Fall 2015

Ability of skin bacteria on the Panamanian frog species, Craugastor fitzingeri, to inhibit the fungal pathogen Batrachochytrium dendrobatidis

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Ability of Skin Bacteria on the Panamanian Frog Species, *Craugastor fitzingeri,* to Inhibit the Fungal Pathogen *Batrachochytrium dendrobatidis*

An Honors Program Project Presented to

the Faculty of the Undergraduate

College of Science and Mathematics

James Madison University

by Tiffany Nichole Bridges

December 2015

Accepted by the faculty of the Department of Biology, James Madison University, in partial fulfillment of the requirements for the Honors Program.

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PUBLIC PRESENTATION

This work is accepted for presentation, in part or in full, at JMU Honors Colloquium on Dec. 4, 2015

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Acknowledgements

I am grateful for the opportunity to help protect and save susceptible populations of amphibians from *Bd*. I would like to thank first and foremost Dr. Reid Harris and Dr. Eria Rebollar for this amazing opportunity. Their passion, determination, and drive to help protect amphibians are beyond contagious. This project would not have been possible without their expertise, technical support, and devotion.

I would also like to thank Kelsey Savage, Molly Bletz, Dr. Idelle Cooper, Oliver Hyman, and previous Harris lab members: Heather Gagné, Rebecca Dickey, Stephen Simonetti, and Emma Bales for their continuous help and motivation throughout this project. Nevertheless, I would also like to express my sincere gratitude to the National Science Foundation Grant 1136602 to Dr. Reid Harris for making this study possible.

Abstract

An emerging infectious disease caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*) is leading to global amphibian declines and is threatening the biodiversity of amphibians. *Bd* susceptibility varies among individuals, species, and populations perhaps due to defensive mechanisms such as symbiotic skin microbes. Some species of amphibians such as *Craugastor fitzingeri*, a terrestrial frog native to Central America, continue to persist in *Bd*-positive environments in Panama. My study focused on identifying antifungal bacterial isolates and determining the culturability of the bacterial community on 15 individuals of *C. fitzingeri*. Morphologically distinct isolates were challenged against *Bd* in inhibition assays to determine an inhibition score for each isolate. The 16S rRNA sequences of all cultured isolates were aligned and grouped in Operational Taxonomic Units (OTUs). The relative abundance of cultured OTUs was compared to that of the entire bacterial community obtained with the culture-independent method. Over 80% of the individuals had at least one morphologically distinct *Bd*-inhibitory isolate at an inhibition score of 80% or greater. In comparing culturability, the cultured community was significantly more relatively abundant than the entire culture-independent community (Wilcoxon test: W=101130, $p= 2.53e^{-14}$). Specifically, *Cellulomonas, Comamonas testosteroni*, and *Acinetobacter johnsonii* were highly relative abundant and were culturable. However, one relatively abundant species of *Pseudomonas* was not culturable. Additionally, I was able to culture 17.92% of the total relative abundance within the entire bacterial community. Identifying inhibitory isolates and the relative abundances of culturable OTUs are crucial steps to designing an ideal probiotic to potentially protect amphibian populations from *Bd*.

Introduction

An emerging infectious disease, chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), is leading to global amphibian declines and is threatening the biodiversity of amphibians (Berger et al., 1999). In the neotropics, *Bd* has spread from Mexico to Panama (Woodhams et al., 2008; Lips et al., 2008; Cheng et al., 2011), colonizing areas such as Gamboa, Mamoni, Nuevo Vigia, and Icunati (Rebollar et al., 2014). Although *Bd* persists throughout Panama, *Bd* susceptibility varies among individuals, populations and species, perhaps due to variations in the innate immune system, such as antimicrobial peptides (AMPs), adaptive immune system, and defensive symbiotic skin microbes. The goal of my study was to identify *Bd*-inhibitory bacterial isolates and determine the culturability of the bacterial community on *C. fitzingeri* as critical steps in discovering effective probiotics for conserving Panamanian amphibians.

Innate and adaptive immune responses to *Bd*

Amphibians have two types of immune responses: the innate and adaptive immune response. The innate immune response is a rapid, non-specific response that serves as a first line of defense until the adaptive immune response can be activated (Carey et al., 1999). Antimicrobial peptides (AMPs), which are part of the innate immune response, are secreted by many species of frogs and are a species-specific mechanism that potentially protects individuals against pathogens such as *Bd.* For example, AMPs from the frog *Limnodynastes tasmaniensis* were more inhibitory in laboratory assays than those from *Litoria chloris, Litoria caerulea*, and *Mixophyes fasciolatus*, which inhabited the same area in Australia (Woodhams et al., 2007a). *Litoria tasmaniensis* had a higher survival rate than the other species where *Bd* is present, which suggested that its AMP mixtures and concentrations were more inhibitory and protective in

nature (Woodhams et al., 2007a). As a result, *Bd* susceptibility was not equivalent among species. If a species does not secrete AMPs that effectively inhibit *Bd*, then the species is likely to decline unless other defenses exist or selection can increase the frequency of effective defenses (Bletz et al., 2013).

Alternatively, the adaptive immune response is an antigen-specific response that requires ample time to become activated (Carey et al., 1999). It is surprisingly ineffective against *Bd* because the fungus impairs lymphocyte generation and induces apoptosis of host cells (Fites et al., 2013). The evasion of host immunity by *Bd* explains why attempts at a vaccine have been less than successful, although repeated vaccinations on the same individual are known to increase *Bd* resistance and allow up to 50% survival (McMahon et al., 2014). Given that AMPs cannot be manipulated and are species specific and that vaccinations have met with limited success, symbiotic skin microbes may be the primary mechanism in conserving amphibians, especially since they can be manipulated (Bletz et al., 2013; Harris et al., 2009a; Lam et al., 2010).

Beneficial skin microbiota

The resident skin microbiota on amphibians play a crucial role in inhibiting *Bd* and enabling amphibians to coexist with *Bd*. Previous studies revealed that amphibian populations persisting with *Bd* had a greater proportion of *Bd*-inhibitory bacterial isolates than declining populations (Woodhams et al., 2007b). A recent study showed that the mucosome of the amphibian, consisting of both AMPs and bacterial metabolites, predicted the individual's survival when exposed to *Bd in vitro* (Woodhams et al., 2014). Further analysis illustrated that variation in AMP effectiveness did not significantly contribute to the amphibian's survival.

Therefore, the resident bacterial community is the most likely mechanism leading to protection **(**Woodhams et al., 2014).

As expected, there is a strong negative correlation between *Bd* survival and anti-*Bd* metabolites produced by resident skin bacteria (Becker et al., 2009). Inhibitory bacteria such as *Janthinobacterium lividium,* which produce the metabolite violacein (Harris et al., 2009a; Becker et al., 2009), and *Pseudomonas fluorescens,* which produce the metabolite 2,4-DAPG (Myers et al., 2012), have been isolated from several amphibian species and are known to inhibit *Bd*. *Rana muscosa* juveniles that were augmented with *J. lividium* had higher concentrations of violacein on their skins than controls and experienced dramatically reduced morbidity and mortality, which suggests bioaugmentation of inhibitory isolates through probiotic therapy can prevent chytridiomycosis (Harris et al., 2009a).

One aim of this study was to identify inhibitory isolates that can be further tested for their suitability as probiotics. The success of probiotic therapy depends on the probiotic's ability to persist on individuals and within a population, while not causing harm to the amphibian host or other species. Furthermore, the amphibian's immune defenses must not inhibit the probiotic nor should the probiotic eliminate important bacteria native to the amphibian host. A successful probiotic is also defined by the culturability of the microbial isolate, such that it can be effectively applied to individual amphibians and to the environment for transmission to individuals (Muletz et al., 2012; Bletz et al., 2013). Specifically in this study, I identified *Bd*-inhibitory bacteria as possible candidates for probiotics.

Focus of this study

This study encompassed three aims within the overall goal of discovering effective probiotics to protect *Bd*-susceptible amphibians. The *first aim* of my study was to identify *Bd*-inhibitory skin bacteria residing on *Craugastor fitzingeri*, a terrestrial frog native to Panama. Although *C. fitzingeri* persists in environments with *Bd*, some individuals could still be negatively affected; however, this needs to be experimentally determined. Therefore, identifying *Bd*-inhibitory bacteria and their relative abundance on the skin bacterial community can be used in future conservation efforts to proactively protect this species and related Panamanian amphibians to enhance their survival. The *second and third aims* were to determine if the frequently cultured OTUs were relatively abundant in the overall community of skin microbiota and whether the more relatively abundant OTUs were more likely to be inhibitory than less relatively abundant OTUs.

Significance of isolates being culturable

Previous studies regarding the culturability of microorganisms implied that culturable microbes are not necessarily the most dominant members of the microbial community but are species that can easily grow on selected media (Hugenholtz, 2002). However, a study investigating the bacterial community residing on *Rana catesbeiana* (bullfrog)*, Notophthalmus viridescens* (eastern newt)*, Pseudarcris crucifer* (spring peeper)*,* and *Bufo americanus* (American toad) near Blacksburg, VA, revealed that most of the relatively abundant OTUs were in fact culturable (Walke et al., 2015). Furthermore, individuals with a greater culture-dependent OTU richness also exhibited a higher culture-independent OTU richness (Walke et al., 2015). The culture-independent method isolates DNA from all bacterial species regardless of their

culturability, whereas the culture-dependent method isolates only bacteria that are able to grow on selective media. One goal of my study was to determine if this relationship between culture-independent and culture-dependent OTU richness is a more general pattern in amphibians such as *C. fitzingeri.*

I hypothesized that the probability of having *Bd*-inhibitory metabolites was likely to be higher among the most relatively dominant members of the resident bacterial community due to natural selection. In the presence of *Bd*, natural selection could act on the skin bacterial community to increase the frequency of inhibitory isolates on frog skins without inducing host mortality. This could lead to the most relatively abundant OTUs being antifungal. Alternatively, natural selection could increase the proportion of frog individuals with the most inhibitory bacterial communities as individuals with more protective communities survive and those with less protective communities have higher mortality. Taking into account natural selection, a focus of my study was to identify whether relatively dominant OTUs had a greater intensity of *Bd* inhibition compared to less abundant OTUs. Thus, Panamanian frogs that do persist with *Bd* are hypothesized to have relatively abundant *Bd*-inhibitory bacteria.

Summary of hypotheses:

1. Individuals of *C. fitzingeri* that persist in an environment with *Bd* will have *Bd*-inhibitory bacteria.

2. The most relatively abundant OTUs will be culturable.

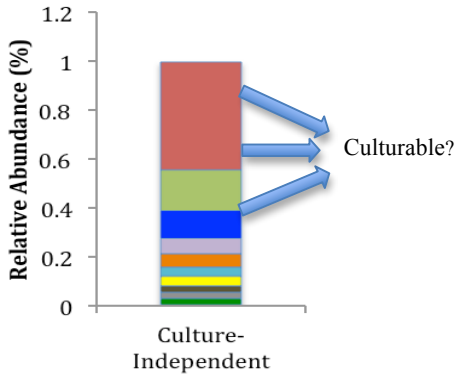

Figure 1: Relative abundance of culture-independent OTUs. Each color represents a different OTU. I hypothesized that the most relatively abundant OTUs are culturable.

3. The probability of having *Bd*-inhibitory metabolites will be higher among the most relatively abundant members of the resident bacterial community.

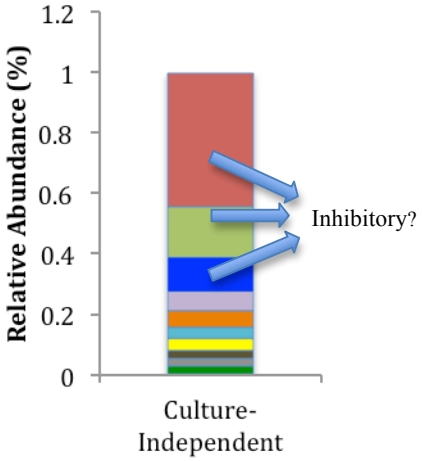

Figure 2: Possible *Bd*-inhibitory isolates. Each color represents a different OTU. I hypothesized that the likelihood of having antifungal properties is higher among the most relatively abundant OTUs.

Study species

Craugastor fitzingeri:

C. fitzingeri is a nocturnal amphibian that usually inhabits leaf litter or the margins of streams (Polaña & Crawford, 2003). Although *C. fitzingeri* is persisting with *Bd* (Rebollar et al., 2015), individuals might be susceptible to *Bd*-induced morbidity. Furthermore, the bacterial community of *C. fitzingeri* is known to consist of Actinobacteria and *Pseudomonas*, although these taxa are less common in *Bd*-naïve areas (Rebollar et al., 2015). Previous studies emphasized the importance of both groups of bacteria as possible antifungal isolates (Harris et al., 2009b; Rebollar et al. 2015). Therefore, characterizing the bacterial community of *C. fitzingeri* and identifying possible *Bd*-inhibitory isolates for probiotics could protect related species, such as *Craugastor punctariolus,* a neotropical stream-breeding frog (Chaves et al., 2014), that are *Bd* susceptible.

Bd:

Bd was first isolated in 1998 from a captive, blue poison dart frog at the National Zoological Park in Washington, D.C. (Longcore et al., 1999). Its lifecycle, prevalence, and virulence have been well documented since its discovery. The life cycle of *Bd* consists of the motile and flagellated zoospore (Van Rooij et al., 2012). After colonization, the zoospore encysts in the host's epidermis and forms a germ tube, where it grows into a cell of the stratum corneum, transfers its cellular material into the host's epidermal cell, and begins to develop into a zoosporangium (Van Rooij et al., 2012). Once the zoosporangium reaches the epidermal layer, it releases its zoospores, which can then infect the host or colonize other potential hosts.

Studies suggest that *Bd* moves in a wave to naïve areas, resulting in rapid and mass mortality of amphibian populations (Lips et al., 2006). Infected amphibians shed zoospores in the

environment, thereby enabling horizontal (frog-to-frog contact) and environmental (environment-to-frog) transmission of the fungal pathogen (Lips et al., 2006). The exact pathogenesis of chytridiomycosis is unknown, but studies show that the pathogen predominantly inhibits electrolyte absorption, thereby disrupting osmoregulatory capacities of the amphibian skin and thus resulting in death (Voyles et al., 2009).

Methods

Sampling and isolation protocol

I investigated the bacterial community found in the culture-dependent samples of 15 individuals of *C. fitzingeri* from Gamboa, Panama and compared the relative abundance of each OTU identified from cultured isolates to that of the culture-independent sample using 16S Illumina sequencing. In 2012, Dr. Myra Hughey from Virginia Tech (VT) swabbed each of the 15 individuals twice in the field: first for culture-independent characterization and second for culture-dependent isolation (Figure 3: step 1). Each individual was swabbed 20 times on its ventral surface and 5 times on its feet and webbing. The first swab was placed into a microcentrifuge tube and kept on ice until it could be stored at -20°C. Dr. Eria Rebollar processed the first swab using 16S Illumina sequencing to determine the bacterial community structure. Sequences that were 97% similar in 16S rRNA gene sequence were clustered together and taxonomically identified using the Quantitative Insights into Microbial Ecology (QIIME) pipeline (Caporaso et al., 2010).

The second swab was preserved in glycerol solution and kept frozen until examination. VT researchers at the Smithsonian Tropical Research Institute in Panama plated the culture-dependent swabs on 1% tryptone and incubated them at 25°C (Figure 3: step 2). Morphologically distinct colonies were further isolated on 1% tryptone agar plates (Figure 3: step 3) and incubated at 25°C. These isolates were inoculated with trypticase soy broth and 40% glycerol cyroprotectant and cryopreserved at -80°C.

I re-isolated each isolate and plated it onto its own mini plate, which provided an additional check for contamination (Figure 3: step 4). If the isolate did not match the original morphological description, I re-isolated it again to check for contamination. If the isolate was in

pure culture, I noted the discrepancy of its description. However, I did not use the isolate if there were two strains. I inoculated each isolate into 2 mL microcentrifuge tubes containing trypticase soy broth and glycerol cyroprotectant and incubated it at room temperature for a maximum of 24 hr (Figure 3: step 5). The microcentrifuge tubes were then cryopreserved at -80°C. After "waking up" each isolate, I taxonomically classified each cryopreserved isolate and challenged it against *Bd* in an inhibition assay.

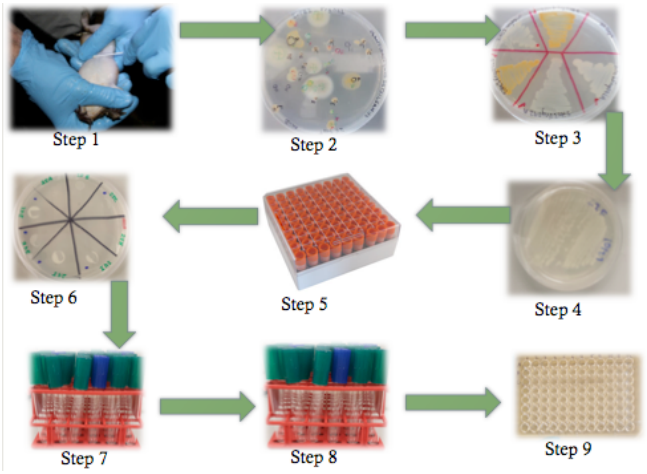

Figure 3: Overview of methods to identify *Bd*-inhibitory isolates. Culturing bacteria from the skin of *C. fitzingeri* began with swabbing the frog (step 1) and generating a mixed culture plate (step 2). Each isolate was reisolated (steps 3 and 4) to axenic culture before cryopreserving (step 5) in an -80°C freezer. The cryopreserved isolates were then plated (step 6) and cultured in tryptone (step 7). Afterwards each isolate was co-cultured with *Bd* (step 8) in preparation for challenge assays (step 9) against *Bd*.

Isolate identification

To prepare cryopreserved isolates for DNA extraction, I plated 15 µL of each isolate onto 1% tryptone agar and incubated it at 25°C for 48 hr. Each isolate was then re-isolated onto individual agar plates and re-incubated at 25°C for 48 hr to ensure pure cultures. I extracted the DNA of each isolate using PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA) or DNeasy Blood & Tissue Kit (QIAGEN, Inc., Valencia, CA, USA). I PCR amplified the 16S rRNA gene sequence with the primers Bac8F (5'-AGA GTT TGA TCC

TGG CTC AG-3') and Univ1492R (5'-GGT TAC CTT GTT ACG ACT T-3'). Each PCR isolate underwent the following amplification protocol: 1 cycle of 94°C for 4 min to denature the DNA and 30 cycles of 94°C for 1 min, 53°C for 1 min, and 72°C for 90 s, and 1 cycle of 72°C for 10 min. To confirm amplification, I ran PCR products on 2% gel electrophoresis and then sent them to Eurofins (Louisville, KY) for sequencing. I taxonomically classified each isolate according to maximum percent similarity with NCBI Genbank identities.

Inhibition assays

I thawed cryopreserved *Bd* at 40°C for 3 min and then incubated it with 20 mL of 1% tryptone broth at 21°C for 7 days. Afterwards, *Bd* was re-cultured by transferring 1 mL of *Bd* into 20 mL of 1% tryptone broth and incubated at 21°C. I re-cultured *Bd* twice in order to generate enough zoospores to co-culture the fungus with each bacterial isolate and challenge the bacterial isolates against the fungus. Four days following the *Bd*-reculture, I thawed the cryopreserved bacterial isolates at room temperature and plated 9 µL of each isolate on 1% tryptone agar and incubated it at 25°C for 2 days (day 0) (Figure 3: step 6).

I cultured each isolate with 2 mL of 1% tryptone broth and placed it on a shaking incubator at 21°C for 2 days to ensure uniform growth conditions (day 2) (Figure 3: step 7). In preparation for using *Bd* for the challenge assays (day 7), I plated and evenly distributed 1 mL of *Bd* onto 1% tryptone agar plates. The *Bd* was slightly dried at room temperature in a laminar flow hood without the lid for approximately 10 minutes and then incubated with the lid at 21°C for 4 days (day 3). I co-cultured 100 µL of each bacterial isolate with 1000 µL of 1% tryptone broth and 100 µL of *Bd* to encourage the formation of bacterial metabolites (Day 4). I also generated a positive control, consisting of 1100 µL of 1% tryptone and 100 µL of *Bd,* and a

negative control, containing 1200 µL of 1% tryptone. Each co-culture and control was placed on a shaking incubator at 21°C for 3 days (day 4) (Figure 3: step 8).

According to the inhibition of *Bd* assay procedure outlined by Bell et al. (2013) and modified by Matt Becker and Molly Bletz, the cell free supernatant (CFS) of each bacterial isolate was generated, as explained below, and then challenged against *Bd* in a 96-well plate inhibition assay (Day 7) (Figure 4: step 9). In order to standardize the amount of *Bd* zoospores used in each challenge, I flooded the *Bd* plates generated on day 3 with two-3 mL portions of 1% tryptone broth and incubated them at room temperature for 10 min. To assess zoospore density, zoospores of *Bd* were filtered and diluted to $2x10^6$ zoospores/mL using a hemocytometer procedure, which consisted of 2 replicate counts of zoospore concentration. Each of the two replicate counts was similar in concentration. I prepared a heat-killed *Bd* control by incubating 500 µL of quantified *Bd* at 60°C for 1 hr. Meanwhile, I prepared the bacterial isolates for challenge by centrifuging 1000 µL of each isolate from day 4 at 10,000 rpm for 5 min to pellet the cells. The supernatant, consisting of bacterial metabolites, was added to the 96-well plate as described below.

To prepare the 96-well plate for the addition of the bacterial CFS, I first added 50 µL of quantified *Bd* ($2x10^6$ zoospores/mL) to all wells, excluding the controls. I then filtered (filter size: 0.22µm) and pipetted 50 µL of bacterial CFS into its corresponding well (bacterial isolate treatment). I also pipetted nutrient depleted, positive, heat-killed *Bd*, and negative controls in the 96-well plate as described in Table 1. There were three replicates for all bacterial isolates and controls within each challenge assay. The nutrient depleted control served as an important comparison for the CFS from the isolates in order to determine whether the bacterial metabolites or the depletion of nutrients in the co-culture conditions inhibited *Bd* growth. Therefore, any

inhibition above that of the nutrient depleted control was evidence of inhibition due to bacterial metabolites (Figure 4). I incubated the 96-well plate at 21°C for 10 days and determined the optical density (OD) using a spectrophotometer on days 0, 4, 7, and 10 (Figure 4). The OD readings correlated with *Bd* growth such that each reading was used to determine the extent to which each isolate inhibited *Bd*, as described below.

Table 1: Treatments used in the inhibition assays

Treatment	Description of Treatment	Purpose
Bacterial isolates	*Bd*, CFS from bacterial isolates, and tryptone	Detect effects of bacterial metabolites on *Bd* growth.
Nutrient depleted control	*Bd* and sterile water	Control for inhibitory effects of nutrient depletion
Positive control	*Bd*, CFS from *Bd*, and tryptone	Control for growth of *Bd* with its own metabolites
Negative control	CFS from *Bd* and tryptone	Control for the effect of nutrients and metabolites from CFS
Heat-killed *Bd*	Heat-killed *Bd* and CFS from *Bd*	Control for the effect of no growth with *Bd*

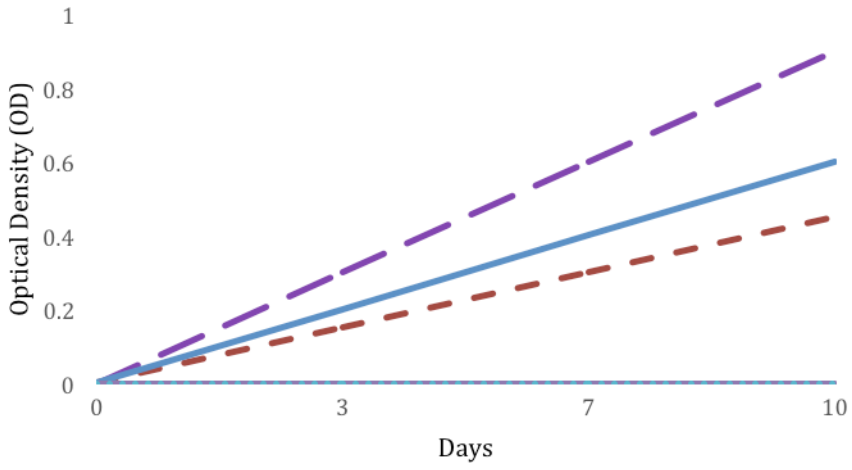

Figure 4: Predicted trends in OD values for the controls. The positive control (purple dash) is expected to have the greatest increase in OD values, following the nutrient depleted control (solid blue line), and inhibitory isolates (dashed orange line). The negative and heat-killed *Bd* controls are expected to have OD readings of zero.

Data analysis

In order to calculate an inhibition score of each isolate, I determined the slope of the OD readings over time using data collected on days 0, 4, 7, and 14 for each replicate (total of 3) and averaged the slopes from each replicate to generate a mean value. The average slope of each isolate was then divided by the average slope of the nutrient depleted control, within that same challenge plate, to determine the proportion of growth. I subtracted the proportion of growth from 1 to determine the inhibition score so that positive values indicated inhibition and negative values indicated facilitation. I considered inhibition scores of greater than or equal to 80%, 85%, 90%, and 95% in the analysis.

To determine whether relatively abundant OTUs were culturable, I first clustered cultured isolates into OTUs. I aligned the 16S rRNA sequence of each isolate and calculated the percent similarity between the sequences using Geneious (6.1.8, 2005-2013 Biomatters Ltd.). I generated OTUs from all the sequences based on 97% similarity. Afterwards, I re-aligned the 16S rRNA gene sequences of all isolates from each OTU group that had more than one sequence to generate a consensus sequence. I then taxonomically identified each consensus sequence using NCBI Genbank database. Each cultured OTU was compared to the culture-independent sample using QIIME. I eliminated OTUs that had less than 12 reads across individuals and rarefied the OTUs to the minimum number of reads per individual to normalize the number of reads, resulting in 36,952 reads per individual. I compared the relative abundance of cultured OTUs to the Illumina sample using a nonparametric Wilcoxon test in R (Core Team, 2014).

Results

Presence of *Bd*-inhibitory isolates

Fourteen out of fifteen individuals of *C. fitzingeri* had at least one morphologically distinct *Bd*-inhibitory bacterial isolate at 80% inhibition. Individuals 21 and 35 had the highest proportion of *Bd*-inhibitory isolates, whereas individuals 27 and 30 had the lowest proportion (Table 2). Regardless of percent inhibition (80-95%), individual 21 had 10 inhibitory isolates (Table S1, S2, S3). At an inhibition score of 80%, 36.25% of the cultured isolates were inhibitory towards *Bd*-growth. Specifically, over 50% of the isolates within the genera *Curtobacterium* (Actinobacteria), *Enterobacter* (Gammaproteobacteria), *Microbacterium* (Actinobacteria), and *Comamonas* (Betaproteobacteria) were inhibitory (Figure 5). Alternatively, over 50% of the isolates within the genus *Mycobacterium* (Actinobacteria) were facilitating. Each genus had at least 6 isolates in total in order to determine a valid proportion of isolates that were inhibitory.

Table 2: The proportion and number of inhibitory isolates on individuals of *C. fitzingeri* at 80% or greater inhibition

Inhibition at 80% or Greater	Individual	Number of Isolates Tested	Number of Inhibitory Isolates	Proportion of Inhibitory isolates
	21	13	10	0.769
	22	11	2	0.182
	23	17	9	0.529
	24	10	5	0.500
	25	16	3	0.188
	26	17	5	0.294
	27	6	0	0.000
	28	20	6	0.300
	29	6	1	0.167
	30	11	1	0.091
	31	13	6	0.462
	32	10	3	0.300
	33	8	3	0.375
	34	13	4	0.308
	35	4	3	0.750

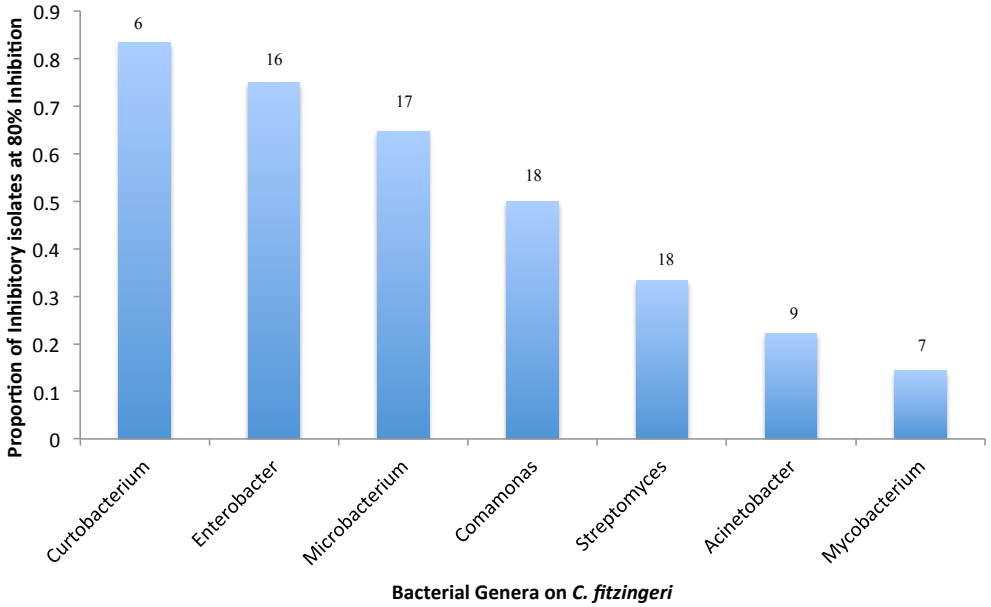

Figure 5: Inhibitory isolates within specific genera on *C. fitzingeri*. Each bar represents the proportion of inhibitory isolates relative to the total number of isolates for that genus. The total number of isolates for each genus is located above each bar.

Culturability of bacteria

On average, the cultured isolates were significantly more relatively abundant than the entire community obtained through Illumina methods (Wilcoxon test: W=101130, p= $2.53e^{-14}$). I cultured 17.92% of the total relative abundance identified by culture-independent methods (Table 3, Table S4). In fact, I determined that most of the relatively abundant OTUs were culturable. Specifically *Cellulomonas*, *Comamonas testosteroni*, and *Acinetobacter johnsonii* were all in high relative abundance within the Illumina community and were culturable (Figure 6). However, one species of *Pseudomonas* was highly abundant (43.75%) but was not culturable (Figure 6).

I also found that some bacterial classes were more culturable than others. OTUs that were found in more than 12 reads across all individuals in the Illumina community were included into the dataset. All cultured Betaproteobacteria met this criterion (Figure 7). Alternatively, only 72.22% Actinobacteria, 77.78% Firmicutes, 62.5% Alphaproteobacteria, and 90.91% Gammaproteobacteria met this criterion (Figure 7). The remaining isolates were found on 12 or fewer reads across individuals.

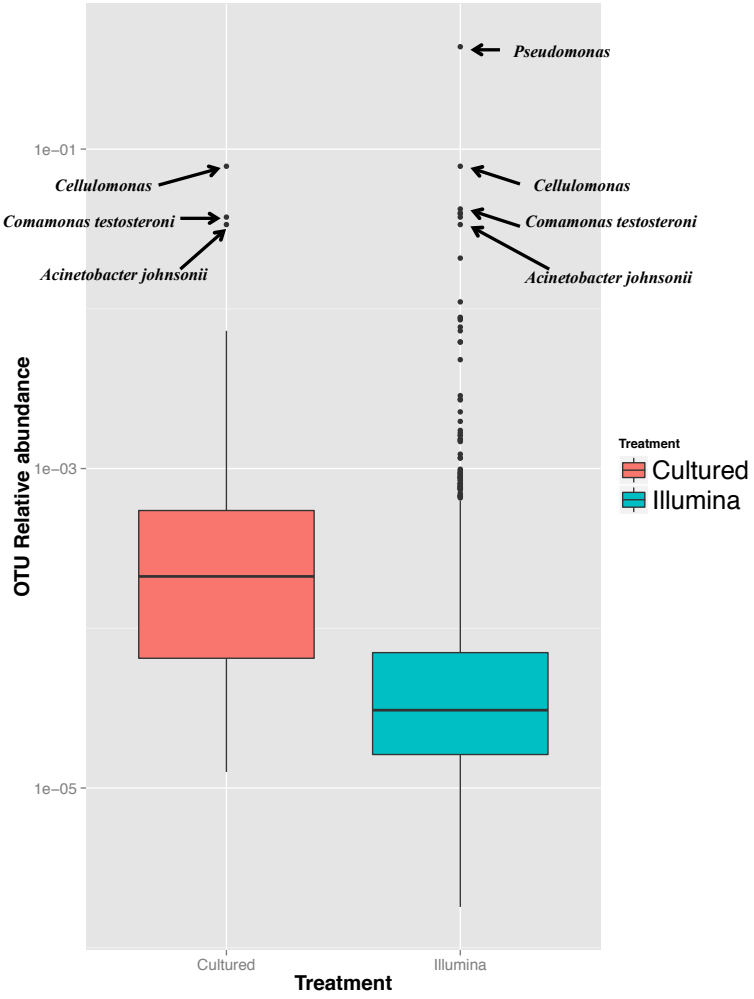

Figure 6: Relative abundance of cultured OTUs compared to the entire Illumina community. The cultured OTU community had a higher relative abundance than the Illumina community (W=101130, p= $2.53e^{-14}$). *Cellulomonas, Comamonas testosteroni,* and *Acinetobacteri johnsonii* were highly abundant and culturable.

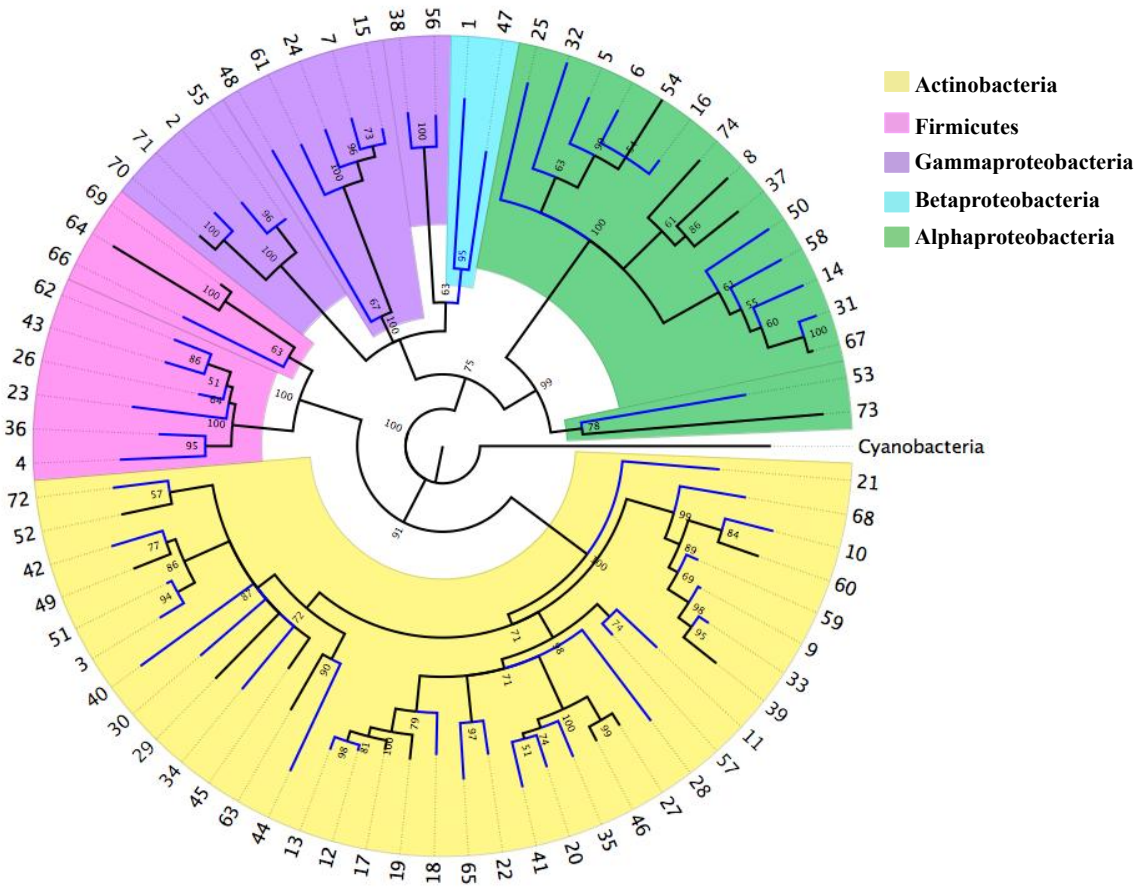

Figure 7: Phylogenetic analysis of the cultured community based on the 16S rRNA gene sequence. Phylogenetic analysis of each bacterial class Actinobacteria, Firmicutes, Gammaproteobacteria, Betaproteobacteria, and Alphaproteobacteria was compared to an out-group 16S rRNA sequence of a Cyanobacterium. Blue branches represent OTUs that were found in at least 12 reads across all 15 individuals, whereas black branches represent OTUs found in fewer than 12 read in the Illumina community. Isolate identification numbers are located outside of each branch and boot strap values are located near each branch node.

Correlation between relative abundance of OTUs and inhibition

I was unable to determine if there was a higher probability of having inhibitory metabolites among the most relatively abundant OTUs. Some of the isolates within the same consensus OTU exhibited different biological properties with respect to *Bd* growth such that some were facilitating, whereas others were inhibiting. Therefore, each consensus OTU was separated into unique strains, depending on its inhibition score such that strain A, strain B, and

strain C had inhibition scores ranging from greater than 0.75, between 0.2 and 0.75, and below 0.20, respectively (Table 3). The relative abundance of each strain within the Illumina community was not obtained. Therefore, I could not determine whether a highly inhibitory strain was in high relative abundance. However, I did find that all of the isolates of Betaproteobacteria were inhibitory towards *Bd* to some extent (Figure 8). In contrast, isolates assigned to OTUs of Alphaproteobacteria (Figure 8), Actinobacteria, Firmicutes, and Gammaproteobacteria (Figure 1S) consisted of various inhibitory and facilitating isolates.

Table 3: Proportion of *Bd*-inhibition for strains within consensus OTUs

OTU ID	Identification	Relative Abundance	Strain	Average Proportion of Inhibition
1	*Comamonas testosteroni*	0.037509923	1A	0.867317319
			1B	0.642068269
2	*Enterobacter aerogenes*	0.002715234	2A	0.947873265
			2B	0.583088086
3	*Mycobacterium brisbanense*	0.000122682	3A	1.082458771
			3B	0.451475354
			3C	-0.134520153
4	*Staphylococcus saprophyticus*	0.006202641	4A	0.935532234
			4C	-0.151529988
5	*Rhizobium sp.*	0.000598975		0.739130435
6	*Rhizobium causense*	9.92E-05	6B	0.557180434
			6C	0.037849393
7	*Acinetobacter radioresistens*	0.007279714		0.586766735
9	*Streptomyces drozdowiczii*	0.000371653	9A	0.932156959
			9B	0.567574869
			9C	0.021992238
10	*Streptomyces*	3.79E-05		0.611591488
11	*Isoptericola variabilis*	0.00023093	11B	0.421095008
			11C	-0.124798712
12	*Microbacterium oleivorans*	0.000487118	12A	0.869526302
			12C	-1.194788394
13	*Microbacterium pumilum*	0.000333766	13A	0.794909902
			13B	0.710144928
			13C	-1.859000557
14	*Methylobacterium radiotolerans*	1.26E-05	14B	0.320621746
			14C	-0.584507042
15	*Acinetobacter johnsonii*	0.033699574	15A	0.935849185
			15B	0.403063787
			15C	-1.983869501
16	*Ochrobactrum pseudogrignonense*	0.000672945	16A	0.850221855
			16B	0.626096759
18	*Curtobacterium oceanosedimentum*	0.000523201	18	0.943155405
20	*Diaminobutyricimonas aerilata*	0.000553872	20	0.980897152

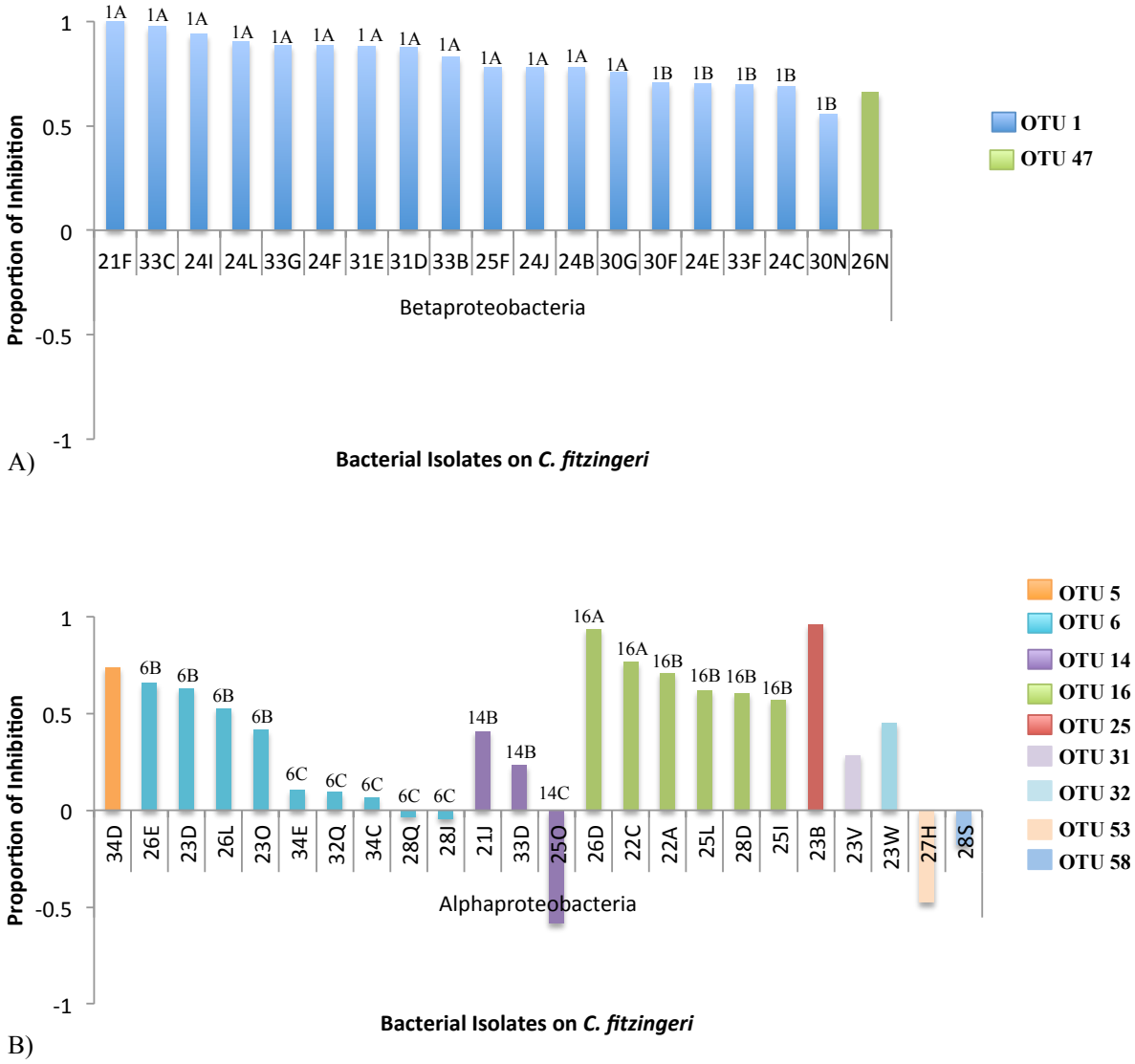

Figure 8: Inhibition scores of bacterial isolates in (A) Betaproteobacteria and (B) Alphaproteobacteria OTUs. Strain identification is located above each isolate. Bacterial isolates are identified by their numbers along the x-axis. Strains A, B, and C had inhibition scores ranging from greater than 0.75, 0.2 - 0.75, and less than 0.20, respectively. OTUs that consisted of one isolate were not differentiated based on strain identification.

Discussion

Presence of *Bd*-inhibitory microbes

Approximately 93.3% of the individuals that persisted in an environment with *Bd* had at least one morphologically distinct *Bd*-inhibitory bacterial isolate (Table 1). Since the proportion of protected individuals exceeds 80%, it is likely that susceptible individuals may be protected from *Bd*-induced mortality through a process analogous to herd immunity (Lam et al., 2010). Herd immunity can indirectly protect susceptible individuals if a large percentage of the population is protected from an infectious disease. For example, previous studies of *R. muscosa* suggested that herd immunity played a crucial role in preventing *Bd* epidemics by reducing *Bd* survival and viability (Lam et al., 2010). Therefore, it is possible that the bacterial community protects and thereby enables *C. fitzingeri* to persist in *Bd*-endemic areas without experiencing drastic population declines.

Greater than 50% of the isolates within the genera *Curtobacterium* (Actinobacteria), *Enterobacter* (Gammaproteobacteria), *Microbacterium* (Actinobacteria), and *Comamonas* (Betaproteobacteria) were inhibitory towards *Bd* growth (Figure 5). Previous studies also confirm that these genera can be inhibitory towards *Bd*-growth (Woodhams et al., 2015). It is possible that individuals of *C. fitzingeri* were enriched with these protective bacteria in relationship to their relative abundance in the environment (Rebollar et al., 2015; Walke et al. 2014)

Individuals of *C. fitzingeri* that had *Bd*-inhibitory isolates and were thus likely protected had dissimilar community structures to those that are predicted to be unprotected from *Bd* (Rebollar et al., 2015). Therefore, *C. fitzingeri* may be undergoing either hologenomic selection or microbial community selection. In hologenomic selection, unprotected holobionts—a single

unit consisting of an individual and its microbiota—are selected against, resulting in host mortality (Zilber-Rosenberg & Rosenberg, 2008). Alternatively, if selection acts on the microbial community, there would be an increase in inhibitory bacterial species without inducing host mortality. For example, individual 27 lacked *Bd*-inhibitory isolates. Therefore it may be under selection to have a skin microbial community similar to that of the protected individuals or the holobiont may be selected against. A previous study showed that *C. fitzingeri* residing in Serrania del Sapo, Panama—a *Bd* naïve area—had a greater microbial diversity compared to *C. fitzingeri* in *Bd*-occupied sites such as Mamoni, Gamboa, and Soberania, Panama (Rebollar et al., 2015). Rebollar et al. (2015) suggested that the microbial community on Sapo individuals would be selected, without host mortality, to have a protective community similar to individuals within the other sites upon the arrival of *Bd*. Alternatively, individuals without a protective community structure will face a high mortality rate.

Culturability of bacteria

Culturability is a crucial aspect in determining relative abundance estimates of inhibitory members of the bacterial community. Previous studies suggest that culturable OTUs are not necessarily abundant, but are easily grown on selective media (Hugenholtz, 2002). Moreover, most state that 99% of microbes in nature are unculturable (Hugenholtz et al., 1998). For example, studies estimate that only 0.25% of freshwater and sediment, and 0.30% of soil microbes are culturable (Amann et al., 1995). Alternatively, another study involving amphibians showed that the most relatively abundant OTUs were culturable (Walke et al., 2015). I found that the most relatively abundant OTUs, specifically *Cellulomonas*, *Comamonas testosteroni*, and *Acinetobacter johnsonii,* were in fact culturable (Figure 6). I was able to culture 17.92% of the total relative abundance identified by culture independent methods, specifically within the phyla

Actinobacteria, Firmicutes, and Proteobacteria. This is notably higher than previous estimates of culturable OTUs. However, one species of *Pseudomonas* was highly relatively abundant but was not culturable (Figure 6). *Pseudomonas* OTUs have possibly coevolved with the skin microbiota of *C. fitzingeri* and is dependent on the particular chemical characteristics and nutrients of the mucosome for survival. Therefore, this symbiont would not be viable on standard culture media or outside of the amphibian host in general.

Correlation between relative abundance and inhibition

I hypothesized that the most relatively abundant OTUs had a higher probability of having *Bd*-inhibitory isolates. However, I was unable to address this hypothesis due to extensive variability within several consensus OTUs. Most of the consensus OTUs consisted of several bacterial isolates, each with unique biological properties. These properties rendered some isolates within the same OTU as inhibitory, whereas others were facilitating. Therefore, I was unable to assign an overall inhibition score for several OTUs.

Goals for probiotic discovery

Identifying inhibitory isolates and their relative abundances are fundamental steps to designing an ideal probiotic (Bletz et al., 2013). Probiotics that are inhibitory towards *Bd* can help save individuals, species, and populations of amphibians from *Bd*-induced mortality. In order to further understand the role of the microbial community in probiotics, future work should focus on comparing amphibian skin microbiota before and after the arrival of *Bd* at the same site. This study could further illustrate whether microbial community or hologenomic selection could protect susceptible individuals. Additionally, future experimentation should focus on co-

culturing combinations of bacterial isolates with *Bd* to determine whether combinations of bacteria, rather than single isolates, are more inhibitory towards *Bd.*

Appendix: Supplementary Tables and Figures

Table S1: Proportion and number of inhibitory isolates on individuals of *C. fitzingeri* at 95% or greater inhibition

	Individual	Number of Isolates Tested	Number of Inhibitory Isolates	Proportion of Inhibitory isolates
Inhibition at 95% or Greater	21	13	10	0.769
	22	11	1	0.091
	23	17	4	0.235
	24	10	1	0.100
	25	16	2	0.125
	26	17	0	0.000
	27	6	0	0.000
	28	20	5	0.250
	29	6	0	0.000
	30	11	0	0.000
	31	13	2	0.154
	32	10	3	0.300
	33	8	1	0.125
	34	13	2	0.154
	35	4	2	0.500

Table S2: Proportion and number of inhibitory isolates on individuals of *C. fitzingeri* at 90% or greater inhibition

	Individual	Number of Isolates Tested	Number of Inhibitory Isolates	Proportion of Inhibitory isolates
Inhibition at 90% or Greater	21	13	10	0.769
	22	11	2	0.182
	23	17	7	0.412
	24	10	3	0.300
	25	16	2	0.125
	26	17	3	0.176
	27	6	0	0.000
	28	20	5	0.250
	29	6	0	0.000
	30	11	0	0.000
	31	13	3	0.231
	32	10	3	0.300
	33	8	1	0.125
	34	13	2	0.154
	35	4	3	0.750

Table S3: Proportion and number of inhibitory isolates on individuals of *C. fitzingeri* at 85% or greater inhibition

	Individual	Number of Isolates Tested	Number of Inhibitory Isolates	Proportion of Inhibitory isolates
Inhibition at 85% or Greater	21	13	10	0.769
	22	11	2	0.182
	23	17	8	0.471
	24	10	4	0.400
	25	16	3	0.188
	26	17	4	0.235
	27	6	0	0.000
	28	20	6	0.300
	29	6	0	0.000
	30	11	0	0.000
	31	13	6	0.462
	32	10	3	0.300
	33	8	2	0.250
	34	13	4	0.308
	35	4	3	0.750

Table S4: Proportion of *Bd*-inhibition for non-consensus OTUs. The proportion of inhibition could not be determined for OTU 50 due to logistics.

OTU ID	Identification	Relative Abundance	Average Proportion of Inhibition
21	*Frankia sp.*	0.000276034	0.722155893
22	*Agromyces sp.*	0.000050500	0.753709705
23	*Bacillus sp.*	0.000869597	0.638751492
24	*Acinetobacter sp.*	0.000301292	0.606515436
25	*Paracoccus marinus*	0.000126290	0.961623742
26	*Bacillus pumilus*	0.000059500	0.897322190
28	*Leucobacter tardus*	0.000510572	0.769211452
30	*Gordonia terrae*	0.000351808	-0.344548774
31	*Bosea sp.*	0.000117269	0.285863328
32	*Caulobacter segnis*	0.000030700	0.453834116
33	*Streptomyces gramineus*	0.000930937	1.254303599
34	*Gordonia sp.*	0.000907484	0.675534690
35	*Microbacteriaceae*	0.000120878	0.775952008
36	*Bacillus megaterium*	0.000117269	0.282733438
38	*Stenotrophomonas maltophilia*	0.000990474	0.975743349
40	*Williamsia serinedens*	0.000036100	-0.174230569
41	*Leifsonia sp.*	0.000992000	0.683359416
42	*Mycobacterium iranicum*	0.000193043	-0.323682838
43	*Bacillus sp.*	0.000290467	0.338101430
44	*Nocardioides sp.*	0.000187631	0.809957273
47	*Achromobacter xylosoxidans*	0.001513675	0.659297789
48	*Steroidobacter agariperforans*	0.000014400	0.157718744
50	*Methylobacterium*	0.000027100	
51	*Mycobacterium sp.*	0.000030700	-0.040869404
53	*Rhodomicrobium sp.*	0.000021600	-0.475571243
55	*Enterobacter sp.*	0.000281446	0.853055917
56	*Stenotrophomonas sp.*	0.000037900	0.966364812
57	*Cellulomonas sp.*	0.078083279	-0.058214748
58	*Microvirga sp.*	0.000064900	-0.180558122
59	*Streptomyces sp.*	0.000178610	0.452411754
61	*Acinetobacter sp.*	0.000241755	-0.049343929
62	*Bacillus cibi*	0.000064900	0.703382000
65	*Agromyces*	0.000018000	0.900966184
66	*Chonella panacarvi*	0.000014400	0.187600644
68	*Streptacidiphilus jiangxiensis*	0.000137115	0.699677939
71	*Pantoea dispersa*	0.000339179	0.767686659
72	*Gordonia sp.*	0.000120878	0.127294982

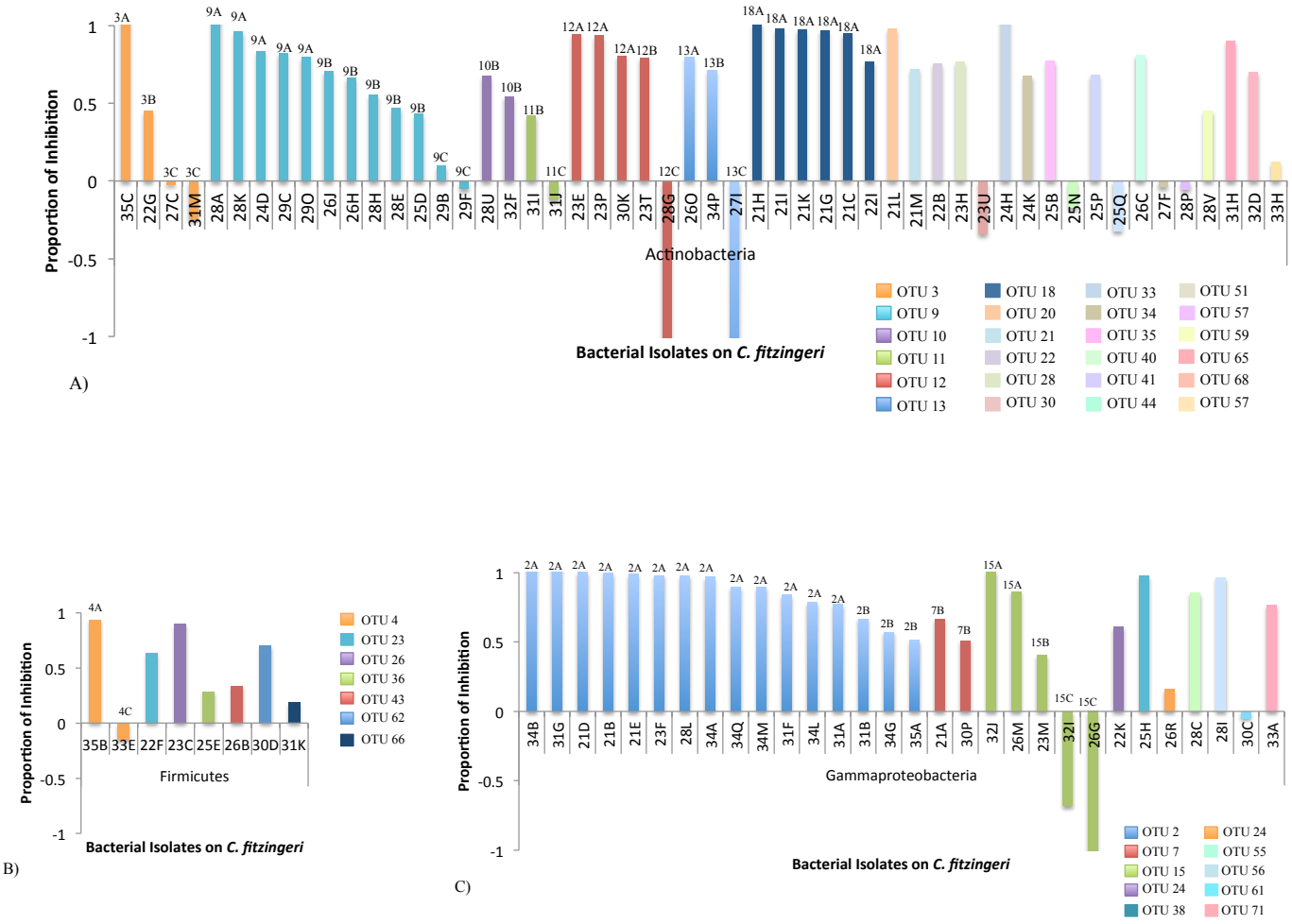

Figure 1S: Inhibition scores of bacterial isolates in (A) Actinobacteria, (B) Firmicutes, and (C) Gammaproteobacteria OTUs. Strain identification is located above each isolate. Bacterial isolates are identified by their numbers along the x-axis. Strains A, B, and C had inhibition scores ranging from greater than 0.75, 0.2-0.75, and less than 0.20, respectively. OTUs that consisted of one isolate were not differentiated based on strain identification.

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