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Comparison of *in vitro* methods to inhibit growth of a virulent strain of *Batrachochytrium dendrobatidis* (Longcore, Pessier, and Nichols 1999)

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***Abstract.*—A highly virulent genotype of *Batrachochytrium dendrobatidis* (*Bd*), the Global Panzootic Lineage (*Bd*-GPL), is implicated as a major cause of global amphibian population declines. *Bd*-GPL strain JEL274 was originally isolated from the skin of Western Toads (*Anaxyrus boreas*) in Colorado, USA, where populations are in decline due to chytridiomycosis, the disease caused by *Bd*. Here, the difficulty in attempting to control *Bd*-JEL274 is shown by challenging the genotype against three methods of inhibition: exposure to the antifungal drug amphotericin B, recombinant *E. coli*-violacein, and *A. boreas* skin-associated bacterial isolates. The Minimum Inhibitory Concentration (MIC) value of amphotericin B on *Bd*-JEL274 was 10-fold higher than in previously tested strains, suggesting that *Bd*-JEL274 is remarkably drug-resistant. Violacein, an antifungal secondary metabolite naturally expressed by some proteobacteria, has been shown to inhibit growth of the fungus. In this study, the difference in fungal inhibition between recombinant *E. coli*-violacein and natural antifungal activities of bacteria isolated from captive *A. boreas* skin was demonstrated using *in vitro Bd*-bacterium inhibition assays. The abundant skin-associated bacterium C*hryseobacterium indologenes* inhibited *Bd*-JEL274 significantly better than recombinant *E. coli*-violacein and this bacterium may have been involved in the natural clearing of *Bd* infections in the toads. Larger studies should focus on using the amphibian skin microbiome for probiotic treatment of chytridiomycosis in *A. boreas* toads, rather than risking lowered fitness or increased mortality from drug treatments.**

Keywords. Amphibian conservation, *Anaxyrus boreas,* Anura, chytridiomycosis, microbial ecology, transformation, violacein, wildlife disease

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Introduction

Loss of biodiversity is one of the most problematic issues facing humans today, and amphibians are the most threatened taxonomic class (Howard and Bickford 2014). The cascading implications of losing the majority of members of a major vertebrate clade are likely to have effects that disrupt entire ecosystems (Briggs et al. 2005). Amphibian populations around the world have dramatically declined in the last four decades, with over 3% of amphibian species already extinct (Alroy 2015). Current projections estimate that more than 7% of all amphibians will become extinct within the next century (Alroy 2015). One of the greatest threats to amphibian populations is infection by the parasitic fungus *Batrachochytrium dendrobatidis* (*Bd*). This pathogen is easily transmitted between hosts because the aquatic zoospores are free-living, flagellated, and substrate independent. An alarming number of amphibian species are known to be susceptible to infection by *Bd* (Olson et al. 2013; Scheele et al. 2019). *Bd* can cause extremely damaging effects on wild amphibian populations and is thought to have been the proximate driver of many amphibian species extinctions throughout the world since the 1970s (Kriger and Hero 2009; Scheele et al. 2019).

Most amphibian population declines that are sufficiently documented are specifically attributed to the Global Panzootic Lineage of *Bd* (*Bd*-GPL) [Farrer et al. 2011; James et al. 2015]. *Batrachochytrium dendrobatidis* is hypothesized to have originated in Asia and disseminated around the world due to amphibian trade, including the use of frogs for pets, food, and medical testing (O'Hanlon et al. 2018), and it is therefore considered an invasive species. Of six known *Bd* lineages,

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Bd-GPL is a hypervirulent genotype that represents the majority of genotypes isolated globally, which may be outcompeting endemic lineages (Schloegel et al. 2012; James et al. 2015). Even within certain *Bd* lineages such as GPL, there is significant variation in virulence between the genotypes (Berger et al. 2005; Fisher et al. 2009; Farrer et al. 2011). *Bd*-JEL274 is responsible for Western Toad (*Anaxyrus boreas*) declines in the United States, therefore this study utilizes *Bd*-GPL genotype JEL274 to evaluate the antifungal effects of *A. boreas* skin-associated bacteria as well as the drug susceptibility of this *Bd*-GPL strain (Muths et al. 2003; Scherer et al. 2005; Pilliod et al. 2010).

Although extensive *A. boreas* declines have occurred due to *Bd* infection, remaining populations that survived the emergent disease may consist of individuals which have some sort of resistance mechanism, such as antifungal skin-associated bacteria or secretions of bufadienolide chemicals (Park et al. 2014; Barnhart et al. 2017). These studies indicate that the individual survival probability of each toad seems to be strongly dependent on the composition of skin microbiota, host stress level, and virulence level of the *Bd* genotype. Therefore, understanding the microbiomes of *A. boreas* toads, as well as the drug resistance level of the *Bd* genotype associated with this species, could lead to treatment strategies that may prevent additional *Bd*-related die-offs.

In areas of high *Bd* prevalence, surviving populations of amphibians tend to have higher numbers of antifungal bacteria in their skin microbiomes (Harris et al. 2009). Thus, one promising treatment involves augmenting an amphibian's skin bacteria to fight pathogens (Harris et al. 2009). *Bd* is susceptible to the cutaneous, Gram-negative bacterium *Janthinobacterium lividum*, one of the native microbes that can be cultured from skin swabs of some amphibians (Harris et al. 2009). *Janthinobacterium lividum* secretes protective secondary metabolites violacein and indole-3-carboxaldehyde, which have been shown to inhibit pathogen growth (Brucker et al. 2008).

Current evidence suggests that epithelial cells of some amphibians are incapable of supporting *J. lividum* or other non-native bacterial strains, therefore simply inoculating *Bd*-susceptible amphibians with foreign bacteria may not be a plausible approach to treating or preventing chytridiomycosis (Becker et al. 2011). For example, amphibian skin peptides and alkaloids may be toxic to invading bacteria if the host and foreign microbe did not co-evolve. Bacteria that are associated with frogs which secrete toxins from their skin can tolerate these toxins, while artificially introduced microbes may not (Becker et al. 2011).

Although a number of experimental treatments for chytridiomycosis have been tried, viable treatment options for *Bd* remain debatable (Woodhams et al. 2012). Experimental solutions include the use of antifungal pharmaceutical drugs, which have their merits, but can also be toxic and have been known to kill or retard the growth of treated frogs (Martel et al. 2011). This study included a minimum inhibitory concentration (MIC) assay that shows the *in vitro* effects of the antifungal drug amphotericin B on *Bd*-JEL274. Violacein-producing microbes were not found from swab cultures in this study therefore, the heterologous host *E. coli*-violacein was used to understand the chemical's potency in comparison to native skin microbes in *Bd* inhibition assays. In order to better understand the basis for bacterial treatment options for chytridiomycosis, this pilot study also compared inhibitory capabilities between *A. boreas* skin-associated microbes. The results of this study can assist herpetologists in understanding how the relationships between an amphibian's own native skin bacteria and *Bd* zoospore levels may be important aspects of chytridiomycosis management in captive or wild populations, which can help guide conservation strategies that involve treatment of *Bd*-infected amphibians.

Materials and Methods

Sample Collection

During this study, the California State Polytechnic University, Pomona herpetological vivarium housed three *A. boreas* toads. This species was chosen based on its availability for repeated swabs and its conservation status. According to the IUCN, this species is declining in population, but it is not federally listed as Endangered. Toads were collected from Menifee, California (33°44'07.75"N, 117°11'59.17"W, near McLaughlin Road). One male (#2) was collected in April 2011, and one male (#1) and one female (#3) were collected in May 2014 and housed with the original male. Toads were swabbed for bacterial collection according to Brucker et al. (2008). Each toad was handled using clean latex gloves and thoroughly rinsed with a minimum of 10 mL sterile water to remove transient bacteria and soil contaminants. Using DNA-free rayon swabs (Medical Wire and Equipment Co., #MW 100-100), toads were swabbed five times at each of the following locations for a total of 25 strokes: ventral surface from mid-abdomen to cloaca; each inner thigh; and one stroke on the ventral side of webbing between each hind leg toe. Two swabs were used per individual toad, one for bacterial culturing and the other for *Bd* detection. For the bacterial collection swabs, the Copan Innovation™ swabbing system was used, in which the swabs were soaked in sterile PBS and the samples were shaken prior to spreading 50 µL of solution onto agar plates using a sterile spreader.

Bacterial Culture and Isolation

Swabs were thoroughly streaked onto 1% tryptone agar and Tryptic Soy Agar (TSA) plates to allow a variety of bacteria to grow in different nutrient conditions. Plates were incubated at 25 °C and colony growth

was characterized after 72 h, when individual colonies were picked from culture plates using a sterile loop and streaked for isolation onto new agar plates. Only the most visually unique and abundant colonies were sub-cultured and isolated. Visually unique colonies included those of differing colors, sizes, and textures. Each bacterial isolate was Gram stained and evaluated microscopically (Coico 2006). Bacterial taxa were then identified using 16S rRNA gene sequencing analysis.

Genomic DNA Extraction and 16S rRNA Gene Sequencing

Genomic DNA was extracted from a total of 18 bacterial isolates using the Invitrogen Easy-DNA Kit (cat No. K1800-01, Life Technologies™, Carlsbad, California, USA) for genomic DNA isolation, according to the manufacturer's instructions. Extracted DNA was amplified using the eubacterial 16S rRNA gene primer set, B27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and B1492r (5′-ACCTTGTTACGACTT-3) [Eurofins Microbiology, Garden Grove, California, USA]. PCR parameters were 5 min at 94 °C; 35 cycles of 30 sec at 94 °C, 30 sec at 55 °C, and 2 min at 72 °C; followed by 2 min at 72 °C. PCR product was purified using QIAquick PCR purification kit (cat. Nos. 28104 and 28106, Qiagen, Hilden, Germany) and checked for DNA quality and quantity using an Implen NanoPhotometer® (Westlake Village, California, USA). Aliquots of samples were analyzed by gel electrophoresis to ensure proper amplification of the 16S rRNA gene region. The amplified products were sent for Sanger sequencing to Source BioScience (Santa Fe Springs, California, USA). Forward and reverse sequence reads were then concatenated using SeqTrace. Identification of the 16S gene in FASTA format was performed using NCBI BLASTn based on sequence similarity to GenBank sequence database entries (Altschul et al. 1990).

Growth and Maintenance of *Bd*

Bd-JEL274, originally isolated from *A. boreas* toads in Clear Creek Co., Colorado (1999), was obtained from Joyce Longcore of the Maine Chytrid laboratories, University of Maine. *Bd* was maintained in Tryptone Glucose hydroLysate (TGhL) broth or 1% (w/v) tryptone broth for seven days until active zoospores were visible under a dissecting microscope, and then sub-cultured periodically to maintain fresh cultures. *Bd* grew best in TGhL broth with incubation at 23–24 °C. Cultures were grown on agar plates and/or in 25-cm^2 Corning cell culture treated flasks and incubated for 5–10 days. Growth progress was viewed under a dissecting microscope. Once maximum zoospore production was observed, plates and flask tops were wrapped in Parafilm®, and stored at 4 °C for up to two months. Zoospores were harvested by scraping the flask walls before aspirating liquid from the flasks, and by flooding plates with 3 mL of 1% tryptone broth before transferring the zoospores and sporangia to new media.

Production of Zoospores

Bd was grown in broth until clumps of sporangia were visible to the unaided eye. A sterile serological pipette was used to add 0.75 mL of this broth culture to tryptone agar in 9-cm culture dishes. Inoculated dishes were left open in a laminar flow hood until the added broth was dry. Covers were replaced on dishes, which were then incubated at 23–24 °C. After 7–10 days, active zoospores could be observed around the periphery of the fungal colonies by inverting the dishes on the stage of a dissecting microscope viewed at 40x. Zoospore concentration was measured by counting spores using a hemocytometer. To harvest zoospores, plates were flooded with 3 mL 1% tryptone broth and after ~10 min, zoospores in liquid were collected by pipetting. Glassware was bleached before washing and sterilization. All materials that contained or came into contact with the pathogenic fungus were autoclaved before disposal.

Determination of Minimum Inhibitory Concentration of Amphotericin B for *Bd*-JEL274

The MIC of amphotericin B (X-Gen Pharmaceuticals, Inc., Big Flats, New York, USA) for *Bd*-JEL274 was determined using a macrodilution method in 24-well plates. The final assay concentrations of the drug were 3.2, 1.6, 0.8, 0.4, 0.2, and 0.1 µg/mL. To each well, 200 µl of TGhL culture broth containing one of the serial dilutions of amphotericin B was added to 200 µl of a five-day-old growing *Bd* culture, containing a mixture of approximately 1 x 10^5 *Bd* sporangia and zoospores. Cells were counted using a hemocytometer, then diluted in TGhL media until a standard 5 x 10^5 concentration of zoospores and sporangia was obtained. The MIC value was determined as the lowest concentration of amphotericin B at which no growth of *Bd* was recorded. Growth was assessed after 5, 7, and 10 days of incubation at 24 °C using stereoscopic and compound microscopic examination of the wells. To obtain final *Bd* cell counts, 10 µL of spores from the appropriate wells were placed in a hemocytometer and five out of 25 grids were counted within the larger grid. The concentration of zoospores was calculated by multiplying the number of cells by five, and again by 10,000 to obtain cells/mL. This experiment was carried out in triplicate.

Recombinant *E. coli*-Violacein

Plasmid cloning vectors containing the violacein gene operon copied from *Chromobactrium violaceum* were obtained from Derek Sarovich (Sarovich and Pemberton

2007). The pPSX-vio++ vector (pPSX-violacein) produces moderate amounts of violacein. pJP1000 (pUC18-violacein), a pUC18 derivative, is multi-copy and produces high levels of violacein. The pPSX-vio+ (pPSX-violacein-opv1) over-produces violacein. The relative degrees of violacein production were indicated by color intensity of the cultures (light to dark violet). The pPSX-vio+, pPSX-vio++, and pJP1000 vectors were transformed into *Escherichia coli* NEB5-alpha (New England BioLabs) using the manufacturer's instructions. All plasmids were stable when cloned into *E. coli*, even under no selection pressure. Visual evidence of violacein production by *E. coli* was observed when cultures showed the deep violet color characteristic of violacein after transformation and throughout the passage of generations.

Bd Inhibition Assays

Inhibition assays followed Harris et al. (2006). *Bd* was grown on 1% tryptone, 1% agar plates for five days, or until maximum zoospore production was observed (up to seven days). Three mL of sterile water was added to each plate, and plates were rocked back and forth to loosen the zoospores from the agar. Plates were tilted so the liquid pooled on the side, and 0.75 mL was pipetted and spread onto four new tryptone plates using a sterile spreader. Each *Bd* plate was used to make four new plates. Plates were left to dry in a laminar flow hood for 45–60 min. After the *Bd* solution had soaked into the fresh agar plate, using a sterile loop one colony from each bacterial isolate (24-h agar cultures) was streaked across the center of the agar plate. Plates were incubated inverted at 24 °C for 5–10 days.

Scoring: Measurement of Zone of Inhibition

Differences between bacterial isolates and between treatments for an isolate in inhibition experiments were tested after **7**–10 days growth on agar medium. The bacterial isolates were tested in triplicate and scored as inhibitory against a lawn of *Bd.* Six points around the bacterial streak reaching to the edge of the zone of inhibition (ZOI) were measured using digital calipers. The average zone of inhibition (the distance between the edge of the bacterial streak where no *Bd* growth occurred and the edge of the *Bd* lawn) and standard deviations were calculated for all bacteria with inhibitory properties. Bacterial isolates were scored as "not inhibitory" if no zone of inhibition developed and the plates were covered with active zoospores after 7–10 days of incubation (ZOI = 0). Bacteria were considered "weakly inhibitory" if a clear ZOI < 10 mm developed between the bacterial streak and the *Bd* culture. Bacterial isolates with a ZOI > 10 mm were considered as having strong antifungal properties. When a bacterial streak overtook the whole plate, the assay result was considered indeterminate. If an indeterminate result was obtained, the experiment was repeated two more times before being recorded as indeterminate. The negative control plates containing *Bd* showed complete lawns of *Bd* (Harris et al. 2006; Park et al. 2014).

Statistical Analysis

A non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons tests were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, California, USA, http://www.graphpad.com). The Kruskal-Wallis rank test was used to test for differences in fungal inhibition by bacterial isolates. Differences were considered significant if $P < 0.05$. A box plot for the amphotericin B *Bd* MIC assays was created in Rstudio (Version 1.0.136, RStudio, Inc.) to show differential growth between the positive control and the experimental groups.

Bd Detection in Toads

Toads were swabbed twice for the presence of *Bd,* nine months apart. Swabs were taken according to Kriger et al. (2006), using a sterile DNA-free fine-tipped rayon swab (Medical Wire and Equipment Co., #MW 100-100). *Bd* swabs were placed in microcentrifuge tubes containing 50 µL 70% ethanol and frozen at -20 °C until they could be brought to the Vredenburg lab at San Francisco State University (San Francisco, California, USA) for DNA analysis. Latex gloves were used during any interactions with housing, tubes, or frogs, and changed as necessary. DNA was extracted using Prepman Ultra (Life Technologies, Carlsbad, California, USA) in accordance with Cheng et al. (2011). DNA extracts were amplified following a standard, probe-based quantitative Polymerase Chain Reaction (qPCR) protocol using the standard *Bd* primer set ITS1-3-CHYTR and 5.8S-CHYTR (Boyle et al. 2004; Hyatt et al. 2007).

Results

Amphotericin B: *Bd*-JEL274 Minimum Inhibitory Concentration (MIC)

MIC assays of the antifungal drug amphotericin B were conducted in the range of 0.1–3.2 µg/mL and indicated the MIC for *Bd*-JEL274 as 1.6 µg/mL. Little to no difference in growth was observed in wells with drug concentrations from 0.1–0.8 µg/mL, however, noticeable inhibition occurred at 1.6 µg/mL, where there were visibly fewer viable cells than in the positive control. The fungicidal effect of amphotericin B was obvious at 3.2 µg/mL, as the vast majority of cells were dead. The MIC value was determined as the lowest concentration of amphotericin B at which no growth of the *Bd* strain was recorded. A boxplot of the triplicate results was created to compare

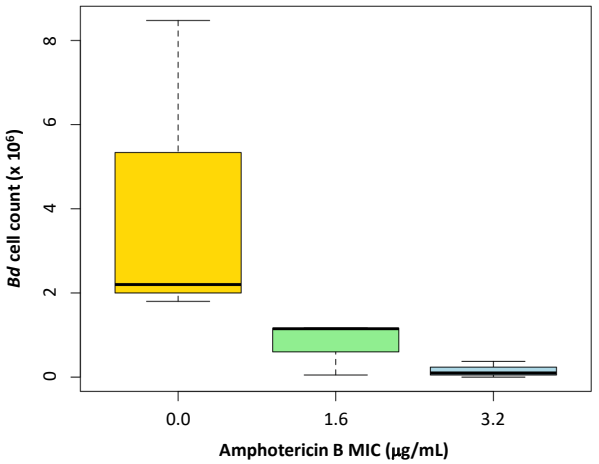

Fig. 1. The Kruskal-Wallis rank sum test for the boxplot returned a chi-squared value = 2, degrees of freedom (df) = 2, and P = 0.3679. The Wilcoxon rank sum test for the positive control and the highest concentration of amphotericin B returned W = 9, P = 0.1. These analyses show that the alternative hypothesis is true and assumes that there is information in the magnitudes and signs of the differences between paired observations, because the mean (location shift) is not equal to 0. Observed differences in mean cell count between the three groups (0.0, 1.6, and 3.2 µg/mL) are not statistically significant ($P > 0.05$).

the positive control (0.0 µg/mL amphotericin B) with concentrations of amphotericin B for which *Bd i*nhibition was detected by microscopy, namely 1.6 µg/mL and 3.2 µg/mL (Fig. 1). For comparison, the result of the MIC of amphotericin B on *Bd*-JEL274 was 10-fold higher than previously reported for five other *Bd* strains (Martel et al. 2011). Although mean differences between cell counts in the experimental groups versus the control group are not statistically significant, this 10-fold increase in drug dose relative to that reported in previous studies is likely to be lethal to amphibians, if tested *in vitro.* Therefore, testing the amphotericin B MIC for *Bd*-JEL274 on *Bd*-infected animals is not recommended.

Bacterial Preparation and Identification

Bacterial isolates from captive *A. boreas* skin swabs were cultured using either 1% tryptone (Trp) agar or Tryptic Soy Agar (TSA) media (Suppl. Fig. 1). Using BLASTn Basic Local Alignment Search Tool for nucleotides (Altschul et al. 1990), B27f and B1492r 16S rRNA gene sequence reads in FASTA format were queried in the NCBI sequence database to obtain the closest relative matches. The identity (Ident %) is recorded as the extent to which two nucleotide sequences have the same residues at the same positions in an alignment within the database, expressed as a percentage. Sixteen bacterial strains were isolated and identified from swabs. Isolates were Gram stained and only potential proteobacteria (Gram negative rods) were identified genetically. Bacterial identities determined by 16S rRNA gene sequencing and NCBI BLASTn included members from six distinct classes: Flavobacteria (3), Gammaproteobacteria (1), Bacilli (4), Actinobacteria (4), Alphaproteobacteria (1), and Betaproteobacteria (2) [Table 1]. Two visually distinct isolates could not be identified by 16S region and subsequent NCBI BLASTn searches. Some genera were isolated from colonies which looked unique and identified more than once using 16S gene results and BLASTn. Duplicate isolates of genera include *Microbacterium* and *Paenibacillus*, while *Chryseobacterium* was isolated three separate times.

No violacein-producing bacteria were isolated from the skin swabs, consequently, the inhibitory action of natural violacein producers could not be evaluated using a *Bd* inhibition assay. Instead, violacein production was introduced using recombinant *E. coli* strains for the purpose of comparing the fungal inhibition capacity of violacein to the bacteria isolated from toad skin swabs. The *E. coli* NEB5-alpha strains included the negative control (unmodified *E. coli*), *E. coli*-vio+, *E. coli*-vio++, and *E. coli*-JP1000. Recombinant *E. coli* expressed the characteristic deep violet color of violacein (Suppl. Fig. 2).

***Bd* Inhibition Assays**

To determine if any of the isolates exhibited natural antifungal activity against *Bd*, the bacterial isolates and recombinant *E. coli*-violacein were challenged against *Bd*-JEL274 and the size of the ZOI around the bacterial streak was measured (Table 2). The violacein-producing *E. coli* strains did not produce significantly larger ZOI than the *E. coli* control. Of the 15 bacterial strains (11 skin isolates and four *E. coli* strains) challenged against *Bd*, 12 had a measurable ZOI surrounding the bacterial streak and the *Bd* clearing zones were well-delineated and clearly visible to the unaided eye (Fig. 2). According to the Kruskal-Wallis test, strong fungal inhibition coincided with bacteria that produced significantly larger ZOI than that of the violacein over-producer *E. coli*-vio+ ($p < 0.05$). A ZOI of 0.0 mm was associated with no inhibition, ZOI < 10 mm with weak inhibition, and ZOI > 10 mm with strong inhibition. *Lysinibacillus fusiformis* swarmed over the agar, overtaking the entire plate, so a ZOI could not be determined, and therefore *L. fusiformis* was considered indeterminate (No Data/ND).

The means and standard deviations of the ZOI for all trials were calculated for each bacterium tested (Table 2). Among them, *C. indologenes*, exhibited the largest ZOI of 11.0 mm, more than twice the size of the ZOI exhibited by the recombinant violacein over-producer *E. coli*-vio+ (4.2 mm). *Bacillus* sp. also exhibited inhibitory action with an average ZOI of 7.6 mm. Four bacterial isolates; two *Microbacterium* isolates, *Rhodococcus* sp., and *Brevundimonas* sp., did not show any distinct zone of inhibition, as zoospores were distributed directly adjacent to the bacterial streaks (Fig. 3).

A non-parametric Kruskal-Wallis test followed by

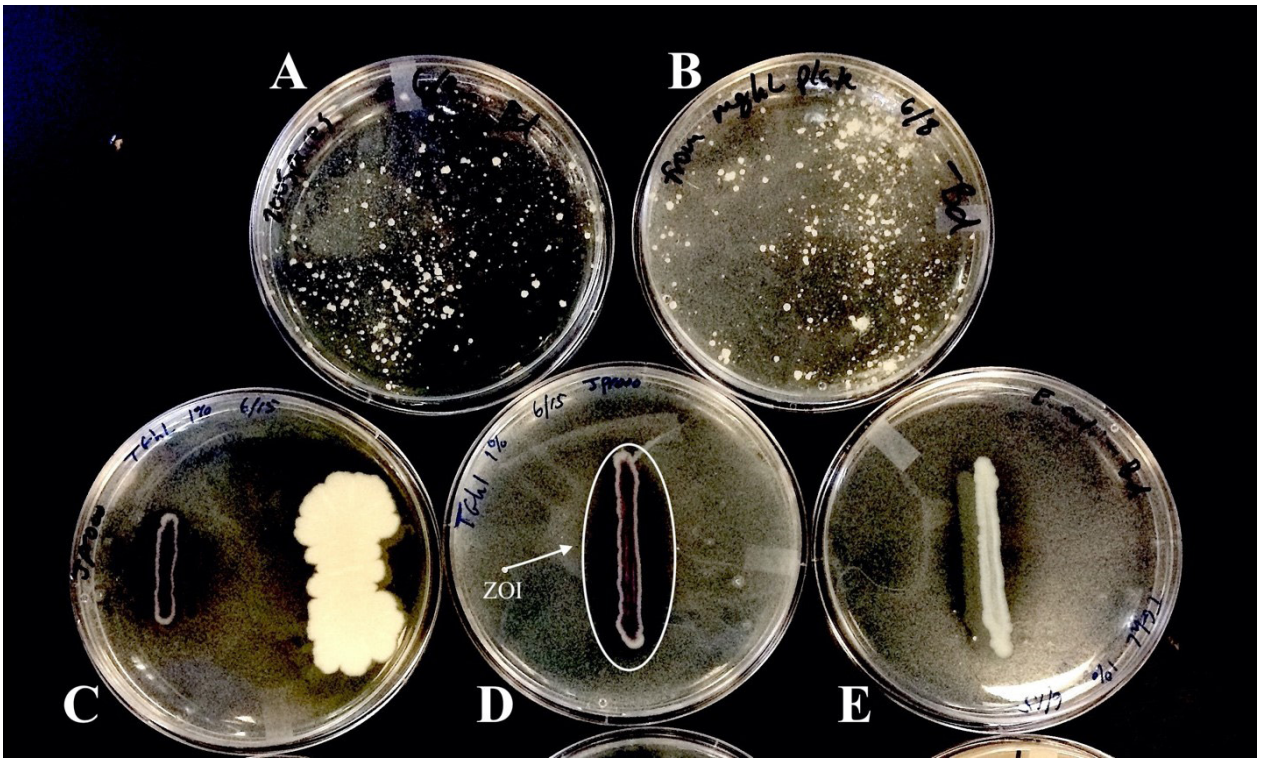

Fig. 2. (A) and **(B)** Duplicate *Bd* negative controls, **(C)** *E. coli*-JP1000 (left bacterial streak) and *Bacillus* sp. (right bacterial streak), **(D)** *E. coli*-JP1000 displaying a clear ZOI surrounding the bacterial streak, **(E)** *E. coli* control.

Table 1. Bacterial isolate identification 16S rRNA gene sequences. *Unclassified Micrococcacae.

I.D.	Toad #	Medium	Gram stain	Identity (%)	Class	Genus/species
2xMD	1	1% Trp	- rod	97	Flavobacteria	*Chryseobacterium indologenes*
4xMD	1	1% Trp	- rod	98	Gammaproteobacteria	*Klebsiella oxytoca*
5xMD	3	1% Trp	+ rod	99	Bacilli	*Paenibacillus* sp.
7xMD	3	1% Trp	+ rod	97	Bacilli	*Paenibacillus pabuli*
8xMD	2	1% Trp	+ rod	95	Actinobacteria	*Microbacterium petrolearium*
A1	1	TSA	+ cocci	97	Actinobacteria	*Micrococcaceae bacterium**
A2L	1	TSA	+ rod	98	Actinobacteria	*Rhodococcus equi*
A3	1	TSA	+ rod	99	Actinobacteria	*Microbacterium* sp.
A5	1	TSA	- rod	99	Alphaproteobacteria	*Brevundimonas* sp.
A6(Y)	1	TSA	- rod	83	Betaproteobacteria	*Comamonas* sp.
A6P	1	TSA	- rod	83	Betaproteobacteria	*Acidovorax ebreus*
A7	1	TSA	- rod	92	Betaproteobacteria	*Ralstonia* sp.
A9	1	TSA	- rod	98	Flavobacteria	*Chryseobacterium tenax*
B1	3	TSA	+ rod	99	Bacilli	*Bacillus* sp.
B3	3	TSA	+ rod	99	Bacilli	*Lysinibacillus fusiformis*
B5	3	TSA	- rod	97	Flavobacteria	*Chryseobacterium tenax*
A2D	1	TSA	+ rod	-	Unidentified	
A8	1	TSA	- rod	-	Unidentified	

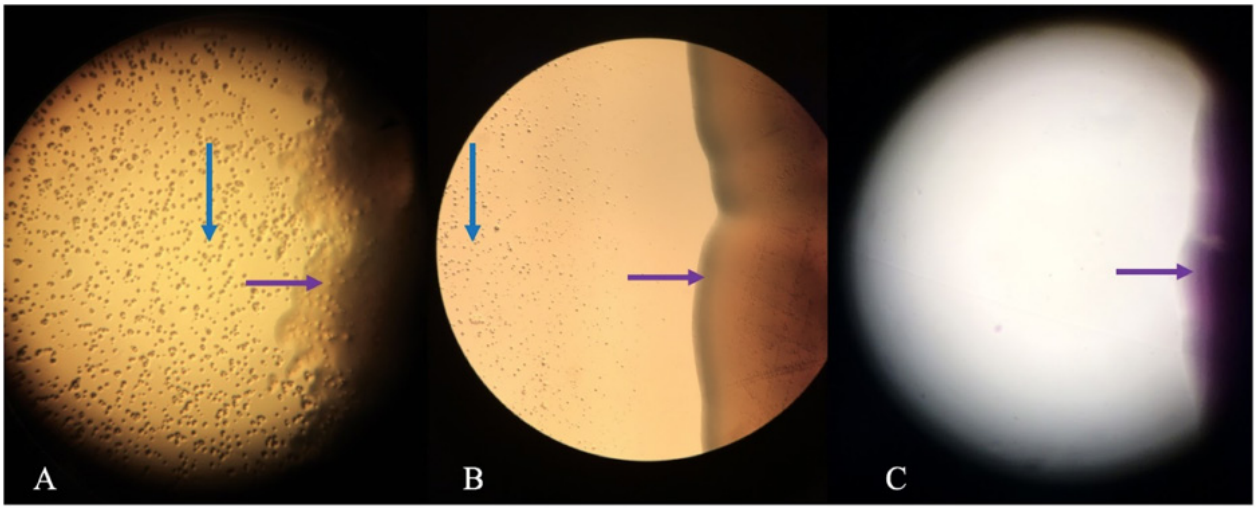

Fig. 3. Microscopic images (40x) of *Bd* challenge assays. **(A)** *Microbacterium*, **(B)** Micrococcaceae, and **(C)** recombinant *E. coli*-vio+. Vertical arrows point to the *Bd* lawn and horizontal arrows point to the left side of the bacterial streak. Absence of *Bd* lawn within field of view indicates the inhibitory effect from the bacterial streak. Approximate diameter of field of view ~0.5 mm.

Dunn's multiple comparisons tests were performed to test the significance of the size of the ZOI (Table 3). When comparing the relative inhibition of the negative control (*E. coli*) to the toad bacterial isolates and the genetically modified *E. coli*-violacein strains, only *C. indologenes* and *Bacillus* sp. (this strain is closely related to *Bacillus cereus*) showed significantly larger ZOI than *E. coli* ($P < 0.0001$). Among pairwise comparisons between isolates, *C. indologenes* inhibited *Bd* significantly more than the violacein overproducer *E. coli*-vio+. Although *Bacillus* sp. also had average ZOI greater than *E. coli*-vio+ there was no significant difference between their inhibitory effects, nor was there was a significant difference between average ZOI of *C. indologenes* and *Bacillus* sp.

Bd Detection by qPCR

Toads #1 and #3 (see Materials and Methods) initially tested positive for *Bd,* while toad #2 tested negative. The zoospore equivalent (ZE) scores indicated low level infections (ZE scores of 37.68 and 4.96, respectively). Nine months later, the infected toads were swabbed again and qPCR analysis revealed a complete clearance of infection in toad #1, and substantial reduction of zoospore load in toad #3 (ZE scores of 0.0 and 0.0808, respectively). Toad #2 was euthanized due to extensive peripheral edema before the second *Bd* swab was taken.

Discussion

To gain a better understanding of chytridiomycosis treatment options, three *in vitro Bd* inhibition methods were tested: the antifungal drug amphotericin B for which the MIC value was determined, *Bd* inhibition assays using skin-associated microbes, and inhibition assays using the antifungal compound violacein in recombinant *E. coli.* The results of this study favor of augmentation of the natural skin microbiota as the most feasible, efficient, and biologically relevant approach for chytridiomycosis treatment.

Amphotericin B works by binding directly to ergosterol (a chemical in fungi which is functionally equivalent to cholesterol in animals), which disrupts fungal cell membrane permeability and causes leakage. This drug has a stronger binding affinity for ergosterol than for cholesterol, but still binds to cholesterol, making it potentially lethal for host cells as well as fungal cells. Amphotericin B has low selectivity and can potentially cause nephrotoxicity in vertebrate hosts (Odds et al. 2003; Martel et al. 2011). However, this drug has been

Table 2. Zone of inhibition (ZOI) for *Bd* inhibition assays**.**

Bacterial strain	Avg. ZOI (mm)	S.D.
C. indologenes	11.00	4.72
Bacillus sp.	7.60	3.26
E. coli-vio+	4.21	4.55
E. coli-JP1000	2.55	2.87
Comamonas sp.	2.11	2.95
Micrococcacae bacterium	1.87	2.35
E. coli-vio++	1.16	1.08
A. ebreus	0.82	1.37
Ralstonia sp.	0.77	1.56
E. coli	0.46	1.11
K. oxytoca	0.21	0.41
Brevundimonas sp.	0.0	0.0
Microbacterium sp.	0.0	0.0
M. petrolearium	0.0	0.0
R. equi	0.0	0.0
L. fusiformis	ND	ND

Table 3. Dunn's multiple comparison test for zones of inhibition (ZOI). Symbols and definitions: ns (not significant) = $P > 0.05$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$.

Bacterium	ZOI (mm) ± SD	*E. coli*	*E.c.*v++	*E.c.*JP	*E.c.*v+	*B.*sp.	*C.i.*
C. indologenes (*C.i.*)	11.00 (± 4.72)	****	****	**	*	ns	
Bacillus sp. (*B.*sp.)	7.60 (± 3.26)	****	*	ns	ns		
E. coli-vio+ (*E.c.*v+)	4.21 (± 4.55)	ns	ns	ns			
E. coli-JP1000 (*E.c.*JP)	2.55 (± 2.87)	ns	ns				
E. coli-vio++ (*E.c.*v++)	1.16 (± 1.08)	ns					
E. coli	0.456 (± 1.11)						

found to cause fewer grossly observable side effects than itraconazole, such as reduced growth, which can diminish host fitness. Carey et al. (2006) showed that *Bd* zoospore load caused chytridiomycosis-induced death in *A. boreas* toadlets with exposure to *Bd*-GPL genotype JEL275, and found that exposure to 1 x 10^6 zoospores for three days was sufficient to cause mortality in the toads. Each MIC assay in this study used a standard starting concentration of 1 x 10^5 zoospores, a 10-fold lower concentration than the Carey study, and this MIC value would be too high to be safely applied to *in vivo* animal studies. The MIC assay included in this study showed that *Bd*-JEL274 has a high tolerance for amphotericin B, inhibiting *Bd* growth at a dosage of 1.6 µg/mL, and killing *Bd* at 3.2 µg/mL. Previous amphotericin B MIC assays using different *Bd* strains have shown lower effective MIC levels (Martel et al. 2011). High resistance to amphotericin B by *Bd*-JEL274, in conjunction with previously established evidence of toxicity of amphotericin B to amphibians, suggests that this drug may not be a practical treatment option for *in situ* treatment of chytridiomycosis in *A. boreas* toads, as the dosage needed for significant effects could cause negative side effects such as nephrotoxicity, reduced growth, death, or other unforeseen outcomes.

This study included a modest survey of the skin-associated bacteria from only three toads, and this approach corroborated the positive findings from previous amphibian microbiome studies. Although this study was narrow in terms of subjects, many of the same genera of skin-associated bacteria from these few captive toads are those also found throughout wild North American amphibian skin microflora (Roth et al. 2013). For example, the assemblage of microbes found was similar to that of wild-caught Colorado *A. boreas* toads, even though this pilot study used captive animals (Park et al. 2014). Previous studies on *A. boreas* have found bacteria closely related to those that were isolated from the captive toads here, such as *Bacillus*, *Lysinibacillus*, *Rhodococcus*, and *Chryseobacterium*, indicating that established skin-associated bacteria may persist through environmental changes (McKenzie et al. 2012). Of the bacterial isolates screened for potential anti-*Bd* activity *in vitro*, one of the 11 toad isolates (*C. indologenes*) inhibited *Bd* significantly better than the recombinant violacein over-producer, *E. coli*-vio+, suggesting that commonly occurring skin bacteria may be more efficient for use in chytridiomycosis treatment than the process of seeking out native violacein-producing bacteria in the amphibian population of interest. Two species of interest in this study, *C. indologenes* and *Bacillus* sp. (*B. cererus*), have previously been isolated from wild North American amphibians and have shown anti-*Bd* activity (Roth et al. 2013; Park et al. 2014). The antifungal properties of these bacteria provide insight into the ongoing investigations of using natural skin-associated bacteria as a bioaugmentation treatment for chytridiomycosis.

Chryseobacterium indologenes is a Gram-negative, lactose non-fermenting, oxidase-positive, rod-shaped bacillus with a distinct yellow to orange pigment. This bacterium is found ubiquitously in soil, on plants, and in water sources (Wauters et al. 2015). The antifungal properties of *C. indologenes* have not been studied; however, a closely related species, *Chryseobacterium aquaticum*, is known to secrete proteases and chitinases, which break down chitinous fungal cell walls (Gandhi et al. 2009). *Bacillus cereus* is known to protect agricultural crops from fungal infections, and it naturally secretes the antibiotics zwittermicin A and kanosamine which inhibit the growth of fungal plant pathogens. The antifungal compounds of *B. cereus* strains have been developed as useful biological control agents in the suppression of fungi and crop diseases (Silo-Suh et al. 1994). The synergistic application of *C. indologenes* and *B. cereus* in the biocontrol of *Bd* should be further explored as a chytridiomycosis treatment.

This study demonstrates that antifungal micro-symbionts can be found even within a small subset of bacteria cultured from amphibian skin, and that large-scale skin microbiota studies may not be necessary for finding antifungal bacteria that could potentially be used for disease treatment.

Chryseobacterium indologenes, and possibly *Bacillus* sp., may have assisted the hosts in clearing *Bd* infection. The three toads used in this study were captured from the wild in 2011 (#2), and 2014 (#1 and #3), and initially had contact with other *A. boreas* toads in the vivarium, preventing a clear determination of whether the *Bd* infections were contracted in the wild, or after the toads were brought into the vivarium. Despite *Bd* infection, the toads did not exhibit grossly observable symptoms of

chytridiomycosis, such as red legs and ventral surface, or skin sloughing. Two of the toads initially tested positive for *Bd* (#1 and #3) and either naturally cleared (#1) or reduced (#3) their infections. Having cleared the *Bd* infection within nine months suggests that the toads were infected recently at the vivarium and were not harboring the infection in the wild. The fact that *C. indologenes* was abundantly cultured from the skin swabs, and that it had significantly better capacity to inhibit *Bd in vitro* than all other isolates tested, circumstantially suggests that *C. indologenes* may have facilitated the observed clearing of *Bd.* One possible explanation for this is that the microbes were providing protection against *Bd* infection, but aside from this correlative evidence, other factors (such as general good health of the individuals or bufanolide production) could have also contributed to the clearing of infection.

Before commencement and during the course of the study, toad #2 had peripheral edema of the left front leg. Toad #2 initially tested negative for *Bd* but was euthanized due to signs of lethargy and a severely swollen leg before the second swab was taken. As a known opportunistic pathogen of amphibians, *C. indologenes* can cause peripheral edema among other symptoms in captive and wild animals, especially if the pathogen can enter the body through a lesion (Olson et al. 1992; Mauel et al. 2002). The effect of *C. indologenes* presence in the enclosure was not measured, however it is conceivable that there may have been an effect of inhibitory bacteria on the health status of the toads. The peripheral edema in one toad and the low level *Bd* infection in the other two toads that naturally cleared over time may have been correlated with the abundance of *Chryseobacterium* that was cultured from the skin swabs. Such knowledge of antifungal bacteria as shown in this study is encouraging for the future of amphibian microbiome research, and for the control of *Bd* without introducing pharmaceuticals to the natural environments, which could negatively affect non-target organisms.

Chryseobacterium indologenes, a common bacterium that seems to be highly abundant in the skin microbiome of North American amphibians, may have a better potential for use in the treatment of chytridiomycosis than the widely studied violacein-producing bacteria such as *J. lividum*. Additional studies could involve *in vivo* bioaugmentation assays using *C. indologenes* to understand the mechanism of its capacity for reducing zoospore load of infected amphibians and identify whether this bacterium can be safely inoculated onto otherwise healthy animals. It may be helpful to determine which antifungal compound(s) *Chyrseobacterium* secretes, and test whether these compounds can be isolated and utilized to treat chytridiomycosis.

The use of single isolates in a sterile experimental environment can be useful for establishing baseline observational data, but microbes do not exist in isolation in the natural world. Recombinant *E. coli* that expresses violacein may not fully represent how native microbes deliver or utilize this compound. For example, violacein is secreted by *C. violaceum* in extracellular membrane vesicles to both solubilize the hydrophobic pigment and transport the compound to other microorganisms in aqueous solutions, a mechanism that is partially responsible for its bactericidal effects (Choi et al. 2019). The secretion mechanism of violacein produced synthetically by plasmid DNA in recombinant *E. coli* is unknown and could impact violacein's biocidal effects.

Native microbial communities may also shape chytridiomycosis treatment strategies as amphibian skin supports a complex microecosystem. Isolated bacteria may not behave in the same way or excrete the same chemical compounds that they would in community settings. Antifungal bacteria have the potential to be augmented and used as natural biocontrols for *Bd* infection, however multi-species communities are known to inhibit *Bd* growth more than monocultures of constituent species, an important consideration for designing probiotic treatments (Piovia-Scott et al. 2017).

Interbacterial complementarity and synergy are important for healthy community function, and multispecies interactions should be studied in depth before performing *in vivo* trials of single isolate bioaugmentation. For example, the presence of *C. indologenes* has been shown to increase the survival of *C. violaceum* when the two organisms are co-cultured. When grown in co-culture with other sympatric bacteria, the production of violacein by *C. violaceum* can be reduced or decolorized, though violacein production is not reduced when grown in co-culture with *C. indologenes* (Shiau and Lin 2011). To achieve maximum *Bd* inhibition, future studies should include the selection of probiotic mixtures of bacteria that each have antifungal properties and are complimentary to each other's growth and production of antifungal compounds, perhaps a combination of *C. indologenes* and *J. lividum*.

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Supplementary Figures

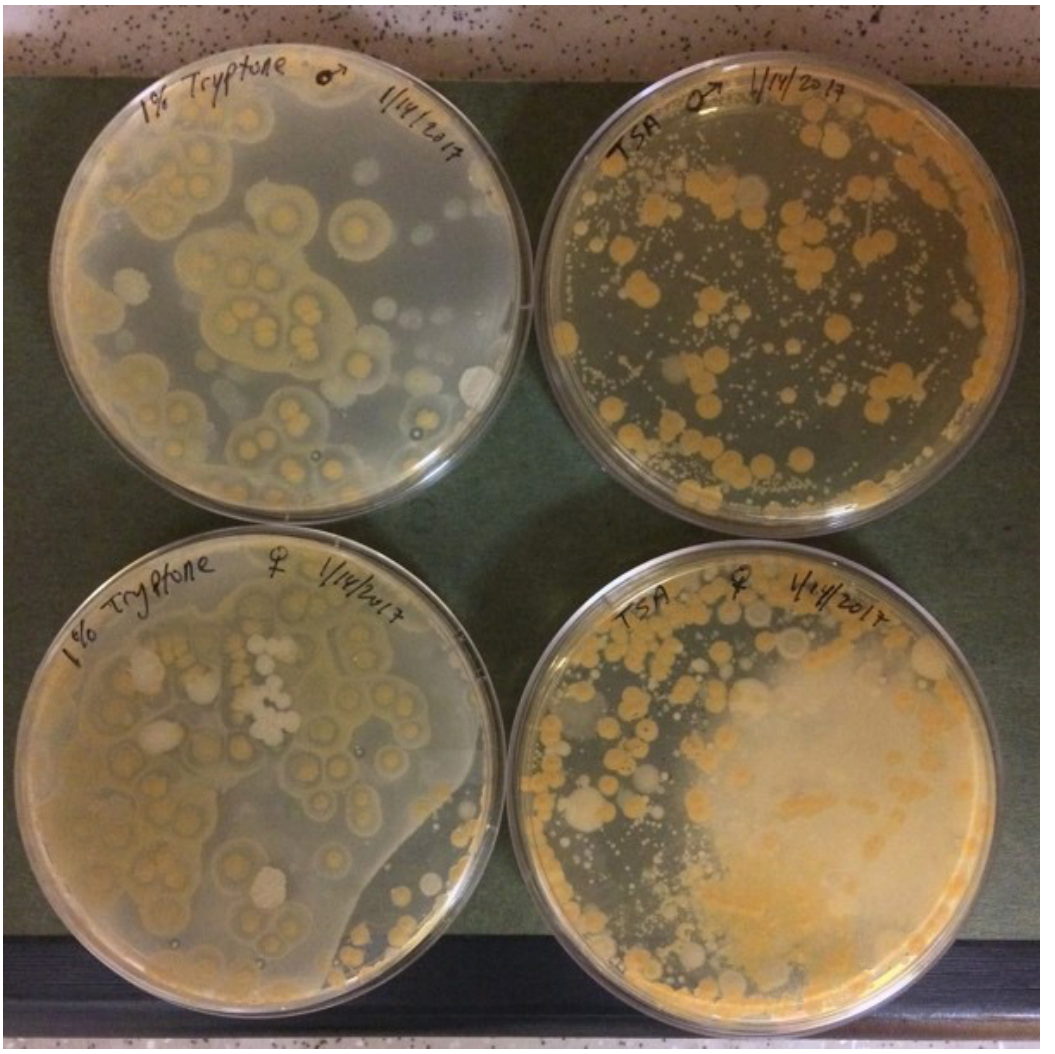

Fig. S1. Toad skin swab cultures. Culture plates from *A. boreas* skin swabs after three days incubation at 25 °C.

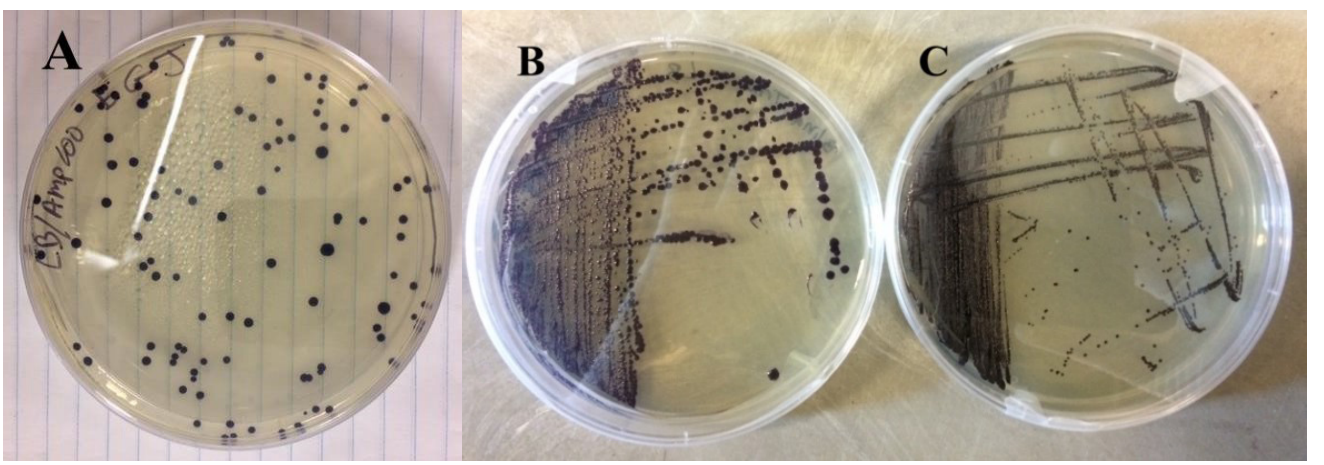

Fig. S2. *E. coli*-violacein transformations. Violacein gene transformations of *E. coli.* **(A)** Example of NEB5-alpha-pJP1000 colony growth after 24 h post heat shock transformation. Each week, transformants were passed by re-streaking onto fresh agar and incubated at 37 °C for 24 h. **(B)** NEB5-alpha-pPSXvio+, **(C)** NEB5-alpha-pPSXvio++.