and MDCK. As shown in Figure 4, CM taken after 2 days (CM2 (A)) from SMO cultures of *H. boans* inhibited bacterial activity even when only 5% of CM was added to the test system. Yet, as shown in Figure 4b CM2 even at a concentration 10 times higher was found to have no effect on cell growth of any of the cell lines tested. Similarly CM4 (data not shown) from the same species was found not to inhibit cell growth of the CHO-K1 cell line, but had a roughly 50% decrease in the number of COS-7 and MDCK cells as compared with untreated controls. Figure 5 shows that CM obtained from SMOs derived from *D. truncatus* after 3 days in culture (CM3) (Figure 5a) inhibited bacterial activity in a dose-dependent manner. Yet no effect was observed with a 50% concentration of the same conditioned medium (CM3) in the viability of any of the somatic cell lines tested (Figure 5b). Comparison of Figures 6a and b show that CM obtained from *P. pipa* after 1 day in culture (Figure 6a) had a powerful antibacterial activity. This activity was found not only not to be toxic to the somatic cell lines tested but also to be stimulatory to MDCK cells (Figure 6b).

SMOs from *P. bicolor* transcribe a dermaseptin gene

We were particularly interested in determining if the frog-derived SMOs were de-novo transcribing tissue-specific genes. To that extent and in order to obtain a measure of integrity and viability of the cultures, SMO samples obtained from *P. bicolor.*, *S. lacteus*, *H. boans* and *D. truncatus* (data not shown), were cultured as described above, samples removed every 24 h and total RNA prepared. Viability was confirmed by the integrity of the RNA during 1 week in culture. All four species transcribed at steady levels the housekeeping gene actin for the whole culture period of 6 days (data not shown). Furthermore, Figure 7 shows that SMO cultures continue to transcribe mRNAs at sustained levels for a whole week *in vitro*.

Using primers directed to highly conserved regions of Dermaseptin B4, bands were amplified, as expected, from SMOs derived from *P. bicolor.* Interestingly, a band was also amplified in samples obtained from *S. lacteus*. The identities of the bands obtained were confirmed by sequencing. Sequences from both species showed 98% identity on the conserved region. The sequence of the variable region obtained from *P. bicolor* was found to be 93% similar both in size and in sequence to those reported for the Phyllomedusinae subfamily as expected. However, the variable region obtained from *S. lacteus* was found to be considerably shorter and only 49% similar to that of the Phyllomedusinae subfamily (data not shown).

Patterns of bacterial inhibition do not seem to be related to specific habitats

Activities of the different species tested have been summarized in Table 1. The table shows concentration of conditioned medium required to bring about 50% inhibition of bacterial cell growth (bacterial inhibition LD_{50}). Antibacterial activities are organized in Table 1 by species and their habitat and site of origin. No specific patterns of activities could be assigned to particular habitats. For

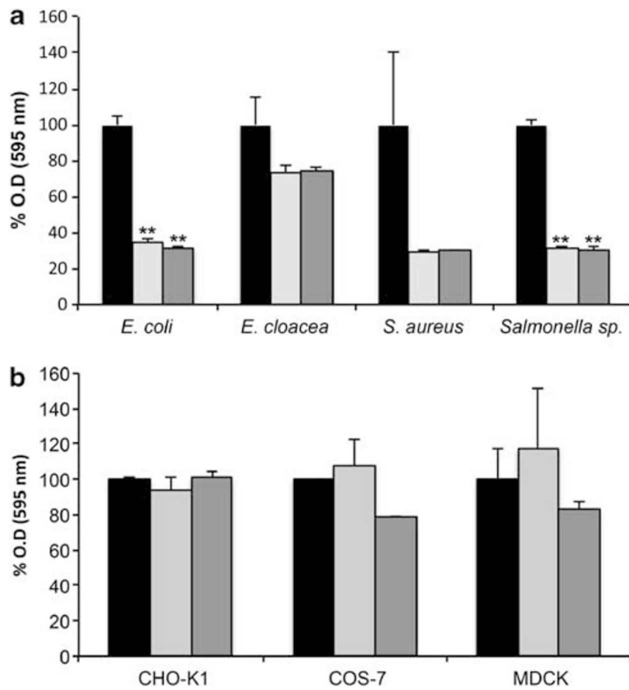

Figure 5 Secretions from SMOs of *Dendrobates truncatus* CM3 (**a**) inhibit bacterial in a dose-dependent manner and (**b**) are not toxic to somatic cell lines. Antibacterial assay—no CM3 added—(solid bar), increasing amounts of CM3 5% (light bar) and 25% (gray bar). Cytotoxic assays: 10% of CM3 (light bar), 50% of CM3 (gray bar). The data are presented as the mean of two replicate samples from two independent experiments. $^{**}P<0.001$.

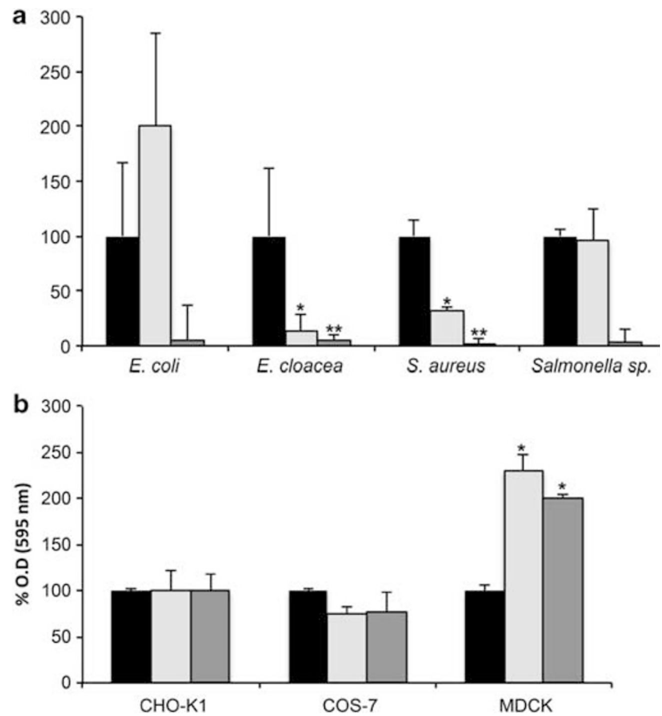

Figure 6 Secretions from SMOs of *Pipa pipa* (**a**) inhibited bacterial activity while at the same time (**b**) stimulated growth of MDCK cells in culture. CM was taken after first day of SMO culture (CM1). Antibacterial assay: no CM1 added (solid bar), increasing amounts of CM1: 5% (light bar) and 25% (gray bar). Cytotoxic assay: 10% of CM1 (light bar), 50% of CM3 (gray bar). The data are presented as the mean of two replicate samples from two independent experiments. $^{*}P<0.05$, $^{**}P<0.01$.

example, CM1 of *H. lanciformis* found in Leticia (Amazon) displayed an LD_{50} of 5% in *S. aureus* and *salmonella* and of 25% in *E. Cloacae*. Yet, 70% CM1 was required to bring about the same level of inhibition in *E. coli*. In contrast, a CM1 concentration of 35% was required from *Hypsiboas hobssi*, found in the same habitat as *H. lanciformis* in order to achieve the same LD_{50} in all four bacterial species tested. CM1 derived from cultures of *Scinax cruentommus* obtained from the same area on the Amazon basin was found to have a much weaker antibacterial activity on all bacterial species tested (data not shown). If we now look at species collected in other different and varied habitats we see that some like *Centrolene sp.* were highly active in all species tested while *Scinax ruber* showed varied but generally lower activities. Only 5% of CM3 of *D. truncatus* (from the same habitat) was required for an LD_{50} for three of the species tested, and more than 70% was required to bring about the same LD_{50} in *E. cloacae*. In contrast, *P. pipa* obtained in Leticia (Amazon) displayed high activity for *S. aureus* and *E. cloacae*, and intermediate activity against *Salmonella* and against *E. coli* (see Table 1). Clearly, there does not seem to be any specific pattern that assigns specific activities to certain habitats.

DISCUSSION

We have shown in the past that preservation of the epithelial–mesenchymal interactions in mammalian SMOs allows keratinocytes to continue to proliferate, and to transcribe epidermal-specific genes for long periods when cultured in defined medium in the absence of serum or exogenous factors.[9,12] SMOs were found to retain those properties irrespective of whether they were derived from new born or adult skin even though they were cultured in serum-free medium. In the present work we show that in frog-derived SMOs, not only do epidermal cells continue to transcribe housekeeping and tissue-specific genes, but also that whole secretory glands remain functional for several days in culture. In fact we show that secretions from frog SMOs in culture were, in several cases, more powerful antibacterial agents than those obtained from *in vivo* secretions. These results taken together indicate that frog SMO culture can provide a powerful method to identify secretory molecules from frog skin. As the SMOs act as bioreactors, the amount of secretion that can be obtained per animal is amplified thus minimizing the number of specimens required for the characterization process.

Our strategy has been first to create a bank of substances secreted by amphibian skin from a variety species living in far and varied habitats of Colombia. This bank is of particular importance as some of the species are in danger of disappearing. It is has been suggested that every species harbors a unique, specific collection of antimicrobial peptides, tuned to defend the organism against microorganisms that it is likely to encounter.[13]

As shown in Table 1, we do not seem to find any specific pattern, which assigns specific activities to certain habitats. In fact what seems to be the case is the opposite. On second thoughts, this may be understood on the bases that the habitats visited are extremely rich in microbial diversity. Around 8000 species of prokaryotes have been described but they form only a very small fraction of the true diversity.[20] Techniques based on analysis of environmental DNA let to suggest that the total number of species of bacteria (as based on the current broad species definition) may be in the order of 10^9–10^{12}.[21] Thus, it is unlikely that amphibian species have become specialized but rather have developed a broad-spectrum strategy in order to cope

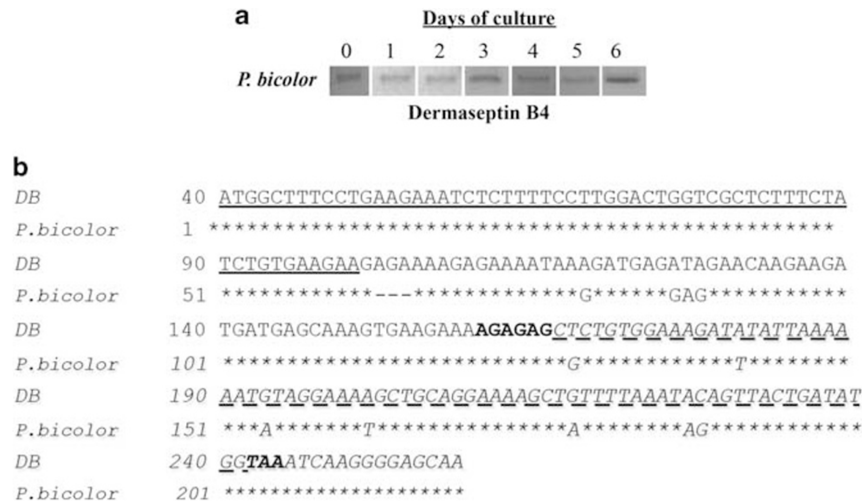

Figure 7 Gene expression were produced in SMOs from *Phyllomedusa bicolor*. (**a**) Amplification for specific antimicrobial gene dermseptin B4 (GI:3256038). (**b**) Alignment of nucleotide sequence of encoding precursor of dermaseptin B4 (DB4), data base (db) and *P. bicolor* (ClustalW Tool). The putative signal peptide (single-underlined), mature processed peptide (dashed line), processing site KR, stop codon (bold) and nucleotides conserved (asterisks) are indicated.

Table 1 Comparison of the antibacterial activities from frog skin secretions of different regions of Colombia

	Site of origin	*Antibacterial inhibition LD_{50}*			
Frog species	*Type of habitat*	*S. aureus*	*Salmonella*	*E. cloacae*	*E. coli*
Hypsiboas lanciformis	Leticia (Amazone)	5	5	25	>70
Hypsiboas hobssi	Tropical moist lowland forests	35	35	35	35
Cochranella punctulata endemic	Mariquita (Tolima)	5	5	5	20
Scinax ruber	Tropical dry forests and rainforest	70	35	35	35
Pleurodema brachyops	Palomino (Guajira)	70	5	20	>70
Pseudis paradoxa	Tropical dry forests	5	35	5	5

with such variety. It is this line of though that directed our approach not towards individual substances but rather to a whole repertoire of substances that act in synergism, with the mixture having up to a 10-fold greater antibiotic activity than the peptides separately.[13,22]

The methodology presented here allows for *in vitro* production, analysis and characterization of the 'natural mixture' of compounds secreted by different anuran species in order to cope with the highly hostile environment in which they live. In this respect it should be pointed out that some of the activities reported here, as shown in Figures 5 and 6, were very potent against various pathogenic bacteria without having any toxic effects on somatic cells even at 10-fold higher concentrations.

It is becoming clear that each ranin or hylid frog species produces its own set of antimicrobial peptides. Some of these peptides differ by only a few amino acid substitutions or deletions and have similar biochemical characteristics (that is, dermaseptins B1 and B2).[23]

Many infections that would have been cured easily by antibiotics in the past now are resistant, resulting in sicker patients and longer hospitalizations. The economic impact of antibiotic-resistant infections is estimated to be between $5 and $24 billion per year in the United States alone.[24] There is therefore an urgent need for novel antibiotics, many peptides with antibacterial properties have been identified in the past from other species and several are in stage III of clinical trials. A recent review lists seven companies involved in the development of antibacterial peptides as drugs.[25] There are, however, many challenges awaiting solution: in order to be a good candidate for therapeutic use, a drug needs to show appropriate function, low toxicity, have stability *in vivo* and be reasonably inexpensive to manufacture. So far only a few cationic peptides have made their way to clinical trials and only two are used in topical creams and solutions. A problem associated with administering cationic peptides as a treatment for infection is the ability to direct the peptides to the appropriate locations with the accuracy of white blood cells after crossing the epidermal barrier.[2] When injected i.v. the peptides are required to infiltrate healthy tissue in order to reach the appropriate locations, which can be a very slow process. The host's system can also act on the peptides; for example, the presence of proteases can inactivate peptides before they reach their destination.[26] A major problem that limits the systemic use of these compounds is toxicity.[19] In the present work, we have started to address this latter point. We believe the reason that we found, on the one hand a strong antibacterial activity and on the other, hardly any toxicity is because of the approach taken where the whole repertoire of skin secretions instead of individual components was tested for activity. Thus, we have screened for activities, which are likely the result of synergism between various substances secreted by the skin of the different frog species. The next step is to work with those species where the most powerful and specific activities (that is, no toxicity to somatic cells) have been obtained. Peptide composition of CM secreted by these species is being characterized by LC MS/MS (data not shown).

Once the sequence identity of the various peptides is obtained, the peptides will be synthesized and different cocktails tested again for antimicrobial activity. Furthermore, it is believed that comparison of combinations of peptides obtained in the different secretions vis a vis their biological activity will help formulate better and more specific antibiotics.

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