Skin bacteria provide early protection for newly metamorphosed southern leopard frogs (*Rana sphenoccephala*) against the frog-killing fungus, *Batrachochytrium dendrobatidis*

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Abstract

An important concern for conservation biologists is the continuing loss of global biodiversity. This is especially true for amphibians, which are disappearing at an alarming rate. The emerging fungal pathogen, *Batrachochytrium dendrobatidis* (*Bd*), causes the skin disease chytridiomycosis and is a major contributor to amphibian population declines. Recent studies suggest that symbiotic skin bacteria provide some protection against *Bd*. Possible mitigation strategies include skin microbiome manipulation and bioaugmentation with potentially beneficial bacteria. Such approaches may employ a method to reduce resident bacteria on the skin to allow for new colonization. Using the southern leopard frog, *Rana (Lithobates) sphenoccephala*, we show here that the skin of this species harbors a rich array of culturable bacterial species that inhibit *Bd* growth *in vitro*. Use of a potent antibiotic cocktail dramatically reduced culturable skin bacteria within 48 hours. Reduction of bacteria on post-metamorphic juvenile skin using this antibiotic cocktail resulted in an increased *Bd* skin burden within two weeks of *Bd* exposure and a higher *Bd* burden in those frogs that died. However, efforts to maintain a normal bacterial skin community using a semi-natural mesocosm-derived source of microbes failed to provide long term protection of these vulnerable frogs. Regardless of presence or absence of mesocosm-derived bacteria, *Bd* loads decreased over time in surviving frogs, suggesting the development of an adaptive immune defense. In this species, development of an antimicrobial peptide defense is

delayed, and thus, the microbial community and adaptive immune defenses may provide protection at this critical period of development.

Keywords: amphibian skin microbiome; antibiotic depletion; antimicrobial peptides; chytridiomycosis; southern leopard frogs; skin defenses

1. Introduction

Batrachochytrium dendrobatidis (*Bd*) is an emerging fungal pathogen linked to catastrophic amphibian population declines and extinctions (reviewed in Skerratt *et al.*, 2007; Kilpatrick *et al.*, 2010; Fisher *et al.*, 2012). This fungus infects the skin causing chytridiomycosis (Berger *et al.*, 1998; Longcore *et al.*, 1999; Pessier *et al.*, 1999) with symptoms that include appetite loss, lethargy, excess skin sloughing and imbalances in essential ions that can result in cardiac arrest (Berger *et al.*, 1998; Parker *et al.*, 2002; Voyles *et al.*, 2009).

B. dendrobatidis infection begins when a motile zoospore migrates into host cells via a germ tube (Greenspan *et al.*, 2012; Van Rooij *et al.*, 2012). During this critical step, zoospores encounter chemical defenses in host mucus, including bacteria competing for space and producing antifungal metabolites, antimicrobial peptides (AMPs), and mucosal antibodies (reviewed in Rollins-Smith *et al.*, 2011). In newly metamorphosed juveniles, the adaptive immune response to this pathogen is immature (reviewed in Rollins-Smith, 1998) and factors produced by the fungus can interfere with a lymphocyte-mediated responses (Fites *et al.*, 2013, 2014). As a result, understanding innate defenses such as symbiotic skin bacteria and AMPs is critical to understanding effective anti-*Bd* defenses in young frogs.

As a robust test of the protective capacity of skin bacteria, we chose to expose a cohort of *Bd*-naïve *R. sphenoccephala* juveniles with an intact skin microbiome to *Bd* at a time when they do not yet express AMPs and compare their responses to those of a matched group that lacked a skin microbiota. To assess the capacity of the bacterial defenses, we examined the bacterial community in these young frogs by isolation and identification of individual culturable members of the skin microbiome and by testing their capacity to inhibit *Bd* growth *in vitro*. We developed an effective method to deplete this skin community and assessed effects of short-term antibiotic depletion on overall health. We confirmed that antimicrobial peptide defenses were missing in the newly metamorphosed frogs, and we evaluated the ability of the skin microbiome to inhibit *Bd* infection and development of chytridiomycosis in this early postmetamorphic period when the AMP defenses are immature.

2. Materials and methods

2.1 *Rana sphenoccephala* care and husbandry

In 2012 and 2013, *R. sphenoccephala* juveniles were raised from locally-collected eggs in polyethylene tank mesocosms (diameter = 1.83 m) at the University of Memphis Edwards J. Meeman Biological Field Station, Shelby County, TN (35° 22' N / 90° 01' W) by S.M.H. and M.J.P.

Juveniles raised in 2012 were used for: (1) isolation of skin bacteria; (2) pilot experiments to optimize bacterial reduction by antibiotics; and (3) as positive controls for AMP expression at age 52 weeks post-metamorphosis. Juveniles raised in 2013 were used for: (1) evaluation of AMP production following norepinephrine injection immediately after metamorphosis and (2) a bacterial reduction and infection experiment described below. Norepinephrine-induced individuals were not used in the infection studies.

Mesocosm tanks were prepared in February each year as previously described (Holden *et al.*, 2015). Briefly, each tank was filled with ~613 L of well water. About 300 g of dry deciduous leaf litter and one 500 ml aliquot of concentrated plankton from a nearby pond were added. Fiberglass mesh screens (1-mm mesh) were used as lids to prevent colonization by *Bd*-infected adults or predators and to provide shading. A fiberglass composite ramp was placed in each tank at a 30° angle to simulate the margin of a pond. Tanks were allowed to condition for 14 days before the addition of eggs (N= 8 egg clutches) collected from water bodies at the field station. [From previous experience, the water, eggs, and added plankton from these local sources were not a source of *Bd*, and emerging juveniles were later shown to be *Bd*-free.] The egg clutches were evenly distributed between two tanks, and tadpoles were reared to Gosner stage 25 (Gosner, 1960). At that time, tadpoles were randomly selected from both tanks and distributed into nine mesocosm rearing tanks at densities of ~30/tank. The rearing tanks were left undisturbed until the first metamorphs were observed in June. Newly metamorphosed juveniles were removed and placed into terrestrial enclosure tanks placed at a 25° slant. The lower half was filled with water taken from the tadpole rearing tanks while the upper half was filled with sand and leaf litter, allowing metamorphs to assume a semi-terrestrial life style. Fiberglass ramps were placed over the water portion to provide structure and shade, and mesh screens remained securely fastened as lids. Rearing tanks were checked every other day, and new metamorphs were evenly distributed into the terrestrial tanks.

Metamorphs were removed from the terrestrial tanks and transported to the laboratory at Vanderbilt University in mid-summer. Once in the laboratory, frogs were kept at 21–25°C and fed live, vitamin-dusted crickets three times weekly, with water changed four times weekly. Some frogs were kept in mesocosm water as described below. Mesocosm water contained environmental bacteria, zooplankton, and plant matter removed from mesocosms on the same day that juveniles were transferred. Mesocosm water was stored at 4 to 7°C until its use in the experiment, beginning immediately after transfer of frogs from the terrestrial tanks and continuing for a period of five weeks. For the *Bd*-exposure study, metamorphs were housed individually in sterile 280 ml plastic containers. Metamorphs used to assess the antimicrobial peptides were housed in polystyrene tanks measuring 44 × 24 × 20 cm (10 frogs per tank). The Institutional Animal Care and Use Committee (IACUC) at Vanderbilt University Medical Center approved all procedures involving *R. sphenoccephala* except the raising of metamorphs from eggs in mesocosms, which was approved by the IACUC at the University of Memphis.

2.2 Isolation, identification, and characterization of bacterial isolates

To collect bacterial isolates, 15 newly metamorphosed frogs removed from the terrestrial mesocosms were rinsed with 50 ml sterile water and swabbed with sterile cotton swabs (Medical Wire & Equipment Co., Advantage Bundling, Durham, NC) at about 18 hours after their arrival in the laboratory. During the brief time between collection and swabbing, metamorphs were housed in sterile polystyrene containers to assure that they did not acquire laboratory bacteria before swabbing.

After sampling, swabs were streaked across R2A agar plates (Becton, Dickinson and Co., Sparks, MD, USA) to isolate individual colonies. Phenotypic characteristics of each colony including size and color were recorded, and frozen cultures in Luria-Bertani broth and glycerol were stored at -80°C. To identify bacteria by 16S rDNA sequencing, isolates were grown in 96-well plates with R2A media. DNA was extracted using an UltraClean 96-Well Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) with the centrifugation protocol.

The PCR reaction contained: 12 μ L PCR water, 10 μ L 5 Prime Master Mix, 1 μ L of the forward and reverse primers at 10 μ M concentration, 1 μ L $MgCl_2$, and 1 μ L genomic DNA. PCR universal primers consisted of 515F (5'-GTG CCA GCM GCC GCG GTA A-3') and 1391R (5'-GAC GGG CRG TGW GTR CA-3') for 16S rRNA amplification. The PCR profile included an initial denaturation step of 94°C for 2 minutes, followed by 29 cycles of 94°C for 30 seconds, 55.5°C for 60 seconds, and 72°C for 90 seconds and final extension at 72°C for 12 minutes. Amplicons were sent to Beckman Coulter Genomics for Sanger sequencing. Geneious version 6.1 was used to create contigs by combining forward and reverse reads. The Quantitative Insights Into Microbial Ecology (QIIME) v.1.7 was used to identify isolates based on RDP classification (http://qiime.org/scripts/assign_taxonomy.html#index-0) (Caporaso *et al.*, 2010). Sequence data were further used to identify the closest species match, where available, using standard nucleotide BLAST with default search parameters.

2.3 *Batrachochytrium dendrobatidis* (Bd)

Batrachochytrium dendrobatidis isolate JEL197 (Longcore *et al.*, 1999) was used to test the antifungal activity of supernatants from individual bacterial isolates. We chose this isolate for the *in vitro* tests because it is the original type strain and is among global panzootic lineage isolates (Schloegel *et al.*, 2012). For *Bd* exposure studies we chose a recently isolated strain of *Bd*, designated "Section Line". This isolate was highly virulent in infection studies and is also among the global panzootic lineage isolates (Piovia-Scott *et al.*, 2014). The Section Line isolate had been passaged approximately 60 times before use in the exposure experiments. Cultures of both strains were maintained in 1% tryptone broth and on tryptone agar at 19-21°C and passaged weekly.

2.4 Growth inhibition of *B. dendrobatidis* by bacterial supernatants

Individual bacterial isolates were streaked onto R2A agar plates and allowed to grow at room temperature (about 23 °C). A small sample of the growing bacteria was inoculated into 1% tryptone broth, incubated at room temperature, and allowed to grow to optical densities of 0.20 $\pm$ 0.02 at a wavelength of 630 nanometers (OD₆₃₀). We assumed that at this final OD, most isolates would have reached optimal growth and maximal secretion of possible inhibitory molecules. The bacterial cells were pelleted by centrifugation at 15,000 $\times$ g, and supernatants were harvested and filtered through 0.2 μ m pore filters to remove bacterial cells.

Batrachochytrium dendrobatidis zoospores were harvested from agar plate cultures and cultured with bacterial supernatants in replicates of five for seven days at 19-21°C in 96-well flat-bottom microtiter plates (BD Falcon, Franklin Lakes, NJ, USA) using a previously published protocol (Rollins-Smith *et al.*, 2002) with minor modifications. Specifically, each well contained 5×10^4 zoospores in 50 μ l tryptone broth with 50 μ l bacterial supernatant. Five replicate wells of positive and negative controls of either living or heat-killed (10 minutes at 60°C) zoospores in tryptone broth were included on each plate along with five replicate wells of 100 μ l tryptone broth without zoospores or bacterial supernatant to serve as a blank. Optical densities at a wavelength of 490 nanometers (OD₄₉₀) were measured by an MRX Microplate Reader (Dynex Technologies, Inc., Chantilly, Virginia, USA) at days 0 and 7 with a change in OD indicating *Bd* growth. Day 0 values were subtracted from the day 7 values to correct for baseline absorbance due to any color or consistency difference in the bacterial supernatants. Each supernatant's effect on *Bd* growth was expressed as percent inhibition or percent enhancement. This measure was calculated as % inhibition or enhancement = [(OD of experimental well minus day 0 value)

÷ (Av OD of positive control wells minus day 0 value)] x 100. The mean and standard error (SEM) for each set of replicates were computed for each supernatant.

2.5 Antibiotic reduction and quantification of skin bacteria

We modified a previously reported method (Becker & Harris, 2010) for reduction of amphibian skin bacteria by adding penicillin, streptomycin and enrofloxacin. The final antibiotic cocktail contained 60 mg/L enrofloxacin (Fluka, Sigma, St. Louis, MO, USA), 24 mg/L cephalexin (Teva Pharmaceuticals, North Wales, PA, USA), 14.5 mg/L sulfamethazine and 2.9 mg/L trimethoprim (Mardel Maracyn Plus, Sergeant's Pet Care Products, Inc., Omaha, NE, USA), 100 mg/L streptomycin, and 10⁵ I.U./L penicillin. At approximately 7-10 days post-metamorphosis, metamorphs were placed in sterile 280 ml plastic containers in a volume of 20 ml of the antibiotic cocktail at a temperature of 21-25°C for 48 hours. This amount was sufficient to cover their dorsal surfaces except the head. The antibiotic cocktail was refreshed at 24 hours to ensure continued efficacy. Control metamorphs were placed in the same volume of mesocosm water that was also changed at 24 hours. Prior to and immediately after this 48 hour treatment, metamorphs were rinsed with 50 ml sterile water and swabbed with sterile cotton swabs. Swabs were added to 1 ml sterile dechlorinated water and vortexed gently (5 seconds) to dislodge bacteria from the swab. The water containing frog skin bacteria was then plated onto Difco R2A agar, incubated at 19-21°C for seven days, and individual colonies counted as colony-forming units (cfu). A separate plate count was performed to assess the number of cfu per ml of mesocosm water by plating multiple dilutions on R2A plates.

2.6 Collection, quantification, and MALDI-TOF mass spectrometry analysis of amphibian antimicrobial skin peptides

Natural peptide mixtures were collected from a group of metamorphs separate from those used in the infection experiment at one week (2013 cohort) and one year (2012 cohort) post-metamorphosis by injection of 40 nmol/g body weight norepinephrine-HCl in amphibian phosphate-buffered saline (APBS) as previously described (Gammill *et al.*, 2012). Recovered peptides were partially purified with C18 Sep-Paks (Waters Corporation, Milford, MA, USA) and quantified by MicroBCA™ assay (Pierce, Rockford, IL, USA) as previously described (Gammill *et al.*, 2012). Peptide concentrations were calculated as µg /ml of mucus as previously described (Ramsey *et al.*, 2010; Gammill *et al.*, 2012).

The presence or absence of individual peptides in the skin secretions of each frog was determined by matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF) mass spectrometry as previously described (Rollins-Smith *et al.*, 2006; Gammill *et al.*, 2012). Briefly, 0.6 µl peptides at 1 mg/ml were spotted onto a stainless steel MALDI plate with the 0.6 ml of matrix [α -cyano-4-hydroxycinnamic acid (CHCA, Fluka, Sigma, St. Louis, MO, USA), 60% acetonitrile, 39.6% HPLC-grade water, and 0.4% trifluoroacetic acid (v/v/v)] and allowed to air dry. The following peptide standards facilitated calibration of the mass spectrometer: bradykinin fragment 1-7 (*m/z* 757.3997), human angiotensin II (*m/z* 1046.5423), P₁₄R synthetic peptide (*m/z* 1533.8582), adrenocorticotrophic hormone fragment 18-39 (*m/z* 2464.1989), and bovine oxidized insulin chain B (*m/z* 3494.6513). An Ultraflex III mass spectrometer (Bruker Daltonics, Billerica, MA, USA) in delayed extraction, positive ion, reflector mode was used to collect 1000 laser shots. Spectra were analyzed with Data Explorer v4.4 software (Applied Biosystems, Foster City, CA, USA).

A previous study in juvenile *Rana pipiens* noted that an unknown component within the peptide samples prevented desorption or ionization of known peptides within the samples (M. Groner, personal communication). To rule out this potential problem in our one-week post-metamorphosis samples, we spiked synthetic brevinin-1Sb (>90% pure, MW =2535.22, obtained from Lifetein, South Plainfield, NJ, USA) into the samples at a 1:1 ratio, such that the final concentrations of brevinin-1Sb and peptides were each at 500 µg/ml. A negative control of HPLC-grade water and positive controls (N = 5) containing 500 µg/ml brevinin-1Sb in HPLC-grade water were also prepared. These mixtures and standards were analyzed by MALDI-TOF mass spectrometry to compare the amount of brevinin-1Sb signal in the positive controls to the amount of brevinin-1Sb signal in samples containing the one-week post-metamorphosis peptides with added brevinin-1Sb. Data Explorer v4.4 software (Applied Biosystems, Foster City, CA) was used to calculate the area under the brevinin-1Sb peaks, including the monoisotopic mass, [M+H]⁺, and the sodium adduct [M+Na]⁺ of that peptide.

2.7 Bacterial reduction, *Bd* exposure, frog survival, and *Bd* infection loads

Frogs used for *Bd* exposure experiments tested negative for the presence of *Bd* by qPCR (described below) prior to the start of the experiment.

The design of this experiment is shown in Figure 1. On day 0, metamorphs were transferred from terrestrial rearing tanks to the lab at Vanderbilt. The next day, they were weighed, swabbed to assess bacterial counts and *Bd* infection status, and treated with antibiotics or maintained in mesocosm water. On day 2, the antibiotic cocktail or mesocosm water was refreshed. On day 3, the frogs were weighed and swabbed again to assess bacterial counts, and antibiotic-treated frogs were placed in sterile de-chlorinated tap water containing 100 µg/ml streptomycin and 100 I.U./ml penicillin. Frogs that had not been treated with antibiotics were maintained in fresh mesocosm water. Zoospores of the *Bd* isolate “Section Line” were harvested and counted as previously described (Rollins-Smith *et al.*, 2002). On days 4, 11, and 18, twenty metamorphs in mesocosm water and nineteen metamorphs with reduced levels of skin bacteria were exposed to zoospores (*Bd*+ groups). Thirty nine control frogs (*Bd*- groups) were given the same volume of tryptone broth without zoospores. Frogs were weighed and swabbed to assess the level of *Bd* on days 6, 13, 20, 27, and 34. The metamorphs in individual sterile containers (*Bd*+ groups) were exposed to 10⁶ zoospores/ml in 20 ml of either dechlorinated tap water containing 100 µg/ml streptomycin and 100 I.U./ml penicillin (shown as Bacteria- for Bacteria-reduced groups) or mesocosm water (shown as Bacteria+ to indicate Bacteria-maintained groups). Each exposure lasted 24 hours, and then the water was changed and zoospores were removed. Metamorphs were examined for signs of chytridiomycosis or death each day.

To assess *Bd* infection intensity, frogs were swabbed with sterile cotton swabs. DNA was extracted from swabs following manufacturer’s directions (DNeasy Blood and Tissue DNA Extraction Kit, Qiagen, Valencia, CA, USA, animal tissue protocol) along with a swab containing a known amount of *Bd* zoospores (Section Line isolate) as a positive control. A sterile swab without added zoospores provided a negative control. A real time quantitative PCR assay was used to quantify *Bd* DNA for each sample (Boyle *et al.*, 2004; Hyatt *et al.*, 2007). A no template (no DNA) control containing PCR reagents without DNA was also included in each PCR. Zoospore equivalents in each sample were quantified by comparing fluorescence intensity to a standard curve of known *Bd* zoospore DNA dilutions equivalent to 1000, 100, 10, and 1 *Bd* zoospores. PCR assays were conducted with an Mx3000P Real-Time PCR machine (Stratagene, La Jolla, CA, USA) using default Stratagene conditions (10 minutes at 95°C; 40 cycles of 30

seconds at 95° C, 1 minute at 55°C, and 1 minute at 72° C). [From past work, we have found that these cycling conditions give more consistent results than those cycling conditions originally described by Boyle *et al.*, 2004.] All controls, standards, and experimental samples were run in triplicate, and individual wells were averaged to obtain a final value per sample. DNA standard for quantification of *Bd* zoospore equivalents were kindly provided by Dr. Alex Hyatt, CSIRO, Geelong, Victoria, Australia.

2.8. Statistical comparisons

Values for percent of *Bd* growth inhibition or enhancement are presented as mean $\pm$ standard error of the mean (SEM) and compared by two-tailed Student's *t*-test (p-value of $< 1.43 \times 10^{-3}$ after Bonferroni correction was considered significant). These data met assumptions for parametric tests including normal distribution and homogeneity of variance, and thus the Student's *t*-test is appropriate. Whenever more than one Student's *t*-test was performed within the same data set, p-values were adjusted to satisfy the Bonferroni correction (Bland & Altman, 1995) using a sequential Bonferroni procedure (Holm, 1979). Bacterial counts and frog masses following bacterial reduction experiments are presented as mean colony forming units (cfu) per swab $\pm$ SEM or average mass (g) $\pm$ SEM and were compared by ANOVA with Tukey post hoc tests. Percent survival of all *Bd* exposed frogs versus unexposed frogs was compared by a Fisher's exact test. *Bd* infection intensity on frog skin swabs determined by qPCR is presented as average zoospore equivalents $\pm$ SEM and compared by ANOVA with Tukey post hoc tests. Zoospore loads on the same frogs at several sampling times were compared by repeated-measures ANOVA with Tukey post hoc tests following log-transformation of the data to meet the assumptions of parametric tests. While zoospore burdens can often follow a non-normal distribution, after data transformation we found that the residual error distribution of zoospore burden at each time point was normally distributed (Normal p-p plot of Regression Standardized Residual, Shapiro-Wilk test, $p > 0.05$) and variances were not significantly heterogeneous among treatment groups (Levene statistic, $p > 0.05$). Thus, our data met assumptions for parametric tests. Survival of all groups of frogs in the bacterial depletion and *Bd* exposure experiment were compared by The Kaplan-Meier analysis using GraphPad Prism® software (GraphPad Prism Inc. San Diego, CA, USA) with the Log-rank (Mantel-Cox) Test that weights all deaths equally and the Gehan-Breslow-Wilcoxon Test that assigns higher weights to earlier deaths (Machin *et al.*, 2006). In each figure legend, descriptions of statistical analyses conducted are listed and significant relationships are shown in the symbols. Rarely, outliers detected by Dixon's Q test were removed. The mass of each frog in the *Bd* exposure study was determined at multiple time points and the slope of mass change was calculated. Mass changes by group were compared using a Kruskal-Wallis test.

3. Results

3.1 Characterization of the culturable microbiome of newly metamorphosed *R. sphenoccephala*

Multiple isolates of skin bacteria were identified by 16S rDNA sequencing and classified into 39 operational taxonomic units (OTUs) (Table 1). [Sequence data was added to a recently published database (Woodhams *et al.*, 2015).] In total, 65 isolates from 15 metamorphs were screened for antifungal activity, and the isolates tested were grouped into three categories: inhibitors, enhancers, and isolates whose supernatants had no significant effect (Fig. 2a, summarized in Table 1). Inhibitory isolates were collected from 87% of individuals sampled,

and isolates with enhancing supernatant activity were collected from 33% of metamorphs, reflecting the level of species richness and diversity in the culturable amphibian skin microbial community.

The most prominent phyla observed were Proteobacteria (49%), Actinobacteria (23%), Bacteroidetes (15%), and Firmicutes (8%) (Fig. 2b), with greater than 95% of isolates in these phyla. Isolates characterized as inhibitory (Table 1, Figure 2a) fell into the Proteobacteria (61%), Actinobacteria (17%), and Bacteroidetes (17%) (Fig. 2c); suggesting that these are critical phyla comprising the protective amphibian skin microbiota. Two OTUs, including one that was inhibitory, were also identified from the phylum *Deinococcus-Thermus*, an unexpected occurrence as this phylum is home to several extremophiles (Tian and Hua, 2010). Analysis of the bacterial classes identified on *R. sphenocephala* indicates a range of taxonomic diversity, especially among Proteobacteria, with members identified from α -, β -, and γ -Proteobacteria (Fig. 2d). Interestingly, every isolate identified within the class γ -Proteobacteria was a significant inhibitor of *Bd* growth (Table 1), making this class the single largest contributor to *Bd* inhibition identified in this study (Fig. 2e). The isolates which produced the most strongly inhibitory supernatants were identified as *Pseudomonas geniculata*, *Pseudomonas hibiscicola*, *Enterobacter aerogenes*, *Stenotrophomonas maltophilia*, and *Brevundimonas nasdae* (Table 1).

3.2 Bacterial reduction on *Rana sphenocephala* skin

In a pilot experiment with *R. sphenocephala* in 2012, the antibiotic cocktail significantly reduced levels of skin bacteria (Fig. 3a). In the same experiment, we also observed reduction in the number of bacterial cfu collected from control, non-antibiotic-treated frogs (Fig. 3a) compared to their bacterial counts when first brought into captivity. For this reason, we used mesocosm water for the 2013 study in an effort to maintain the natural microbial community during the month-long infection experiment. A previous study suggested that providing captive amphibians with a reservoir of environmental bacteria would allow for continued colonization of natural symbionts (Loudon *et al.*, 2014b). Our antibiotic cocktail eliminated virtually all culturable bacteria from the skin following a 48-hour exposure as assessed by plate counts. At the same time, treatment with mesocosm water resulted in the retention of equal numbers of bacteria as detected prior to the treatments (Fig. 3b). The number of cfu detected on frogs following antibiotic exposure were 0.3 ± 0.1 with zero cfu detected on 33/39 (85%) of frogs. This process had no detrimental impact on immediate frog health, as all frogs survived at least one week post-treatment. Additionally, animal masses among groups did not differ significantly before or after treatment or between control (Bacteria+) and bacterially-reduced (Bacteria-) groups (Fig. 3c), demonstrating that groups were not significantly different at the beginning of the experiment or following the bacterial reduction. Culturable bacterial numbers in the mesocosm water determined by plate counts indicated that the number of cfu/ml was $12,180 \pm 2,710$.

3.3 Effects of bacterial reduction on *B. dendrobatidis* infection and survival of frogs

All *R. sphenocephala* metamorphs from the 2012 and 2013 cohorts tested negative for *Bd* by qPCR. The use of naïve metamorphs for these experiments was essential to eliminate the possibility of preformed mucosal antibodies against *Bd* that can develop following *Bd* exposure (Ramsey *et al.*, 2010). Frogs that were not exposed to *Bd*, with or without bacteria (Bacteria+/Bd- and Bacteria-/Bd-), tested negative for *Bd* at the beginning and end of the experiment. Additionally, all non-exposed metamorphs that died during the experiment tested negative for *Bd* by qPCR. All frogs exposed to *Bd* tested positive by qPCR for this pathogen at

every time point examined, although infection intensity decreased in frogs which survived to the end of the experiment. On days 6 and 13 (2 and 9 days following *Bd* exposure respectively), a significantly increased number of zoospore equivalents was observed in the Bacteria-/Bd+ frogs in comparison with the Bacteria+/Bd+ frogs (Fig. 4a). At later time points, no significant differences were observed. Furthermore, there was a significant difference in the zoospore burden of those individuals lacking bacterial protection which died in comparison with those with intact bacteria (Fig. 4b). Approximately 10^5 more zoospores were detected on metamorphs without mesocosm water-derived skin bacteria as compared to those with mesocosm-supplemented skin bacteria. Exposure to *Bd* reduced survival regardless of treatment within six days following first exposure, and the difference in survival was significant at both days 10 and 13 (one-tailed Fisher's exact test, $p < 0.05$). The trend was observed at later time points, but differences were not significant (Fig. 5a). Despite the significantly lower zoospore loads observed in bacteria-maintained groups at early time points, longer survival of these post-metamorphic juveniles was not significantly improved (Fig. 5b, Kaplan-Meier analysis, $p = 0.578$). Among *Bd*-exposed frogs, seven with bacteria maintained and nine with bacteria reduced survived the thirty-five day experiment. In these survivors, we examined the *Bd* burden at days 27 and 34 following three exposures at days 4, 11, and 18. In comparison with the *Bd* burdens at day 20, *Bd* levels were significantly reduced one and two weeks after the final exposure (days 27 and 34) (repeated measures ANOVA, Tukey post-hoc test) (Fig 5c).

In addition to being swabbed for *Bd*, metamorphs were weighed on days 1, 3, 6, 13, 20, 27, and 34 and each individual's slope of mass change was calculated. There were no significant differences in mass change between treatment groups (Kruskal-Wallis test, $p = 0.491$) and on average, metamorph mass decreased slightly by 2.4 mg/day throughout the experiment. Each group included metamorphs from a range of masses and initial mass was found to predict survival. That is, metamorphs surviving to the end of the experiment had significantly greater mass on day 1 (mean = 0.79 ± 0.03) than metamorphs that died (0.65 ± 0.02 g; Student's *t*-test, $t = 4.329$, $df = 76$, $p < 0.001$).

3.4 Assessment of antimicrobial peptide defenses in newly metamorphosed frogs

Known AMPs reported in this species (brevinin-1Sa, brevinin-1Sb, and brevinin-1Sc) (Conlon *et al.*, 1999) were consistently detected in the mass spectra of peptides from *R. sphenoccephala* (2012 cohort) examined at one year post-metamorphosis (Fig. 6a). However, in pilot experiments conducted in 2012, no AMP signals were detected in newly metamorphosed frogs. Therefore, we examined AMP expression in skin secretions from the 2013 cohort. Mass spectrometry analysis detected no known AMPs at one week post-metamorphosis coincident with the time of the bacterial reduction and *Bd* exposure experiment (Fig. 6b). At this time point, the total amount of hydrophobic skin peptides (μ g) recoverable per frog (110 ± 16) (mean $\pm$ SEM) was less than 10% of the amount recovered from mature frogs (1760 ± 290) (mean $\pm$ SEM). Total peptides recovered from metamorphs at one week post-metamorphosis were significantly reduced compared to older frogs, but increased slightly compared to APBS-injected controls (two-tailed Student's *t*-tests, $*p < 0.05$, $**p < 1 \times 10^{-5}$) (Fig. 6c). In order to confirm that the one-week samples did not contain an unknown component preventing peptide desorption or ionization, we spiked the synthetic peptide brevinin-1Sb into each sample at one-week post-metamorphosis ($N = 10$) and found that the peak area did not differ significantly (Fig. 6d, $p > 0.05$). This indicates that there were no components in the one-week post-metamorphosis samples preventing peptide identification by mass spectrometry.

4. Discussion

4.1 Analysis of *R. sphenocephala* bacterial isolates

Mesocosm-bred frogs were used in this study because they were naïve to *Bd* and would be more likely to contain a natural complement of skin bacteria than lab-reared frogs. The bacterial isolates of *R. sphenocephala* were dominated by four phyla, Proteobacteria (49%), Actinobacteria (23%), Bacteroidetes (15%) and Firmicutes (8%). The same four major phyla were also observed in the microbiome of newts and bullfrogs in the state of Virginia (Walke *et al.*, 2014) and five amphibian species sampled in California (Kueneman *et al.*, 2014). Many skin symbionts of *R. sphenocephala* fall into genera that have also been reported on the skin of other amphibian species including those in the genera *Acinetobacter*, *Bacillus*, *Brevundimonas*, *Comamonas*, *Chryseobacterium*, *Curtobacterium*, *Kocuria*, *Microbacterium*, *Pseudomonas*, *Sphingobacterium*, and *Stenotrophomonas* (Harris *et al.*, 2006; Woodhams *et al.*, 2007; Lauer *et al.*, 2008; Flechas *et al.*, 2012; McKenzie *et al.*, 2012; Roth *et al.*, 2013; Bell *et al.*, 2013; Kueneman *et al.*, 2014; Daskin *et al.*, 2014; Walke *et al.*, 2014; Shaw *et al.*, 2014).

Significant growth inhibition in comparison with the positive control ranged from about 15% to 94% inhibition. Inhibition may be due to release of potential inhibitory metabolites or competition for nutrients. Nutrient depletion has been shown to affect *Bd* growth *in vitro* (Bell *et al.* 2013) and could explain some of the inhibition we observed with less inhibitory isolates. Most inhibitory isolates of *R. sphenocephala* were also classified in the phyla Proteobacteria, Actinobacteria, and Bacteroidetes. Among the strongly inhibitory isolates identified in this study, one species has been identified from the skin of amphibians in four other studies. This species is *Stenotrophomonas maltophilia*, and it has also been identified in amphibians from California in the USA (Woodhams *et al.*, 2007), Colombia (Flechas *et al.*, 2012), Panama (Walke *et al.*, 2011), and Australia (Daskin *et al.*, 2014). In *Hyalinobatrachium colymbiophyllum* of Panama, this bacterial species was found on the skin of the attending male parent and was also found on developing eggs, suggesting possible transfer to protect the developing offspring (Walke *et al.*, 2011). Given that it is often cultured from amphibian skin, it may be among a core of potentially beneficial species able to inhibit growth of *Bd*.

In this study, we tested the inhibitory activity of supernatants from individual bacterial isolates. However, it was recently shown that factors released by more than one isolate can have an additive or synergistic effect as inhibitors of *Bd* growth (Loudon *et al.*, 2014a). Thus, it is likely that bacterial species could cooperate in some fashion to inhibit *Bd* growth on the skin surface. A frequent inhabitant of amphibian skin found in other studies, but not this one, is *Janthinobacterium lividum* which produces the anti-*Bd* metabolites indole-3-carboxaldehyde and violacein (Brucker *et al.*, 2008b). As a first step in bacterial bioaugmentation studies, it was shown that *J. lividum* can be introduced into soil, transferred from soil to an amphibian, and inhibit *Bd* colonization (Muletz *et al.* 2012). Another anti-*Bd* metabolite is 2,4-diacetylphloroglucinol (2,4-DAPG), which is produced by the bacterium *Lysobacter gummosus* (Brucker *et al.*, 2008a) and *Pseudomonas fluorescens* (Myers *et al.*, 2012). A new addition to the anti-*Bd* arsenal produced by co-culture of two inhibitory bacteria (a *Bacillus* sp. and *Chitinophaga arvensicola*) is tryptophol (Loudon *et al.*, 2014a).

Interestingly, OTUs from the phylum *Deinococcus-Thermus* were also identified among our isolates. To our knowledge, this is the first such identification among amphibian skin isolates, representing a novel contribution to the known biogeography of bacteria typically considered to be extremophiles (Tian & Hua, 2010).

Our results confirm the existence of rare isolates capable of enhancing *Bd* growth *in vitro*, a finding suggested by one previous study (Bell *et al.*, 2013). Three such enhancers were identified among *R. sphenoccephala* isolates, each in the phylum Actinobacteria, although they are members of three different families: Microbacteriaceae, Micrococcaceae, and Nocardiaceae. We identified other isolates in two of these families (Microbacteriaceae and Micrococcaceae) capable of significantly inhibiting *Bd* growth. Further, the genus *Microbacterium* was home to one inhibitor and one enhancer. Such diversity within one genus suggests that identification of antifungal isolates at the species level will be very important in future studies with the aim of bacterial identification for bioaugmentation, a conservation strategy involving the addition of beneficial bacteria to the skin of new hosts (Becker *et al.*, 2009; Harris *et al.*, 2009a, 2009b).

Future analyses of the symbiotic skin community defense should focus on the dynamic interactions that make this defense effective. For example, an exploration of whether bacterial signaling through innate pathways (Toll-like receptors, Nod-like receptors, etc.) contributes to eventual AMP or antibody secretion and how these peptides impact the diversity of skin bacteria would be an important addition to the body of knowledge of these defenses. Likewise, a study of whether bacterial skin symbionts are capable of sensing *Bd* in their immediate vicinity and upregulating their production of antifungal metabolites could shed light on the mechanisms at work in effective bacterial defenses.

4.2 Skin bacteria provide some protection of *R. sphenoccephala* metamorphs in the absence of AMPs

To date, very few studies have examined whether depleting skin bacteria of amphibians impacts their ability to fight chytridiomycosis. Becker and Harris (2010) found that short-term (four hours) antibiotic reduction of skin bacteria was associated with increased chytridiomycosis symptoms in the salamander, *Plethodon cinereus*. However, *Bd* burdens measured by qPCR were quite low in that previous study and did not differ between the antibiotic-treated versus the intact control animals. Woodhams *et al.* (2012) found that European water frogs (*Pelophylax esculentus* and *Pelophylax lessonae*), with their skin microbiota and AMP defenses reduced, failed to thrive and gain mass after exposure to *Bd* in comparison with control frogs with both defenses intact. The animals used in that previous study of European water frogs were from populations exposed to *Bd* (i.e. pathogen-primed), and thus possible immunity to this pathogen may have complicated the interpretations.

Our findings add *R. sphenoccephala* to the list of amphibian species with known antifungal skin symbionts and show that this microbial assemblage may provide a critical early defense against *Bd*. Our analysis of the skin peptides showed convincingly that the AMPs previously identified for *R. sphenoccephala* (Conlon *et al.*, 1999) were not detectable at one week post-metamorphosis when our bacterial reduction experiments were initiated. A more detailed study of the ontogeny of development of antimicrobial peptide defenses showed that this defense did not mature in this species raised under identical conditions until 12 weeks post-metamorphosis (Holden *et al.*, 2015). In comparison, the present study was completed at a point when all metamorphs were only 5-6 weeks post-metamorphosis. This observation demonstrates that the AMP defense at this early time point is not yet mature, and thus, any significant defense against *Bd* would be a result of other factors, including the skin microbiome. Because the bacterial depletion and *Bd* exposure experiment was begun at one-week post-metamorphosis, we interpret the differential increase in infection intensity in the bacteria-depleted frogs at days 6 and 13 as being due to the loss of their skin bacteria. When designing the study, we thought that

a role for the adaptive immune response was unlikely because post-metamorphic juveniles have immature adaptive defenses (reviewed in Rollins-Smith 1998 and Rollins-Smith *et al.*, 2011). These young frogs had not been previously exposed to *Bd*, and several studies suggested that the adaptive immune response to this pathogen is weak (Berger *et al.*, 1998; Pessier *et al.*, 1999; Fites *et al.*, 2013, 2014). However, after analysis of the skin burden of *Bd* after three exposures, *Bd* levels had declined significantly at days 27 and 34, suggesting the development of a lymphocyte-mediated adaptive immune response in the skin.

Rana sphenocephala were chosen for these experiments for their reported resistance to *Bd* (Peterson *et al.*, 2007). In fact, a recent study demonstrated that *Bd* was detected in this species in Illinois as early as the period of 1900-1920 (Talley *et al.*, 2015). Additionally, the range of this species clearly overlaps with that of *Rana catesbeiana*, a species long suspected to act as reservoirs of this pathogen (Daszak *et al.*, 2004; Schloegel *et al.*, 2009, 2012). Despite these facts, there are no reported *R. sphenocephala* declines due to chytridiomycosis. Prior to this study, it was unclear what role symbiotic skin bacteria might play in protecting this species from *Bd*. Here, we present evidence for the importance of the skin bacterial defense in providing some protection of juvenile *R. sphenocephala* against chytridiomycosis. Our results support previous studies in other amphibian species (Woodhams *et al.*, 2007; Becker & Harris, 2010; Lam *et al.*, 2010), with the added benefit of assessing the bacterial contribution to defense prior to the development of a skin peptide defense and at a time when the adaptive immune defense is emerging. Our protocol also added a more robust bacterial depletion protocol. Because this skin bacteria defense is present at metamorphosis prior to the maturation of other known immune defenses, it may represent an important defense against *Bd* in species which lack peptide defenses altogether or, like *R. sphenocephala*, do not secrete peptides immediately following metamorphosis. Thus, skin bacteria may be a critical contributor to the persistence of multiple amphibian populations and comprise an important part of the ecosystem. It should also be noted that the mechanism through which skin bacteria are protective may be a combination of factors, including microbial competition for space and resources in addition to the secretion of antifungal metabolites.

Previous studies have observed metamorphosing frogs to be at a particularly susceptible life stage to *Bd* infection in multiple species and populations (Rachowicz & Vredenburg, 2004; Tobler & Schmidt, 2010; Walker *et al.*, 2010). This conclusion is further supported by a study reporting that *Bd* infection of *Anaxyrus americanus* was more severe for newly metamorphosed juveniles than for those first exposed at 28 days post-metamorphosis (Ortiz-Santaliestra *et al.*, 2013). This disparity could be due to development of a more effective peptide defense and a rapidly expanding lymphocyte-mediated defense in older individuals, leading us to conclude that the additional time necessary for other immune defenses to mature also applies to other amphibian species.

4.3 Possible explanations for reduced metamorph survival

Despite the significant differences in fungal infection level between metamorphs with and without skin bacteria at early time points, presence of skin bacteria did not significantly correlate with increased survival. Protective bacteria, which may have been able to limit zoospore colonization, may have been lost over time even though we tried to maintain normal microbiota from the mesocosm water. Alternatively, re-colonization of the skin by bacteria from the food source or other laboratory sources in the bacteria-depleted group may explain the lack of difference in survival between groups. Restored bacteria could begin to compete for space.

This highlights the difficulties in simultaneously maintaining or depleting natural skin microbiota in laboratory conditions on a long-term basis. Further, smaller metamorphs were less likely to survive in general. As each of the four experimental groups contained a mixture of small, medium, and large metamorphs, reduced survival of the smaller metamorphs may explain the reduced survival in all groups. Finally, the difficulty of rearing recently metamorphosed juveniles cannot be overstated, as this period in which the immune system development is still incomplete (reviewed in Rollins-Smith, 1998 and Rollins-Smith *et al.*, 2011). As a result, close monitoring of amphibians is important during studies using reduction of skin bacteria in metamorphs, including future bioaugmentation trials that may introduce this protocol to open niches on the skin in order to increase the likelihood of persistence of introduced antifungal skin bacteria.

4.4 Uses and potential modifications of the bacterial reduction protocol

We report an antibiotic protocol that significantly reduced skin bacteria levels following 48 hours of treatment for the species *R. sphenoccephala*. This protocol and others reported (Becker & Harris, 2010) will be of interest to anyone undertaking future studies aimed at bioaugmentation, as short-term reduction of skin bacteria may aid long-term colonization of added bacteria. We suggest that our protocol could be further modified to include antifungal drugs if the desired goal is reduction of skin bacteria on individuals already infected by *Bd*. This modification could prevent *Bd* colonization on skin once natural bacterial competitors were removed and prior to bioaugmentation with more beneficial species.

In our experiments, one limitation in maintaining bacteria-reduced metamorphs became apparent. We used penicillin and streptomycin in sterile water in an effort to maintain reduced skin bacteria levels and prevent colonization of the skin by environmental bacteria present in the lab, introduced food crickets, and metamorph excrement. However, long-term exposure to these drugs appeared to be detrimental to survival, possibly because of reduction of non-target microbiota such as those in the gut that are essential for proper digestion and nutrient absorption (Stevens & Hume, 1998). Further studies should address the colonization and persistence of probiotic bacteria with and without bacterial reduction by antibiotics to determine whether bacterial reduction is necessary to support bioaugmentation.

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867 metamorphosis (significant differences by two-tailed Student's *t*-tests, * $p < 0.05$, ** $p < 1 \times 10^{-5}$),
868 *p*-values were multiplied by two to correct for two *t*-tests in one experiment. (d) Peak area for
869 Brevinin-1Sb spiked into HPLC-grade water ("Brevinin-1Sb Only", $N = 10$) or into the same
870 volume of samples from one-year post-metamorphic frogs in HPLC-grade water ("1-Week
871 Samples + Brevinin-1Sb", $N = 10$) (no significant differences by two-tailed Student's *t*-test, $p >$
872 0.05).

Table 1. The culturable microbiome of newly metamorphosed *R. spheonocephala*

Taxonomy (GenBank accession number of closest match in parentheses)	Activity	% Change	p-value
Actinobacteria			
Microbacteriaceae			
1. <i>Curtobacterium flaccumfaciens</i> (KF818635)	Inhibitor	-39.1%	6.1E-06
2. <i>Microbacterium lacus</i> (KF057952)	Enhancer	16.2%	4.5E-07
3. <i>Microbacterium</i> sp. KBKU19 (KF751800)	Inhibitor	-26.7%	1.8E-13
Micrococcaceae			
4. <i>Arthrobacter</i> sp. strain SD41 (KC415036)	No Effect	0.9%	8.6E-01
5. <i>Arthrobacter globiformis</i> strain B2S5 (EU221365)	Enhancer	18.0%	9.6E-06
6. <i>Arthrobacter globiformis</i> strain cp45 (JN082276)	No Effect	4.1%	9.3E-01
7. <i>Arthrobacter aurescens</i> (FM213390)	Inhibitor	-15.3%	1.0E-05
8. <i>Kocuria kristinae</i> (HF548363)	No Effect	0.1%	9.5E-01
Nocardiaceae			
9. <i>Rhodococcus fascians</i> (HG796188)	Enhancer	11.3%	9.1E-04
Bacteroidetes			
Flavobacteriaceae			
10. <i>Chryseobacterium defluvii</i> (JX500178)	Inhibitor	-22.2%	1.9E-10
11. <i>Chryseobacterium hispalense</i> (EU336941)	No Effect	3.1%	6.4E-01
12. <i>Chryseobacterium chaponense</i> (JX287903)	Inhibitor	-18.6%	5.4E-08
13. <i>Chryseobacterium</i> sp. strain L7-15 (AB819816)	No Effect	-5.9%	5.7E-02
14. <i>Chryseobacterium indologenes</i> (AB517708)	No Effect	-10.8%	9.5E-02
15. <i>Chryseobacterium</i> sp. strain PanRB004 (AB581570)	Inhibitor	-19.0%	5.6E-09
Firmicutes			
Bacillaceae			
16. <i>Bacillus</i> sp. strain SG2 (HM057848)	No Effect	6.7%	7.5E-02
Enterococcaceae			
17. <i>Enterococcus</i> sp. CD23 (AB673465)	No Effect	0.1%	8.9E-01
18. Uncultured <i>Enterococcus</i> sp. clone SL26 (HQ264086)	N/A	N/A	N/A
Proteobacteria			
Caulobacteraceae			
19. <i>Brevundimonas nasdae</i> (KF006808)	Inhibitor	-79.9%	9.3E-26
20. <i>Brevundimonas</i> sp. strain 266XY5 (KF818659)	No Effect	-18.4%	5.9E-02
21. <i>Brevundimonas vesicularis</i> (KF818658)	Inhibitor	-27.2%	4.4E-06
Methylobacteriaceae			
22. <i>Methylobacterium aquaticum</i> (EU977594)	N/A	N/A	N/A
23. <i>Methylobacterium extorquens</i> (KF572999)	N/A	N/A	N/A
Rhizobiaceae			
24. <i>Agrobacterium</i> sp. strain CRR1-74_SB13A (KF840387)	No Effect	-21.5%	3.1E-02
25. <i>Rhizobium</i> sp. strain M37C698A00 (JX292614)	N/A	N/A	N/A
Sphingomonadaceae			
26. Uncultured clone 300C-C03, related to <i>Novosphingobium</i> spp. (AY662023)	Inhibitor	-21.0%	5.0E-06
27. Uncultured clone 2.31, related to <i>Novosphingobium</i> spp. (JN256101)	No Effect	-13.7%	9.5E-03
28. Uncultured clone ncd09h10c1, related to <i>Sphingomonas</i> spp. (HM251127)	Inhibitor	-32.9%	5.0E-11
29. Uncultured clone 16slp96-1f07, related to <i>Sphingomonas</i> spp. (GQ158667)	No Effect	10.2%	6.3E-03
Comamonadaceae			
30. <i>Comamonas</i> sp. ZYM5 (AB847926)	No Effect	13.8%	7.8E-02
Enterobacteriaceae			
31. <i>Citrobacter freundii</i> (FN997616)	Inhibitor	-42.8%	7.2E-05
32. <i>Enterobacter aerogenes</i> (AB844449)	Inhibitor	-90.4%	1.8E-25
Moraxellaceae			
33. <i>Acinetobacter calcoaceticus</i> (KF843714)	Inhibitor	-22.6%	1.0E-19
34. <i>Acinetobacter rhizosphaerae</i> (JX133182)	Inhibitor	-15.8%	8.1E-06
Xanthomonadaceae			
35. <i>Pseudomonas geniculata</i> (KF254513)	Inhibitor	-93.6%	4.7E-24
36. <i>Pseudomonas hibiscicola</i> (KC172017)	Inhibitor	-90.3%	5.3E-46
37. <i>Stenotrophomonas maltophilia</i> (KC581677)	Inhibitor	-89.6%	8.5E-38
Deinococcus-Thermus			
Deinococcaceae			
38. <i>Deinococcus</i> sp. SA1 (KF790633)	Inhibitor	-17.8%	1.8E-08
39. <i>Deinococcus aquaticus</i> (NR_043472)	No Effect	-4.0%	9.3E-02

Isolates were collected from *R. spheonocephala* metamorphs (N = 15) and identified by 16S rDNA sequencing (Woodhams *et al.*, 2015). Each p-value was calculated by comparing *Bd*

growth in the presence and absence of each isolate's supernatant by two-tailed Student's t-test. All isolate sequences matched sequences of closest BLAST matches with percent identities of $\geq 99\%$. In order to determine significance for each isolate's inhibition of *Bd* growth ($n = 35$), the Bonferroni correction was used to establish a conservative p-value cut-off to reduce the likelihood of false positives: $p < 1.43 \times 10^{-3}$. Use of a sequential Bonferroni procedure confirmed the significance or non-significance of each p-value. Isolates labeled 'N/A' did not grow in tryptone broth.

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