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Screening bacterial metabolites for inhibitory effects against *Batrachochytrium dendrobatidis* using a spectrophotometric assay

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ABSTRACT: Certain bacteria present on frog skin can prevent infection by the pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*), conferring disease resistance. Previous studies have used agar-based *in vitro* challenge assays to screen bacteria for *Bd*-inhibitory activity and to identify candidates for bacterial supplementation trials. However, agar-based assays can be difficult to set up and to replicate reliably. To overcome these difficulties, we developed a semi-quantitative spectrophotometric challenge assay technique. Cell-free supernatants were prepared from filtered bacterial cultures and added to 96-well plates in replicated wells containing *Bd* zoospores suspended in tryptone-gelatin hydrolysate-lactose (TGhL) broth medium. Plates were then read daily on a spectrophotometer until positive controls reached maximum growth in order to determine growth curves for *Bd*. We tested the technique by screening skin bacteria from the Australian green-eyed tree frog *Litoria serrata*. Of bacteria tested, 31% showed some degree of *Bd* inhibition, while some may have promoted *Bd* growth, a previously unknown effect. Our cell-free supernatant challenge assay technique is an effective *in vitro* method for screening bacterial isolates for strong *Bd*-inhibitory activity. It contributes to the expanding field of bioaugmentation research, which could play a significant role in mitigating the effects of chytridiomycosis on amphibians around the world.

KEY WORDS: Chytridiomycosis · Challenge assay · Bacterial metabolite · Amphibian disease · Bioaugmentation

INTRODUCTION

One of the many causes of worldwide amphibian declines and associated biodiversity loss is the emergence of chytridiomycosis, an infectious disease caused by the pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*; Berger et al. 1998, 1999). Amphibians have well-developed immune systems, with both innate and adaptive components (Voyles et al. 2011, Rollins-Smith & Woodhams 2012). Research into the role of the adaptive immune system in defending against *Bd* is in its infancy (Richmond et al. 2009, Ramsey et al. 2010, Savage & Zamudio 2011). In contrast, innate immune defences are known to be effective against *Bd* in many species (Rollins-Smith 2009). Anti-microbial peptides (AMPs), produced in skin glands, may be an important part of this innate immune defence (Rollins-Smith et al.

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2002a,b, Rollins-Smith & Conlon 2005, Woodhams et al. 2006a,b, 2007a). Microbes that colonise amphibian skin can also confer disease resistance on their hosts (Bettin & Greven 1986, Harris et al. 2006, Lauer et al. 2007, 2008, Woodhams et al. 2007b, Banning et al. 2008). Symbiotic microbes can therefore be regarded as another component of amphibians' innate immune defence mechanisms (Woodhams et al. 2007b, Walke et al. 2011). Symbiotic bacteria resident on anuran skin may sometimes play a greater role than AMPs in defence against *Bd*, and AMP potency may be attenuated in species that rely heavily on their beneficial microbiota (Conlon 2011).

Harris et al. (2006) first demonstrated *in vitro* inhibition of *Bd* by a number of bacterial species isolated from the skin of salamanders, and suggested that *Bd*-inhibiting bacteria could be used as a bioaugmentation tool to confer disease resistance to vulnerable amphibian populations. Laboratory trials involving inoculation with a *Bd*-inhibiting bacterium, prior to pathogen exposure, have subsequently demonstrated that this is possible (Harris et al. 2009a,b, Becker & Harris 2010).

Historically, investigations of the antimicrobial activity of microbes have used challenge assays. These *in vitro* techniques, which test the effectiveness of microbial metabolites against a particular microorganism, are typically undertaken using an agar or broth medium. Agar-based methods include disk diffusion, well diffusion and direct inoculation, while broth-based techniques use a liquid medium and are often coupled with spectrophotometry to quantitatively measure microbial growth (Jenkins et al. 1998). Agar diffusion techniques are usually non- or semi-quantitative screening methods that aim to detect the formation of a zone of inhibition, often around an antibiotic-impregnated disk or well. This zone can be measured, enabling ordinal comparisons of results among different test organisms. However, antibiotic compounds can vary in their rate of diffusion through agar (Barry 1980, du Toit & Rautenbach 2000). Broth microdilution techniques are more time-consuming to perform and typically require some knowledge of the test compound concentration. Challenging the target organism with a range of dilutions of the test compound can facilitate determination of a minimal inhibitory concentration (Rollins-Smith et al. 2002b).

Published studies investigating bacterial antagonism against *Bd* have used agar plate assay methods (Harris et al. 2006, Woodhams et al. 2007b, Brucker et al. 2008a,b). In these, *Bd* zoospores are spread evenly across an agar plate surface, which is subsequently streaked directly with a bacterial isolate. The quantities of *Bd* and bacteria inoculated onto plates are not standardised, and live cultures of both of the organisms interact directly. Although this method can be used to screen large numbers of bacterial isolates over a relatively short time, it has potential disadvantages. Moisture requirements can vary between *Bd* strains, and these are not always compatible with those of the test bacteria, which can easily overgrow the *Bd*. In addition, because a distinct zone of inhibition must be observed, agar plate assays may fail to detect isolates that produce antibiotic compounds that diffuse slowly through agar. Finally, they could be subject to priority effects (Kennedy & Bruns 2005, Peay et al. 2012); *Bd* is established first, and may itself inhibit the growth of some bacteria, including isolates that would otherwise show strong activity against it.

To address the problems identified above, we developed a semi-quantitative broth-based assay technique. The method combines attributes of a fully-quantitative assay used to screen anuran AMPs for effectiveness against *Bd* (Rollins-Smith et al. 2002b), where both the concentration of *Bd* and AMPs are known, and a non-quantitative broth-based assay used to detect antifungal compounds produced by cutaneous bacteria from salamanders (Walters 2007). In our technique, the concentration of *Bd* zoospores is known, while the concentration of the substance being assayed is unknown. The technique allows us to visually distinguish between fungistatic and fungicidal effects, and eliminates the possibility of direct competitive or priority effects.

In this paper, we describe the challenge assay methodology, including techniques and analysis of results. We applied this new challenge assay method to bacteria collected from wild frogs, and we report results that demonstrate the potential benefits of this technique.

MATERIALS AND METHODS

Field site and species

We collected bacterial samples from the skin of green-eyed tree frogs *Litoria serrata* found on rocks and vegetation bordering Frenchman's Creek (17° 18′ 32.8″ S, 145° 55′ 04.2″ E) at an elevation of 40 m. The study site is located in rainforest within Wooroonooran National Park, Queensland, Australia. Four adult frogs were captured, held briefly for sampling, and then released at the point of capture.

Sampling resident bacteria

We hand-captured frogs using clean plastic bags and rinsed their skin twice using sterile distilled water to remove transient bacteria that may not be part of the resident microbiota (Lauer et al. 2007). A damp sterile rayon swab (MW112, MW&E) was wiped over the dorsal and ventral skin of the frog from knee to neck 5 times. Bacteria were then transferred to a low nutrient agar plate (R2A, Becton-Dickinson) by rotating and smearing the swab on the plate surface in a wide zigzag pattern. Plates were sealed with Parafilm 'M' (Pechiney Plastic Packaging), stored at ambient temperature (15 to 25°C) and returned to the laboratory within 72 h. After swabbing, we released all frogs at the point of capture.

Isolation and purification of microbial cultures

Inverted R2A agar plates were incubated at ambient temperature (18 to 26°C) in the laboratory for 48 to 72 h until growth was observed. Colonies with different morphological characteristics were identified using a dissecting microscope. Each unique colony was then isolated to axenic (pure, uncontaminated) culture using standard microbiological techniques (Salle 1961). Briefly, each isolate was selected from a single colony using a sterile toothpick, streaked multiple times on non-overlapping sections of a R2A agar plate to thin out the number of bacterial cells, and allowed to grow for 24 to 48 h. This process was repeated until no contaminating organisms were observed. Axenic isolates were held on R2A agar slants at room temperature until challenge assays were conducted, typically between a few weeks and 4 mo. We found that almost all isolates remained viable for more than 6 mo in this form without the need for regular passaging which might have altered the properties of the bacterial sample. For long-term preservation and assurance, a sample of each newly isolated bacterial sample was frozen at –80°C in trypticase soy yeast broth with 20% glycerol (2 g trypticase soy broth, 1 g yeast extract, 200 ml glycerol, to 1 l deionised water, autoclaved).

Preparation of *Bd* cultures

We selected *Bd* isolate 'Gibbo River, L. Les, 06-LB-1' as the strain to be challenged for its known virulence in frog infection trials (Berger et al. 2005). The isolate was maintained in sterile tryptone-gelatin hydrolysate-lactose (TGhL) broth medium (8 g tryptone, 1 g hydrolysed gelatin, 2 g lactose, to 1 l deionised water, autoclaved), passed to TGhL agar plates (as above with 10 g bacteriological agar) after 7 d growth, and incubated at 23°C. On Day 3 after inoculation, plates were flooded with 3 ml sterile TGhL broth to create a suspension of zoospores, which was collected and vacuum filtered through a sterile 20 µm nylon filter (Spectra Mesh, Spectrum Laboratories) to remove sporangia. The filtered zoospores were counted on a haemocytometer and resuspended in sterile TGhL to a concentration of 2×10^6 ml^{-1}.

Preparation of bacterial cell-free supernatants

Axenic isolates were each inoculated into 1 ml sterile TGhL medium in 24-well plates (Costar 3524, Corning) and incubated at 23°C for 48 h. We then transferred each culture to a sterile 1.5 ml microtube that was centrifuged at 7500 × *g* (5 min) to pellet the cells. The supernatant was then filtered through a sterile 0.22 µm syringe filter (Millex GV, Millipore) to remove all cells, leaving bacterially produced metabolites in TGhL medium.

Challenge assay

We set up challenge assays in 96-well microplates (Costar 3595, Corning). Experimental wells contained 50 µl of *Bd* zoospores at a concentration of 2×10^6 ml^{-1} and 50 µl of a bacterial cell-free supernatant sample. Positive control wells contained 50 µl *Bd* and 50 µl TGhL medium. Negative controls contained 50 µl heat-killed *Bd* (60°C for 60 min) and 50 µl TGhL. An additional (medium-only) control consisted of wells containing 100 µl replicates of TGhL alone. A complete plate contained 10 replicates of each positive control, 5 replicates of each negative control, 19 wells containing the medium-only control and 5 replicate experimental wells for each of 12 bacterial isolates.

Microplates were incubated at 23°C until maximum growth in the positive controls was observed (typically after 6 to 8 d of growth); readings were taken using a spectrophotometer (Multiskan Ascent, Thermo Scientific) with a 492 nm filter immediately after plate set-up was complete (Day 0) and every 24 h thereafter. After a minimum of 3 d of growth, we examined the plate visually using an inverted microscope, to provide an independent record of the culture appearance and to identify and exclude wells where bacterial or fungal contamination developed.

Nutrient depletion in cell-free supernatants

Experimental wells in challenge assays contained 50 µl of cell-free supernatants in which bacteria had previously been cultured, while controls contained 50 µl of previously unused medium. Because of this, the growth of *Bd* in experimental wells could have been affected by depletion of nutrients used by the bacteria, as well as by bacterial metabolites. To determine the range of effects that could be produced by nutrient depletion alone, we grew *Bd* in 96-well microplates with various concentrations of TGhL medium to simulate nutrient depletion in cell-free supernatants. Experimental wells contained 50 µl of *Bd* zoospores at a concentration of 2×10^6 ml^{-1} and 50 µl of TGhL broth at 11 different concentrations from 100 to 0%, where 100% was undiluted TGhL medium and 0% was sterile distilled water. Five replicates of each dilution had live *Bd* zoospores and 3 replicates had heat-killed *Bd* (60°C for 60 min) controls. Four replicate microplates, with independent zoospore counts, were incubated and read on a spectrophotometer as described in the previous sub-section.

Data analysis

Challenge assay

For each day's data, we first calculated the mean daily absorbance values of all replicates of each type of control and experimental sample. Then, to remove baseline absorbance, we subtracted the mean negative control value on each day from each of the mean sample and positive control values on that day, to obtain corrected absorbance values. To adjust the dataset further for background colour, which was present in some samples, we subtracted the corrected mean sample values on Day 0 from each daily mean sample value. We repeated this process by subtracting the positive control corrected mean absorbance value on Day 0 from each daily mean positive control value. We then plotted growth curves for each sample and positive control. If positive control growth curves did not follow the standard growth curve shape with a lag, log and stationary phase, the assay was repeated. The proportion of inhibition or enhancement of each sample relative to the positive control was then calculated by dividing the corrected absorbance value for each sample on its maximum growth day by the corrected absorbance value of the positive control on its maximum growth day. A value of 1 was subtracted from all proportional values so that positive values represented growth above that of the positive control and negative values represented potential growth inhibition. Data adjustments are summarized in Table 1.

Nutrient depletion

Each dilution of TGhL was treated as a different sample, and heat-killed replicates were used as negative controls for that sample. The 100% TGhL live *Bd* sample was considered the positive control. Data corrections were performed as described in the previous sub-section, except that colour corrections were not necessary as no cell-free supernatants were used, and negative control data for each sample for each day were subtracted from the corresponding sample containing live *Bd*. For each microplate, the proportion of growth relative to the positive control was calculated for each dilution.

Table 1. Summary of terms and their definitions, and of calculations performed on raw spectrophotometric absorbance data

Term	Definition	Calculation
i	Individual isolate	
j	Individual day from 0 to n	
P_j	Mean measured absorbance of all positive controls on day j	
N_j	Mean measured absorbance of negative controls on day j	
S_{ij}	Mean measured absorbance of samples of isolate i on day j	
PA_j	Baseline absorbance-corrected positive controls on day j	$PA_j = P_j - N_j$
SA_{ij}	Baseline absorbance-corrected samples of isolate i on day j	$SA_{ij} = S_{ij} - N_j$
PAC_j	Colour-corrected positive controls on day j	$PAC_j = PA_j - PA_0$
SAC_{ij}	Colour-corrected samples of isolate i on day j	$SAC_{ij} = SA_{ij} - SA_{i0}$
PAC_{max}	Corrected positive control value on maximum growth day	
SAC_{imax}	Corrected sample value on maximum growth day	
X_i	Proportion inhibition or enhancement relative to positive control	$X_i = (SAC_{imax}/PAC_{max}) - 1$

RESULTS

We isolated and tested a total of 94 bacterial isolates from the 4 *Litoria serrata* sampled in our study. As a result of visual observation of the replicate microplate wells (see Fig. 3), together with the calculated proportion of inhibition and examination of each growth curve (see Fig. 2B), cell-free supernatants were considered totally inhibitory if no growth was observed. Totally inhibitory isolates occurred on all 4 frogs. Of all isolates tested, 23% were totally inhibitory to *Bd.*

Nutrient depletion of medium by bacterial isolates can account for some of the partial inhibition observed in challenge assays. Results from the 4 replicate microplates showed that a progressively greater proportion of apparent *Bd* inhibition, relative to the positive control, occurs with decreasing nutrient concentrations. The proportion of inhibition for the four 100% nutrient depleted samples was 54.6, 63.5, 44.7 and 31.7%. This range of results is due to unavoidable slight variation in zoospore counts among assays. We therefore chose to use the maximum inhibition observed (63.5%) as our cut-off value; isolates that produce inhibition beyond this value must exhibit true inhibition against *Bd*. This figure is conservative because it is unlikely that complete nutrient depletion during bacterial culture always (or even frequently) occurs; if it did, there should be a peak in the frequency distribution at 50 to 60% inhibition (Fig. 1); in fact, there is a local minimum at that point.

Once the cut-off value of 63.5% inhibition was applied to the data to account for nutrient depletion effects, an additional 8% of cell-free supernatants were partially inhibitory to *Bd*, while 70% were non-inhibitory. Of those non-inhibitory isolates, 34% enhanced the growth of *Bd* by more than 25%. Cell-free supernatant effects on *Bd* growth showed a bimodal distribution with 1 peak of inhibitory isolates and 1 of non-inhibitory isolates (Fig. 1). The identification of totally inhibitory isolates is shown in Table S1 in the supplement at www.int-res.com/articles/suppl/d103p077_supp.pdf.

Fig. 2 presents a set of challenge assay growth curves that demonstrate the range of possible outcomes for each isolate tested. Uncorrected mean absorbance values are plotted in Fig. 2A and the corrected absorbance values in Fig. 2B. The corrections align the negative control along the *x*-axis and remove the effect of colour, which could in some cases mask inhibition in our spectrophotometric assays. We present error bars indicating ± 1 SD; these

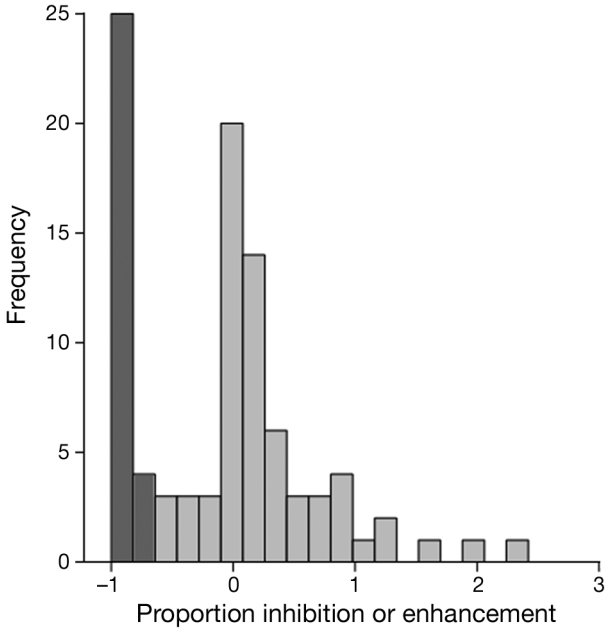

Fig. 1. *Batrachochytrium dendrobatidis.* Bimodal distribution of cell-free supernatant effects upon *B. dendrobatidis* growth. Negative (positive) values indicate growth less (greater) than that of the positive control. Dark grey shading: all cell-free supernatants with inhibitory effects >63.5% and ≤100%; pale grey shading: non-inhibitory cell-free supernatants

should be a standard part of the presentation of outcomes for this assay. They indicate the degree of dispersion of replicates of each treatment or control on each microplate. Excessive variance would indicate contamination or inaccurate pipetting. We also suggest that comparisons of mean positive control values among plates set up at the same time should be carried out to identify and eliminate plates that did not grow as expected. In addition, comparison of mean positive control values among batches of plates set up at different times should be conducted to check accuracy of zoospore cell counts. Our mean maximum positive control absorbance values across all plates in this study was 0.17 (SD = 0.024, n = 18).

Visual inspection of microplates revealed a range of cell-free supernatant effects on *Bd,* from inhibitory to enhancing. Our photos of positive and negative control wells (Fig. 3A,B) are typical of those treatments and provide a reference for visual categorization of other effects. Visual inspection is important as it demonstrates that the effects we observed result from a variety of mechanisms. Total inhibition can result from a simple lack of growth, as seen in the negative control, and can also result from *Bd* cell lysis, as shown in Fig. 3F. Partially inhibitory effects range from the very low-level growth shown in

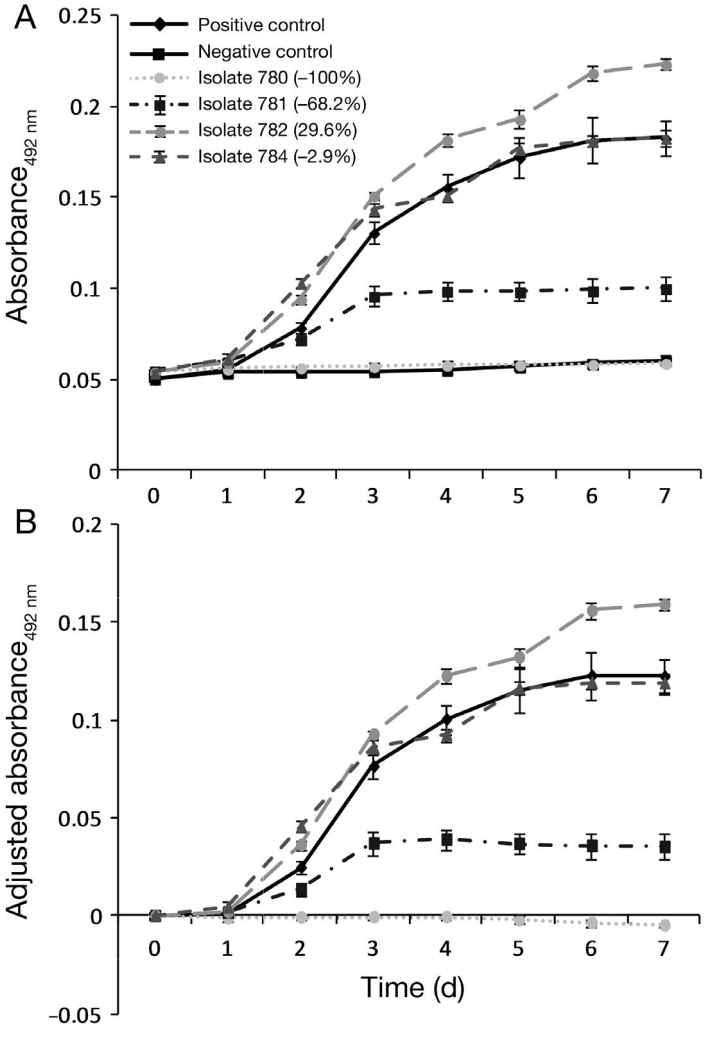

Fig. 2. *Batrachochytrium dendrobatidis*. Growth curves showing spectrophotometric absorbance values for selected isolates demonstrating a range of effects on *B. dendrobatidis* growth, along with positive and negative controls. Selected isolate ID numbers are accompanied by percentage values in parentheses showing growth relative to the positive control, calculated as described in Table 1. (A) Uncorrected mean daily absorbance values (error bars represent ±1 SD from the mean). (B) After corrections to subtract background absorbance and removal of the effect of colour from the sample. Where not visible, error bars are smaller than the symbols used

Fig. 3C to the formation of sporangia without zoospore development (Fig. 3D). Growth enhancement results from increased numbers of zoospores and sporangia as shown in Fig. 3E.

DISCUSSION

We have developed a semi-quantitative challenge assay for testing bacterial cell-free supernatants for activity against *Bd* that reliably distinguishes totally inhibitory bacterial isolates. Our technique improves on the previous method used to screen bacteria for activity against *Bd*, as it removes some potential confounding effects and allows trials to be run in a more controlled manner. We have validated the technique through identification of totally inhibitory isolates; isolate identities (shown in the supplement at www.int-res.com/articles/suppl/d103p077_supp.pdf) are in line with those found on frogs in other studies (Woodhams et al. 2007b, Walke et al. 2011).

Previous studies using agar plates have scored challenge assay results only as either inhibitory or non-inhibitory (Harris et al. 2006). Our study demonstrates that bacterial cell-free supernatants can have a number of different effects on growing *Bd* cultures, from lysis of cells to growth enhancement (Fig. 3). The mode of inhibitory action is most probably through antibiotic production, but other components such as bacteriocins (peptides produced by bacteria), bacterial degradation products, organic acids, lysozymes and proteases may also play a role (see Verschuere et al. 2000). Enhancement of *Bd* growth may occur when bacterial metabolites serve as nutrients, or through hormetic effects, where very low concentrations of antibiotics promote growth that is inhibited by higher concentrations (Southam & Ehrlich 1943, Stebbing 1982). Both of these possibilities may be potentially important in nature, and are worthy of further investigation. However, the analysis of this effect is beyond the scope of this study, which is primarily designed to screen for isolates that can exhibit strong inhibition against *Bd*. Therefore, until further investigation is undertaken, enhancing effects should probably be best considered as non-inhibitory.

Benefits and limitations

Our challenge assay offers a number of advantages over the agar plate technique used to date. The use of a fixed quantity of *Bd* zoospores, instead of the non-standardised quantity used in the agar plate method, removes one source of variation from the resulting data, making our technique semi-quantitative. The agar plate assay can also be difficult to set up. For example, we found that the strain of *Bd* we used would

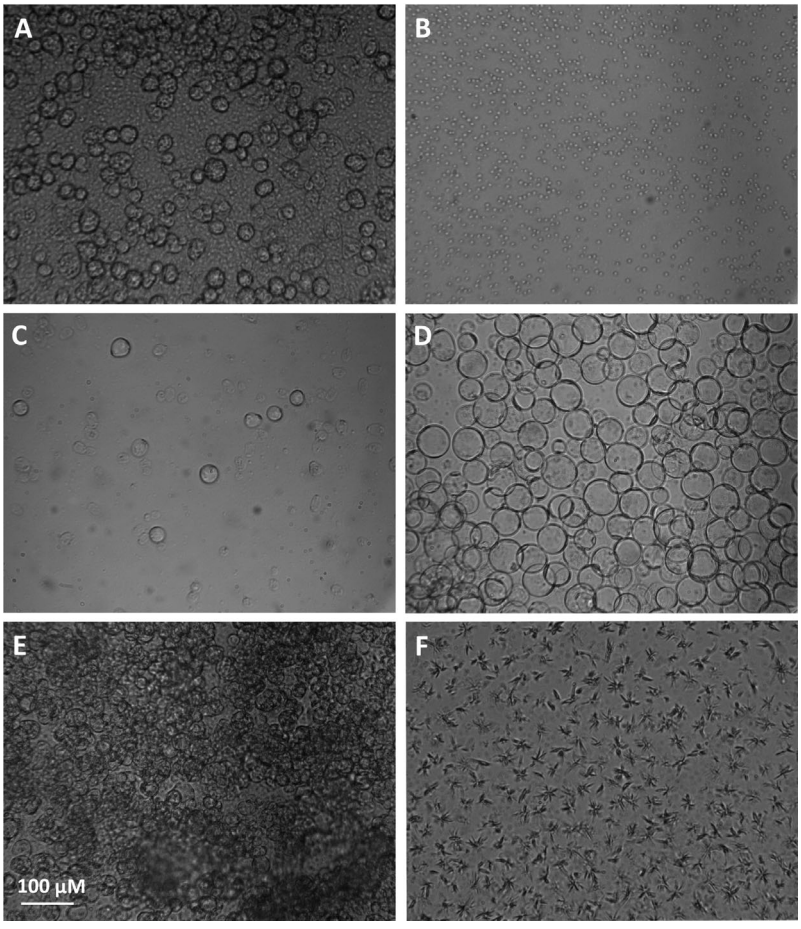

Fig. 3. *Batrachochytrium dendrobatidis.* Light microscope photographs (×250) showing growth of cultures 7 d post inoculation. (A) Positive control. (B) Negative control. (C) Partial inhibition. (D) Partial inhibition where large sporangia develop but no zoospores form. (E) Enhanced growth. (F) Total inhibition showing cell lysis. Scale bar in (E) applies to all panels

not grow well on agar unless the plate was left slightly damp, and under those conditions, bacteria always overgrew the *Bd*. In addition, antibiotic compounds can diffuse at a variety of rates through agar due to the nature of the agar matrix (Kunin & Edmondson 1968, du Toit & Rautenbach 2000), which may mean that some strongly inhibitory compounds produce only a narrow zone of inhibition, leading to inaccurate classification. Our broth-based assay eliminates these problems. The agar-plate technique used to date allows direct interaction between the bacterium and *Bd* and does not account for the possibility that priority effects (sensu Kennedy & Bruns 2005, Peay et al. 2012) may occur. Finally, interaction between growing cultures of test organisms also allows direct competition for space and nutrients, which could result in apparent zones of inhibition that are not due to inhibitory compound production (Jenkins et al. 1998).

Our technique allows visual differentiation between fungicidal and fungistatic effects. Fungistatic effects block growth at different stages of *Bd* development as seen in Fig. 3C,D, while fungicidal effects completely destroy the fungal cells (Fig. 3E). The agar plate assay allows rapid screening of large numbers of bacterial isolates, while our spectrophotometric technique is slightly more labour intensive to set up. However, it still facilitates the screening of reasonably large numbers of isolates at a time, and we feel that the benefits our technique offers are worth the additional time required to set up and run the assays.

Obtaining reliable results from this assay technique on isolates that exhibit total inhibition against *Bd* is based on the satisfaction of 3 assumptions: (1) that the *Bd* culture is growing optimally when the assay is set up; (2) that haemocytometer counts of *Bd* are conducted carefully and therefore reasonably reliably replicated; and (3) that 48 h of bacterial culture growth are likely to be sufficient for production of antimicrobial compounds to have occurred. We believe that the first 2 assumptions were satisfied while developing this assay, and subsequent investigation has shown that the third assumption is highly likely to be correct when assaying for isolates that show total inhibition against *Bd*, as this effect appears reliably repeatable (K. Yasumiba unpublished).

In our assay, isolates were grown and tested individually with the aim of detecting those that could exhibit strong inhibition against *Bd*. However, the interaction of 2 or more different bacteria, or the interaction of a bacterium with *Bd*, may initiate antibiotic production that is not observed in single isolates. In nature, it is probable that metabolite production may have a signaling role (see Yim et al. 2006), and this may only be triggered in response to the presence of other microorganisms. For example, de Boer et al. (2007) demonstrated that competitive interaction between different soil bacteria in culture resulted in stronger fungal inhibition than demonstrated by each bacterium alone. Therefore, in our assay, some isolates that can produce metabolites

active against *Bd* only in the presence of other microorganisms may not have been detected.

We can be certain that cell-free supernatants exhibit genuine inhibitory results when inhibition greater than the maximum possible value for nutrient depletion occurs. Lesser levels of inhibition might result from nutrient depletion alone or from partial inhibition with or without nutrient depletion. The growth enhancement we observed for some isolates cannot be caused by nutrient depletion and appears likely to be genuine.

Because nutrient depletion in cell-free supernatants could contribute to partially inhibitory results, we recommend that a minimum level of inhibition for genuine partially inhibitory effects be determined for each medium and concentration of *Bd* zoospores tested, as outlined in 'Materials and methods' above. Alternatively, 100% nutrient-depleted controls could be included on each 96-well microplate.

An additional simple improvement to this assay method would be to allow growth of bacterial cultures until a minimum, empirically determined, optical density is reached before preparing the cell-free supernatant for assay. This would reduce variation between isolates in terms of starting material available, almost certainly eliminate hormetic effects and ensure that any genuine partially inhibitory effect was not simply due to low bacterial cell numbers. However, we recognize that optical density is not solely dependent on cell number but also on cell motility, cell size and cell membrane composition.

Future directions

The development of this technique has provided numerous opportunities for further research. Alternative broth media can be substituted for TGhL if desired, and comparisons can now be easily made among isolates tested at different temperatures and with mixed microbial communities rather than in isolation. Further research to determine whether hormetic effects are responsible for the growth promotion phenomenon observed can be easily conducted using our technique. The range of effects observed using this assay technique also provides an opportunity to study gene expression in *Bd* to identify the genes involved in growth and development of the pathogen. Furthermore, with large datasets derived from wild animals, categorized data could also be used to statistically test for differences among frogs grouped by species, site, elevation, sex, age class or *Bd* infection status. There are many possibilities for experiments that will contribute knowledge on the diversity and interaction of microbial species found on amphibian skin.

As the field of bioaugmentation research progresses, it will become increasingly important to relate observed *in vitro* effects to the interactions occurring on amphibian skin, possibly through assessment of the contribution of the most dominant microbial community members (Harris et al. 2006, Woodhams et al. 2007b). Characterisation of bioactive compounds will also be useful (Brucker et al. 2008a,b) and, coupled with development of techniques for detection of ambient levels of antibiotics on frog skin without sacrifice of the animal, should aid in elucidating a fuller picture of the contribution of microorganisms to immune defense against this widespread and serious pathogen.

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LITERATURE CITED

Banning JL, Weddle AL, Wahl GW, Simon MA, Lauer A, Walters RL, Harris RN (2008) Antifungal skin bacteria, embryonic survival, and communal nesting in four-toed salamanders, *Hemidactylium scutatum.* Oecologia 156: 423–429

Barry AL (1980) Procedure for testing antibiotics in agar media: theoretical considerations. In: Lorian V (ed) Antibiotics in laboratory medicine. Williams and Wilkins, Baltimore, MD, p 1–23

Becker MH, Harris RN (2010) Cutaneous bacteria of the redback salamander prevent morbidity associated with a lethal disease. PLoS ONE 5:e10957

Berger L, Speare R, Daszak P, Green DE and others (1998) Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. Proc Natl Acad Sci USA 95: 9031–9036

Berger L, Speare R, Hyatt A (1999) Chytrid fungi and amphibian declines: overview, implications and future directions. In: Alastair C (ed) Declines and disappearances of Australian frogs. Environment Australia, Canberra, p 23–33

Berger L, Marantelli G, Skerratt LF, Speare R (2005) Virulence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* varies with the strain. Dis Aquat Org 68: 47–50

Bettin C, Greven H (1986) Bacteria on the skin of *Salamandra salamandra* (L) (Amphibia, Urodela) with notes on their possible significance. Zool Anz 216:267–270

Brucker RM, Baylor CM, Walters RL, Lauer A, Harris RN, Minbiole KPC (2008a) The identification of 2,4-diacetylphloroglucinol as an antifungal metabolite produced by cutaneous bacteria of the salamander *Plethodon cinereus.* J Chem Ecol 34:39–43

Brucker RM, Harris RN, Schwantes CR, Gallaher TN, Flaherty DC, Lam BA, Minbiole KPC (2008b) Amphibian chemical defense: antifungal metabolites of the microsymbiont *Janthinobacterium lividum* on the salamander *Plethodon cinereus.* J Chem Ecol 34:1422–1429

Conlon JM (2011) The contribution of skin antimicrobial peptides to the system of innate immunity in anurans. Cell Tissue Res 343:201–212

de Boer W, Wagenaar AM, Klein Gunnewiek PJA, Van Veen JA (2007) In vitro suppression of fungi caused by combinations of apparently non-antagonistic soil bacteria. FEMS Microbiol Ecol 59:177–185

du Toit EA, Rautenbach M (2000) A sensitive standardised micro-gel well diffusion assay for the determination of antimicrobial activity. J Microbiol Methods 42:159–165

Harris RN, James TY, Lauer A, Simon MA, Patel A (2006) Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. EcoHealth 3:53–56

Harris RN, Brucker RM, Walke JB, Becker MH and others (2009a) Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. ISME J 3: 818–824

Harris RN, Lauer A, Simon MA, Banning JL, Alford RA (2009b) Addition of antifungal skin bacteria to salamanders ameliorates the effects of chytridiomycosis. Dis Aquat Org 83:11–16

Jenkins KM, Jensen PR, Fenical W (1998) Bioassays with marine microorganisms. In: Haynes KF, Millar JG (eds) Methods in chemical ecology: bioassay methods, Book 2. Kluwer Academic Publishers, Norwell, MA, p 1–38

Kennedy PG, Bruns TD (2005) Priority effects determine the outcome of ectomycorrhizal competition between two *Rhizopogon* species colonizing *Pinus muricata* seedlings. New Phytol 166:631–638

Kunin CM, Edmondson WP (1968) Inhibitor of antibiotics in bacteriologic agar. Proc Soc Exp Biol Med 129:118–122

Lauer A, Simon MA, Banning JL, Andre E, Duncan K, Harris RN (2007) Common cutaneous bacteria from the eastern red-backed salamander can inhibit pathogenic fungi. Copeia 2007:630–640

Lauer A, Simon MA, Banning JL, Lam BA, Harris RN (2008) Diversity of cutaneous bacteria with antifungal activity isolated from female four-toed salamanders. ISME J 2: 145–157

Peay KG, Belisle M, Fukami T (2012) Phylogenetic relatedness predicts priority effects in nectar yeast communities. Proc R Soc Lond B Biol Sci 279:749–758

Ramsey JP, Reinert LK, Harper LK, Woodhams DC, Rollins-Smith LA (2010) Immune defenses against *Batrachochytrium dendrobatidis*, a fungus linked to global amphibian declines, in the South African clawed frog, *Xenopus laevis.* Infect Immun 78:3981–3992

Richmond JQ, Savage AE, Zamudio KR, Rosenblum EB (2009) Toward immunogenetic studies of amphibian chytridiomycosis: linking innate and acquired immunity. Bioscience 59:311–320

Rollins-Smith LA (2009) The role of amphibian antimicrobial peptides in protection of amphibians from pathogens linked to global amphibian declines. Biochim Biophys Acta 1788:1593–1599

Rollins-Smith LA, Conlon JM (2005) Antimicrobial peptide defenses against chytridiomycosis, an emerging infectious disease of amphibian populations. Dev Comp Immunol 29:589–598

Rollins-Smith LA, Woodhams DC (2012) Amphibian immunity: staying in tune with the environment. In: Demas GE, Nelson RJ (eds) Ecoimmunology. Oxford University Press, New York, NY, p 92–143

Rollins-Smith LA, Carey C, Longcore JE, Doersam JK and others (2002a) Antimicrobial peptide defenses against *Batrachochytrium dendrobatidis*, the chytrid fungus associated with global amphibian declines. FASEB J 16: A291

Rollins-Smith LA, Doersam JK, Longcore JE, Taylor SK, Shamblin JC, Carey C, Zasloff MA (2002b) Antimicrobial peptide defenses against pathogens associated with global amphibian declines. Dev Comp Immunol 26: 63–72

Salle AJ (1961) Laboratory manual on fundamental principles of bacteriology. McGraw-Hill, London

Savage AE, Zamudio KR (2011) MHC genotypes associate with resistance to a frog-killing fungus. Proc Natl Acad Sci USA 108:16705–16710

Southam CM, Ehrlich J (1943) Effects of extracts of western red-cedar heartwood on certain wood-decaying fungi in culture. Phytopathology 33:517–524

Stebbing ARD (1982) Hormesis — the stimulation of growth by low levels of inhibitors. Sci Total Environ 22:213–234

Verschuere L, Rombaut G, Sorgeloos P, Verstraete W (2000) Probiotic bacteria as biological control agents in aquaculture. Microbiol Mol Biol Rev 64:655–671

Voyles J, Rosenblum EB, Berger L (2011) Interactions between *Batrachochytrium dendrobatidis* and its amphibian hosts: a review of pathogenesis and immunity. Microbes Infect 13:25–32

Walke JB, Harris RN, Reinert LK, Rollins-Smith LA, Woodhams DC (2011) Social immunity in amphibians: evidence for vertical transmission of innate defenses. Biotropica 43:396–400

Walters RL (2007) Antibiotic production by cutaneous bacteria isolated from two species of salamanders. MSc thesis, James Madison University, Harrisonburg, VA

Woodhams DC, Rollins-Smith LA, Carey C, Reinert L, Tyler MJ, Alford RA (2006a) Population trends associated with skin peptide defenses against chytridiomycosis in Australian frogs. Oecologia 146:531–540

Woodhams DC, Voyles J, Lips KR, Carey C, Rollins-Smith LA (2006b) Predicted disease susceptibility in a Panamanian amphibian assemblage based on skin peptide defenses. J Wildl Dis 42:207–218

Woodhams DC, Ardipradja K, Alford RA, Marantelli G, Reinert LK, Rollins-Smith LA (2007a) Resistance to chytridiomycosis varies among amphibian species and is correlated with skin peptide defenses. Anim Conserv 10: 409–417

Woodhams DC, Vredenburg VT, Simon MA, Billheimer D and others (2007b) Symbiotic bacteria contribute to innate immune defenses of the threatened mountain yellow-legged frog, *Rana muscosa.* Biol Conserv 138:390–398

Yim G, Huimi Wang H, Davies J (2006) The truth about antibiotics. Int J Med Microbiol 296:163–170

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