THE ROLE OF SYMBIOTIC BACTERIA IN DISEASE RESISTANCE AND CONSERVATION OF THE CRITICALLY ENDANGERED PANAMANIAN GOLDEN FROG

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ABSTRACT

Amphibian populations have undergone unprecedented declines in recent decades. Many of these declines are due to the spread of the cutaneous fungal pathogen *Batrachochytrium dendrobatidis* (Bd), which causes the disease chytridiomycosis. The Panamanian golden frog (*Atelopus zeteki*) has not been seen in the wild since Bd spread through western Panama. In response to initial declines, golden frogs were collected from wild populations and placed in captive colonies with the goal of future reintroductions. An understanding of this species' natural defense mechanisms against Bd is needed for reintroduction to be successful. Previous studies indicate that cutaneous bacteria are an important defense mechanism for some amphibians and applying antifungal bacteria to the skin of Bd-susceptible amphibians (probiotic therapy) can prevent chytridiomycosis. Therefore, the goals of my dissertation were to characterize the bacterial community of *A. zeteki* and determine if probiotic therapy could be used to prevent chytridiomycosis in this species. I initially characterized the bacterial community of wild and captive golden frogs using samples collected prior to the initial declines and after approximately eight years in captivity. I found that the community structure of the microbiota was significantly different between wild and captive frogs; however, the offspring of the original captive frogs still shared 70% of their microbial community with wild frogs. Then, I characterized the Bd-inhibitory properties of 484 bacteria isolated from 11 species of free-living Panamanian amphibians. I found a large proportion of bacteria (75.2%) had the ability to inhibit Bd and this

trait was widely distributed among bacterial taxa, although there was also significant variation within bacterial genera in their ability to inhibit Bd growth. I then experimentally tested the ability of four of these isolates to prevent chytridiomycosis in captive golden frogs. None of them successfully prevented infection; however, there were several frogs that cleared infection and this was correlated with composition of the bacteria initially present on their skin. Overall these results demonstrate that the structure of microbial communities of *A. zeteki* are important to host health and building on this might provide the best hope for reintroducing this iconic species back to its native habitat.

DEDICATION

To my family:

Jen, Lily, and Noah

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CHAPTER I. INTRODUCTION

Matthew H. Becker

LITERATURE REVIEW

Amphibian declines

In the past three decades, amphibians have experienced unprecedented population declines, leading to many species extinctions (Houlahan et al. 2000, Stuart et al. 2004). As of 2004, the number of amphibian species threatened with extinction reached 1,811 (out of 5,748 described amphibian species, International Union for Conservation of Nature 2013) and at least 2,468 species have experienced population declines (Stuart et al. 2004). Amphibian population declines in developed areas have been hypothesized to be a result of habitat destruction, introduced species, and commercial use. However, these hypotheses are not suitable explanations for the large number of amphibian declines and extinctions that have occurred and are still occurring in protected areas, such as national parks and forests (Bosch et al. 2001, Carey et al. 2003). However, the emergence of infectious diseases serves as a plausible explanation for at least some of these events. Emerging infectious diseases (EIDs) are diseases that have recently emerged or have had a recent increase in incidence or geographical distribution. EIDs can have detrimental impacts on species, such as the cases of chestnut blight (Loo 2009) and canine distemper in seals (Kennedy 1998).

Chytridiomycosis is caused by the fungus *Batrachochytrium dendrobatidis* (Bd) which colonizes the skin of amphibians (Berger et al. 1998). This disease was first detected in 1998 in amphibians of Australia and Central America (Berger et al. 1998) and later characterized in 1999 (Pessier et al. 1999). Chytridiomycosis is now associated with population declines and extinctions of over 200 amphibian species, suggesting the pathogen has low host specificity (Skerratt et al. 2007). Bd has been detected in amphibian populations across the globe including North America (Longcore et al. 2007), Central America (Berger et al. 1998, La Marca et al. 2005), South America (Hanselmann et al. 2004, La Marca et al. 2005), Australia (Berger et al.

1998, Kriger and Hero 2008), Africa (Weldon et al. 2004), Europe (Woodhams et al. 2014), and Asia (Goka et al. 2009). Currently, little is known about what factors are responsible for the emergence of Bd. As stated above, many severe declines caused by Bd occur in areas unaffected by habitat loss; therefore, there is a weak link between habitat destruction and disease emergence. Recent work suggests that Bd has emerged from a single genotype (James et al. 2009) and declines are the result of the anthropogenic spread of a hypervirulent strain (Farrer et al. 2011).

Biology of Bd

Since its discovery, many studies have investigated the biology of Bd. The fungus has two major stages during its life cycle: a zoospore stage characterized as the infective phase and a zoosporangium stage characterized as the growth phase (Berger et al. 2005a). The zoospore stage is the only motile part of the life cycle. After a period of motility, the zoospore becomes encysted (Piotrowski et al. 2004, Berger et al. 2005a). As the zoospore encysts in the skin of the amphibian host, it becomes immobile and a cell wall forms (Berger et al. 2005a). Once mature, the zoosporangium produces flagellated zoospores (Berger et al. 2005a). During this process, discharge papillae form where zoospores will eventually leave the zoosporangium (Longcore et al. 1999). Under ambient conditions, plugs which block discharge papillae are dissolved and motile zoospores are released (Berger et al. 2005a). Released zoospores are then able to reinfect the same individual amphibian, or infect another individual, depending on conditions (Rachowicz and Vredenburg 2004).

Bd can grow under a range of conditions. The optimal temperature for chytrid growth is between 17 and 25 C, but it can survive between 4 and 25 C (Piotrowski et al. 2004). This wide tolerance allows the pathogen to live in many habitats. At temperatures above 28 C and below 10 C the fungus grows very slowly or may stop growing completely (Piotrowski et al. 2004). Pathogenicity of Bd may also peak under optimal temperature conditions (Retallick et al. 2004). Additionally, mortality of infected individuals is greatest during cooler months in eastern Australia (Berger et al. 2004). Infected amphibians brought above 37 C for a period of time under captive conditions may be able to rid themselves of infection (Woodhams et al. 2003). In the wild, amphibians may also behaviorally elevate their temperatures to reduce infection

(Richards-Zawacki 2010). Bd can also tolerate a wide range of acidity; growth of the fungus can occur between a pH of 5 and 10, although optimal pH is 6-7 (Piotrowski et al. 2004). There is a lack of evidence for optimal moisture conditions; however, there is evidence that complete desiccation kills Bd (Johnson and Speare 2003).

Course of infection

Amphibian infection begins with the invasion of the keratinized epidermal cells (Longcore et al. 1999). Within these cells, Bd goes through its life cycle as it does in culture (Berger et al. 2005a). Infections most often occur on the ventral skin, but may occur anywhere on the skin (Pessier et al. 1999). Infections are usually clustered in one general region, but in severe infections zoosporangium are found over the entire surface of the amphibian (Berger et al. 2005a). As a consequence of infection, some amphibian species may undergo physical and behavioral changes. Hyperkeratosis, which is characterized as an increase in the thickness of the skin, is most often seen in infected amphibians. Diseased individuals may experience an increased skin thickness of two to five times that of healthy individuals, which is a direct result of an increase in the layers of the stratum corneum (Berger et al. 1998, 2005a). Sloughing of skin is also associated with this disease and has been seen in many species (Berger et al. 2005a). If skin sloughing occurs faster than an amphibian can regenerate new skin, it may lead to exposure of non-keratinized skin (Pessier et al. 1999, Berger et al. 2005a). Behavioral changes, such as anorexia, lethargy, and unresponsiveness to stimuli, have been documented in diseased animals (Nichols et al. 2001, Berger et al. 2005b).

Mortality occurs in many species and frequently takes place between 18 and 48 days after infection (Berger et al. 1998, 2004, Lips 1999, Lips et al. 2006, Rachowicz et al. 2006, Woodhams et al. 2007a). Death most likely occurs as a consequence of thickening of the skin resulting in the disruption of vital functions as the exchange of ions (Carey et al. 2003, Voyles et al. 2007, 2009). Recent evidence suggests that death may occur due to an osmotic imbalance and electrolyte depletion resulting in heart failure (Voyles et al. 2007, 2009).

Transmission and reservoirs of Bd

Bd may spread from amphibian to amphibian during close contact; this may include activities such as mating and during times when larvae school (Piotrowski et al. 2004, Rachowicz and Vredenburg 2004). Rachowicz and Vredenburg (2004) experimentally demonstrated that infected tadpoles transmit the pathogen to non-infected tadpoles and to post-metamorphic amphibians. In addition, since most tadpoles do not develop chytridiomycosis, they act as a reservoir for the pathogen. Studies also indicate that some adult amphibians, such as the American bullfrog *Rana catesbeiana*, can become infected with Bd, but do not develop disease. Therefore, such species may act as reservoirs for Bd (Davidson et al. 2003, Hanselmann et al. 2004, Retallick et al. 2004). Additionally, Bd has been detected on non-amphibian species, such as crawfish and lizards, which may act as reservoirs for the pathogen (Kilburn et al. 2011, McMahon et al. 2013).

Water is essential for Bd dispersal, which occurs during the zoospore phase of the life cycle (Berger et al. 2005b). Zoospores are motile for approximately 24 hours and may be carried by water currents (Piotrowski et al. 2004). Bd can survive and release zoospores in lake water for up to seven weeks, suggesting that water can be a short-term environmental reservoir for Bd (Johnson and Speare 2003). Moist soil may also serve as an environmental reservoir for Bd (Johnson and Speare 2005). Therefore, transmission could occur through direct contact with contaminated soil or water. Additionally, these environmental reservoirs may allow Bd to survive and persist for a short period of time even if there are no available hosts.

Amphibian defenses

The effect of Bd on an amphibian is dependent on host susceptibility and differs among species and populations (Lips et al. 2003, Retallick et al. 2004). Evidence that the adaptive immune system is effective in preventing chytridiomycosis in resistant amphibian species is limited (Richmond et al. 2009). Recent evidence demonstrates that Bd secretes toxins that inhibit the adaptive immune response (Fites et al. 2013). Other defenses exist for some amphibians. For example, susceptibility to Bd has been correlated with a species' assemblage of antimicrobial skin peptides (AMPs, Woodhams et al. 2006a, 2006b), as well as the microbial community associated with the skin of amphibians (Harris et al. 2006, 2009a, Becker et al. 2009).

Evidence of AMP activity against Bd is well supported. AMPs are stored in granular glands in the dermis of amphibian skin and are released in response to stimulation from the sympathetic nervous system, which may be triggered when an amphibian is under stress (Mills and Prum 1984). One such trigger includes the presence of microorganisms (Mangoni et al. 2001). Several AMPs extracted from multiple amphibian species show inhibitory activity *in vitro* against Bd (Rollins-Smith 2009). In addition, the assemblage of AMPs in some species is correlated with susceptibility to chytridiomycosis (Woodhams et al. 2006a, 2006b).

Symbiotic bacteria on the skin of some amphibians also play a crucial role in preventing colonization of Bd and possibly other fungal pathogens (Harris et al. 2006, 2009b, Lauer et al. 2008, Banning et al. 2008, Becker et al. 2009). Research on the microbiota of amphibian skin is in its initial stages, but amphibians' moist, nutrient-rich skin provides a perfect habitat for microorganisms (Guirard and Snell 1962). The microbial community of amphibians appears to be species-specific and, in part, acquired from microbial reservoirs in the local environment (McKenzie et al. 2012, Kueneman et al. 2013, Loudon et al. 2013, Walke et al. 2014).

The hypothesis that cutaneous bacteria protect amphibians against Bd is supported by three lines of evidence. (1) *In vitro* studies and surveys have shown that bacteria isolated from amphibian skin produce strong anti-Bd metabolites, and these metabolites are present on the skin in high enough concentrations to kill Bd zoospores (Brucker et al. 2008a, 2008b, Becker et al. 2009). One of these antifungal compounds, violacein, inhibits Bd growth at relatively low concentrations and is strongly associated with survival of the redback salamander, *Plethodon cinereus*, and *R. muscosa* infected with Bd (Brucker et al. 2008b, Becker et al. 2009, Harris et al. 2009a). (2) Field surveys of the threatened mountain yellow-legged frog, *Rana muscosa*, have shown that populations coexisting with the pathogen have significantly higher proportions of individuals with anti-Bd skin bacteria than declining populations (Lam et al. 2010). (3) Skin bacteria from a non-susceptible amphibian can be isolated and applied as a probiotic to a susceptible species to provide resistance to chytridiomycosis (Becker et al. 2009, Harris et al. 2009a, 2009b).

The beneficial effect provided by this bacterial community could be attributed to a mutualism between the host and its microbes. Similar mutualisms are seen in other host-

symbiont models. For example, the beetle *Dendroctonus frontalis*, the wasp *Philanthus triangulum*, the leaf-cutter ant (Attini: Formicidae), and the lobster *Homarus americanus* all have mutualistic relationships with microorganisms (Gil-Turnes and Fenical 1992, Currie et al. 1999, Kaltenpoth et al. 2005, Scott et al. 2008). In these systems, the host is protected from a pathogenic fungus by antibiotic metabolites secreted by symbiotic microbes. For example, chemical analysis of a mutualistic actinomycetous bacterium isolated from its host, *D. frontalis*, revealed that fungal infestation of the host is prevented by secretion of a polyene peroxide (Scott et al. 2008). In addition, there is overwhelming evidence that human health is dependent on symbiotic microbes that colonize the lining of the gut, skin, and vagina (Bäckhed et al. 2005, Dethlefsen et al. 2007, Relman 2012).

The case for Atelopus

Frogs in the genus *Atelopus* (harlequin frogs) are arguably one of the most threatened taxa (La Marca et al. 2005). Approximately 80% of the 83 formally described frogs in the genus *Atelopus* are either extinct or critically endangered (La Marca et al. 2005, Pounds et al. 2006, Lötters 2007). Additionally, 22 *Atelopus* species have disappeared from protected habitats since 2000 (La Marca et al. 2005). The high number of species lost from protected land suggests habitat loss is a minor factor contributing to these declines (La Marca et al. 2005, Lötters 2007). Instead, most harlequin frog declines appear to be linked to the spread of chytridiomycosis throughout Central and South America (La Marca et al. 2005, Lötters 2007). Evidence gathered from Bd detections in wild frogs and museum specimens suggests that Bd has already spread throughout most of the range of *Atelopus* species, which extends from Costa Rica south into Bolivia and eastward into the Guianas (Lips et al. 2008).

In Panama, the iconic Panamanian golden frog, *Atelopus zeteki*, has not been seen in the wild since 2009, despite intensive search efforts (E. Griffith and C. Richards-Zawacki personal communication). In the species' historical geographical range, *A. zeteki* was present in rainforests in central-western Panama. *A. zeteki* is highly susceptible to chytridiomycosis (Lips et al. 2006, Becker et al. 2012, Langhammer et al. 2013) and as the threat of extinction increased due to the spread of Bd in western Panama, an extensive captive breeding program for *A. zeteki* was initiated by conservationists in the USA and Panama (Gagliardo et al. 2008). Currently, over

2000 *A. zeteki* are being maintained in North American and Panamanian zoos and aquaria (K. Murphy personal communication, Poole 2008). The end goal of this captive breeding program is to prevent the extinction of *A. zeteki* by reintroducing frogs into the wild when circumstances are favorable for the survival of this species. Unfortunately, *A. zeteki* cannot currently be reintroduced because Bd still persists in their native habitat on amphibian species not susceptible to chytridiomycosis. Currently, the only promising method to accomplish a successful reintroduction program for *A. zeteki* is through the use of probiotic therapy with anti-Bd bacteria (Woodhams et al. 2011, Bletz et al. 2013).

In 2009, I conducted an experiment to test the effectiveness of using the anti-Bd bacterial species *Janthinobacterium lividum* in preventing mortality in infected *A. zeteki* individuals (Becker et al. 2012). *Janthinobacterium lividum* was chosen because it was successful in preventing mortality in infected *R. muscosa* and *P. cinereus* individuals (Becker et al. 2009, Harris et al. 2009a). Although *J. lividum* appeared to colonize *A. zeteki* skin temporarily, it did not prevent or delay mortality. After treating golden frogs with *J. lividum*, bacterial cell number on the skin reached a maximum intensity two weeks after probiotic treatment, but declined steadily after that. As *J. lividum* numbers declined, Bd infection intensity increased, causing mortality. From that study, it was evident that the skin of tropical *A. zeteki* is an unsuitable habitat for *J. lividum*. The failure of *J. lividum* to persist on *A. zeteki* skin may be a direct result of interactions of the bacterial species with the host's defenses and other microbes present on the skin. In previous successful probiotic studies, the probiotic species used had been isolated from wild-caught amphibians of the same species treated with probiotics (Becker et al. 2009, Harris et al. 2009a). This evidence suggested that using an anti-Bd bacterial species naturally found on *A. zeteki*, or a close relative, might be more successful in preventing chytridiomycosis and enhancing the success of future reintroductions.

DISSERTATION OUTLINE

My dissertation research contributed to the conservation of *A. zeteki* by characterizing the microbial community associated with the skin of Panamanian golden frogs and by investigating

how the skin communities can be manipulated to benefit the host by preventing chytridiomycosis. In the study presented in Chapter II, I characterized the cutaneous bacterial community of wild (free-living) and captive *A. zeteki*. This was done to identify bacteria that naturally colonize the skin of *A. zeteki* in order to determine anti-Bd bacterial taxa that may have the most success at persisting on the skin of golden frogs. In addition, I examined how long-term captivity has altered the bacterial community of *A. zeteki* and suggest how this may affect future reintroductions. In the study presented in Chapter III, I investigated the ability of bacteria isolated from the skins of Panamanian amphibian species to inhibit Bd. This was done to find suitable anti-Bd bacterial species to use as potential probiotics for *A. zeteki*. In addition, I determined how this functional trait (Bd inhibition) is distributed among bacterial taxa. In Chapter IV, I present results from an experiment where captive *A. zeteki* were treated with one of four different anti-Bd bacterial isolates collected from Panamanian amphibians (Chapter III) and exposed to Bd. In that study, I also examined the role of bacteria naturally found on captive *A. zeteki* to prevent chytridiomycosis, as there was a correlation between initial bacterial community structure and survival. Finally, in chapter V, I provide an overall conclusion to my dissertation research and propose future directions for research in this field.

LITERATURE CITED

Bäckhed, F., R. E. Ley, J. L. Sonnenburg, D. A. Peterson, and J. I. Gordon. 2005. Host-bacterial mutualism in the human intestine. Science 307:1915–1920.

Banning, J. L., A. L. Weddle, G. W. Wahl, M. A. Simon, A. Lauer, R. L. Walters, and R. N. Harris. 2008. Antifungal skin bacteria, embryonic survival, and communal nesting in four-toed salamanders, *Hemidactylium scutatum*. Oecologia 156:423–429.

Becker, M. H., R. M. Brucker, C. R. Schwantes, R. N. Harris, and K. P. C. Minbiole. 2009. The bacterially produced metabolite violacein is associated with survival of amphibians infected with a lethal fungus. Applied and Environmental Microbiology 75:6635–6638.

Becker, M. H., R. N. Harris, K. P. C. Minbiole, C. R. Schwantes, L. A. Rollins-Smith, L. K. Reinert, R. M. Brucker, R. J. Domangue, and B. Gratwicke. 2012. Towards a better understanding of the use of probiotics for preventing chytridiomycosis in Panamanian golden frogs. EcoHealth 8:501–506.

Berger, L., A. D. Hyatt, R. Speare, and J. E. Longcore. 2005a. Life cycle stages of the amphibian chytrid *Batrachochytrium dendrobatidis*. Diseases of Aquatic Organisms 68:51–63.

Berger, L., G. Marantelli, L. F. Skerratt, and R. Speare. 2005b. Virulence of the amphibian chytrid fungus *Batrachochytium dendrobatidis* varies with the strain. Diseases of Aquatic Organisms 68:47–50.

Berger, L., R. Speare, P. Daszak, D. E. Green, A. A. Cunningham, C. L. Goggin, R. Slocombe, M. A. Ragan, A. D. Hyatt, K. R. McDonald, H. B. Hines, K. R. Lips, G. Marantelli, and H. Parkes. 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. Proceedings of the National Academy of Sciences 95:9031–9036.

Berger, L., R. Speare, H. B. Hines, G. Marantelli, A. D. Hyatt, K. R. McDonald, L. F. Skerratt, V. Olsen, J. M. Clarke, G. Gillespie, M. Mahony, N. Sheppard, C. Williams, and M. J. Tyler. 2004. Effect of season and temperature on mortality in amphibians due to chytridiomycosis. Australian Veterinary Journal 82:434–439.

Bletz, M. C., A. H. Loudon, M. H. Becker, S. C. Bell, D. C. Woodhams, K. P. C. Minbiole, and R. N. Harris. 2013. Mitigating amphibian chytridiomycosis with bioaugmentation: characteristics of effective probiotics and strategies for their selection and use. Ecology Letters 16:807–820.

Bosch, J., I. Martínez-Solano, and M. García-París. 2001. Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. Biological Conservation 97:331–337.

Brucker, R. M., C. M. Baylor, R. L. Walters, A. Lauer, R. N. Harris, and K. P. C. Minbiole. 2008a. The identification of 2,4-diacetylphloroglucinol as an antifungal metabolite produced by cutaneous bacteria of the salamander *Plethodon cinereus*. Journal of Chemical Ecology 34:39–43.

Brucker, R. M., R. N. Harris, C. R. Schwantes, T. N. Gallaher, D. C. Flaherty, B. A. Lam, and K. P. C. Minbiole. 2008b. Amphibian chemical defense: antifungal metabolites of the microsymbiont *Janthinobacterium lividum* on the salamander *Plethodon cinereus*. Journal of Chemical Ecology 34:1422–1429.

Carey, C., A. Pessier, and A. Peace. 2003. Pathogens, infectious disease, and immune defenses. Pages 127–136 *in* R. Semlitsch, editor. Amphibian Conservation. Smithsonian Books, Washington, D.C.

Currie, C. R., J. A. Scott, R. C. Summerbell, and D. Malloch. 1999. Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. Nature 398:701–704.

Davidson, E., M. J. Parris, J. P. Collins, J. E. Longcorel, A. P. Pessier, and J. Brunner. 2003. Pathogenicity and transmission of chytridiomycosis in tiger salamanders (*Ambystoma tigrinum*). Copeia 2003:601–607.

Dethlefsen, L., M. McFall-Ngai, and D. A. Relman. 2007. An ecological and evolutionary perspective on human-microbe mutualism and disease. Nature 449:811–818.

Farrer, R. A., L. A. Weinert, J. Bielby, T. W. J. Garner, F. Balloux, F. Clare, J. Bosch, A. A. Cunningham, C. Weldon, L. H. du Preez, L. Anderson, S. L. K. Pond, R. Shahar-Golan, D. A. Henk, and M. C. Fisher. 2011. Multiple emergences of genetically diverse amphibian-infecting chytrids include a globalized hypervirulent recombinant lineage. Proceedings of the National Academy of Sciences 108:18732–18736.

Fites, J. S., J. P. Ramsey, W. M. Holden, S. P. Collier, D. M. Sutherland, L. K. Reinert, A. S. Gayek, T. S. Dermody, T. M. Aune, K. Oswald-Richter, and L. A. Rollins-Smith. 2013. The invasive chytrid fungus of amphibians paralyzes lymphocyte responses. Science 342:366–369.

Gagliardo, R., P. Crump, E. Griffith, J. Mendelson, H. Ross, and K. Zippel. 2008. The principles of rapid response for amphibian conservation, using the programmes in Panama as an example. International Zoo Yearbook 42:125–135.

Gil-Turnes, M. S., and W. Fenical. 1992. Embryos of *Homarus americanus* are protected by epibiotic bacteria. Biological Bulletin 182:105–108.

Goka, K., J. Yokoyama, Y. Une, T. Kuroki, K. Suzuki, M. Nakahara, A. Kobayashi, S. Inaba, T. Mizutani, and A. D. Hyatt. 2009. Amphibian chytridiomycosis in Japan: distribution, haplotypes and possible route of entry into Japan. Molecular Ecology 18:4757–4774.

Guirard, B., and E. Snell. 1962. Nutritional requirements of microorganisms. Pages 33–93 *in* C. Gunsalis and R. Staneir, editors. The Bacteria. A Treatise on Structure and Function: The Physiology of Growth. Academic Press, New York.

Hanselmann, R., A. Rodríguez, M. Lampo, L. Fajardo-Ramos, A. Alonso Aguirre, A. Marm Kilpatrick, J. Paul Rodríguez, and P. Daszak. 2004. Presence of an emerging pathogen of amphibians in introduced bullfrogs *Rana catesbeiana* in Venezuela. Biological Conservation 120:115–119.

Harris, R. N., R. M. Brucker, J. B. Walke, M. H. Becker, C. R. Schwantes, D. C. Flaherty, B. A. Lam, D. C. Woodhams, C. J. Briggs, V. T. Vredenburg, and K. P. C. Minbiole. 2009a. Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. ISME Journal 3:818–824.

Harris, R. N., T. Y. James, A. Lauer, M. A. Simon, and A. Patel. 2006. Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. EcoHealth 3:53–56.

Harris, R. N., A. Lauer, M. A. Simon, J. L. Banning, and R. A. Alford. 2009b. Addition of antifungal skin bacteria to salamanders ameliorates the effects of chytridiomycosis. Diseases of Aquatic Organisms 83:11–16.

Houlahan, J. E., C. S. Findlay, B. R. Schmidt, a H. Meyer, and S. L. Kuzmin. 2000. Quantitative evidence for global amphibian population declines. Nature 404:752–755.

IUCN (International Union for Conservation of Nature). 2013. The IUCN red list of threatened species. http://www.iucnredlist.org.

James, T. Y., A. P. Litvintseva, R. Vilgalys, J. A. T. Morgan, J. W. Taylor, M. C. Fisher, L. Berger, C. Weldon, L. du Preez, and J. E. Longcore. 2009. Rapid global expansion of the fungal disease chytridiomycosis into declining and healthy amphibian populations. PLoS Pathogens 5:e1000458.

Johnson, M. L., and R. Speare. 2003. Survival of *Batrachochytrium dendrobatidis* in water: quarantine and disease control implications. Emerging Infectious Diseases 9:922–925.

Johnson, M. L., and R. Speare. 2005. Possible modes of dissemination of the amphibian chytrid *Batrachochytrium dendrobatidis* in the environment. Diseases of Aquatic Organisms 65:181–186.

Kaltenpoth, M., W. Go, G. Herzner, and E. Strohm. 2005. Symbiotic bacteria protect wasp larvae from fungal infestation. Current Biology 15:475–479.

Kennedy, S. 1998. Morbillivirus infections in aquatic mammals. Journal of Comparative Pathology 119:201–225.

Kilburn, V. L., R. Ibáñez, and D. M. Green. 2011. Reptiles as potential vectors and hosts of the amphibian pathogen *Batrachochytrium dendrobatidis* in Panama. Diseases of Aquatic Organisms 97:127–134.

Kriger, K. M., and J.-M. Hero. 2008. Altitudinal distribution of chytrid (*Batrachochytrium dendrobatidis*) infection in subtropical Australian frogs. Austral Ecology 33:1022–1032.

Kueneman, J. G., L. Wegener Parfrey, D. C. Woodhams, H. M. Archer, R. Knight, and V. J. McKenzie. 2013. The amphibian skin-associated microbiome across species, space and life history stages. Molecular Ecology:1238–1250.

Lam, B. A., J. B. Walke, V. T. Vredenburg, and R. N. Harris. 2010. Proportion of individuals with anti-*Batrachochytrium dendrobatidis* skin bacteria is associated with population persistence in the frog *Rana muscosa*. Biological Conservation 143:529–531.

Langhammer, P. F., K. R. Lips, P. A. Burrowes, T. Tunstall, C. M. Palmer, and J. P. Collins. 2013. A fungal pathogen of amphibians, *Batrachochytrium dendrobatidis*, attenuates in pathogenicity with *in vitro* passages. PloS One 8:e77630.

Lauer, A., M. A. Simon, J. L. Banning, B. A. Lam, and R. N. Harris. 2008. Diversity of cutaneous bacteria with antifungal activity isolated from female four-toed salamanders. ISME Journal 2:145–157.

Lips, K. R. 1999. Mass mortality and population declines of anurans at an upland site in western Panama. Conservation Biology 13:117–125.

Lips, K. R., F. Brem, R. Brenes, J. D. Reeve, R. A. Alford, J. Voyles, C. Carey, L. Livo, A. P. Pessier, and J. P. Collins. 2006. Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. Proceedings of the National Academy of Sciences 103:3165–3170.

Lips, K. R., J. Diffendorfer, J. R. Mendelson, and M. W. Sears. 2008. Riding the wave: reconciling the roles of disease and climate change in amphibian declines. PLoS Biology 6:e72.

Lips, K. R., J. D. Reeve, and L. R. Witters. 2003. Ecological traits predicting amphibian population declines in Central America. Conservation Biology 17:1078–1088.

Longcore, J. E., A. P. Pessier, D. K. Nichols, and J. E. Longcorel. 1999. *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. Mycologia 91:219–227.

Longcore, J. R., J. E. Longcore, A. P. Pessier, and W. A. Halteman. 2007. Chytridiomycosis widespread in anurans of northeastern United States. Journal of Wildlife Management 71:435–444.

Loo, J. A. 2009. Ecological impacts of non-indigenous invasive fungi as forest pathogens. Biological Invasions 11:81–96.

Lötters, S. 2007. The fate of the harlequin toads – help through a synchronous multi-disciplinary approach and the IUCN "Amphibian Conservation Action Plan"? Mitteilungen aus dem Museum für Naturkunde in Berlin, Zoologische Reihe 83:69–73.

Loudon, A. H., D. C. Woodhams, L. W. Parfrey, H. Archer, R. Knight, V. McKenzie, and R. N. Harris. 2014. Microbial community dynamics and effect of environmental microbial reservoirs on red-backed salamanders (*Plethodon cinereus*). ISME Journal 8:830–840.

Mangoni, M., R. Miele, T. Renda, D. Barra, and M. Simmaco. 2001. The synthesis of antimicrobial peptides in the skin of *Rana esculenta* is stimulated by microorganisms. The FASEB Journal 15:1431–1432.

La Marca, E., K. R. Lips, S. Lotters, R. Puschendorf, R. Ibanez, J. V. Rueda-Almonacid, R. Schulte, C. Marty, F. Castro, J. Manzanilla-Puppo, J. E. Garcia-Perez, F. Bolanos, G. Chaves, J. A. Pounds, E. Toral, and B. E. Young. 2005. Catastrophic population declines and extinctions in Neotropical harlequin frogs (Bufonidae: *Atelopus*). Biotropica 37:190–201.

McKenzie, V. J., R. M. Bowers, N. Fierer, R. Knight, and C. L. Lauber. 2012. Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. ISME Journal 6:588–596.

McMahon, T. A., L. A. Brannelly, M. W. H. Chatfield, P. T. J. Johnson, M. B. Joseph, V. J. McKenzie, C. L. Richards-Zawacki, M. D. Venesky, and J. R. Rohr. 2013. Chytrid fungus *Batrachochytrium dendrobatidis* has nonamphibian hosts and releases chemicals that cause pathology in the absence of infection. Proceedings of the National Academy of Sciences 110:210–215.

Mills, J. W., and B. E. Prum. 1984. Morphology of the exocrine glands of the frog skin. The American journal of anatomy 171:210–215.

Nichols, D. K., E. W. Lamirande, A. P. Pessier, and J. E. Longcore. 2001. Experimental transmission of cutaneous chytridiomycosis in dendrobatid frogs. Journal of wildlife diseases 37:1–11.

Pessier, A. P., D. K. Nichols, J. E. Longcore, and M. S. Fuller. 1999. Cutaneous chytridiomycosis in poison dart frogs (*Dendrobates* spp.) and White's tree frogs (*Litoria caerulea*). Journal of Veterinary Diagnostic Investigation 11:194–199.

Piotrowski, J. S., S. L. Annis, and J. E. Longcore. 2004. Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. Mycologia 96:9–15.

Poole, V. 2008. Project golden frog. Endangered Species Bulletin 33:7–10.

Pounds, J. A., M. R. Bustamante, L. A. Coloma, J. A. Consuegra, M. P. L. Fogden, P. N. Foster, E. La Marca, K. L. Masters, A. Merino-Viteri, R. Puschendorf, S. R. Ron, G. A. Sánchez-Azofeifa, C. J. Still, and B. E. Young. 2006. Widespread amphibian extinctions from epidemic disease driven by global warming. Nature 439:161–167.

Rachowicz, L. J., R. A. Knapp, J. A. T. Morgan, M. J. Stice, V. T. Vredenburg, J. M. Parker, and C. J. Briggs. 2006. Emerging infectious disease as a proximate cause of amphibian mass mortality. Ecology 87:1671–83.

Rachowicz, L., and V. Vredenburg. 2004. Transmission of *Batrachochytrium dendrobatidis* within and between amphibian life stages. Diseases of Aquatic Organisms 61:75–83.

Relman, D. A. 2012. The human microbiome: ecosystem resilience and health. Nutrtion Reviews 70 Suppl 1:S2–S9.

Retallick, R. W. R., H. McCallum, and R. Speare. 2004. Endemic infection of the amphibian chytrid fungus in a frog community post-decline. PLoS Biology 2:e351.

Richards-Zawacki, C. L. 2010. Thermoregulatory behaviour affects prevalence of chytrid fungal infection in a wild population of Panamanian golden frogs. Proceedings of the Royal Society B 277:519–528.

Richmond, J. Q., A. E. Savage, K. R. Zamudio, and E. B. Rosenblum. 2009. Toward immunogenetic studies of amphibian chytridiomycosis: linking innate and acquired immunity. BioScience 59:311–320.

Rollins-Smith, L. A. 2009. The role of amphibian antimicrobial peptides in protection of amphibians from pathogens linked to global amphibian declines. Biochimica et Biophysica Acta 1788:1593–1599.

Scott, J. J., D. Oh, M. C. Yuceer, K. D. Klepzig, J. Clardy, and C. R. Currie. 2008. Bacterial protection of beetle-fungus mutualism. Science 322:63.

Skerratt, L. F., L. Berger, R. Speare, S. Cashins, K. R. McDonald, A. D. Phillott, H. B. Hines, and N. Kenyon. 2007. Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. EcoHealth 4:125–134.

Stuart, S. N., J. S. Chanson, N. A. Cox, B. E. Young, A. S. L. Rodrigues, D. L. Fischman, and R. W. Waller. 2004. Status and trends of amphibian declines and extinctions worldwide. Science 306:1783–1786.

Voyles, J., L. Berger, S. Young, R. Speare, R. Webb, J. Warner, D. Rudd, R. Campbell, and L. F. Skerratt. 2007. Electrolyte depletion and osmotic imbalance in amphibians with chytridiomycosis. Diseases of Aquatic Organisms 77:113–8.

Voyles, J., S. Young, L. Berger, C. Campbell, W. F. Voyles, A. Dinudom, D. Cook, R. Webb, R. A. Alford, L. F. Skerratt, and R. Speare. 2009. Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. Science 326:582–5.

Walke, J. B., M. H. Becker, S. C. Loftus, L. L. House, G. Cormier, R. V Jensen, and L. K. Belden. 2014. Amphibian skin may select for rare environmental microbes. ISME Journal:doi:10.1038/ismej.2014.77.

Weldon, C., L. H. du Preez, A. D. Hyatt, R. Muller, and R. Spears. 2004. Origin of the amphibian chytrid fungus. Emerging Infectious Diseases 10:2100–2105.

Woodhams, D. C., R. A. Alford, and G. Marantelli. 2003. Emerging disease of amphibians cured by elevated body temperature. Diseases of Aquatic Organisms 55:65–67.

Woodhams, D. C., K. Ardipradja, R. A. Alford, G. Marantelli, L. K. Reinert, and L. A. Rollins-Smith. 2007. Resistance to chytridiomycosis varies among amphibian species and is correlated with skin peptide defenses. Animal Conservation 10:409–417.

Woodhams, D. C., J. Bosch, C. J. Briggs, S. Cashins, L. R. Davis, A. Lauer, E. Muths, R. Puschendorf, B. R. Schmidt, B. Sheafor, and J. Voyles. 2011. Mitigating amphibian disease: strategies to maintain wild populations and control chytridiomycosis. Frontiers in Zoology 8:8.

Woodhams, D. C., H. Brandt, S. Baumgartner, J. Kielgast, E. Küpfer, U. Tobler, L. R. Davis, B. R. Schmidt, C. Bel, S. Hodel, R. Knight, and V. McKenzie. 2014. Interacting symbionts and immunity in the amphibian skin mucosome predict disease risk and probiotic effectiveness. PLoS One 9:e96375.

Woodhams, D. C., L. A. Rollins-Smith, C. Carey, L. Reinert, M. J. Tyler, and R. A. Alford. 2006a. Population trends associated with skin peptide defenses against chytridiomycosis in Australian frogs. Oecologia 146:531–540.

Woodhams, D. C., J. Voyles, K. R. Lips, C. Carey, and L. A. Rollins-Smith. 2006b. Predicted disease susceptibility in a Panamanian amphibian assemblage based on skin peptide defenses. Journal of Wildlife Diseases 42:207–218.

CHAPTER II. THE EFFECT OF CAPTIVITY OF THE CUTANEOUS BACTERIAL COMMUNITY OF THE CRITICALLY ENDANGERED PANAMANIAN GOLDEN FROG (*ATELOPUS ZETEKI*)

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ABSTRACT

For many threatened vertebrates, captivity may be the only option for species survival. Maintaining species in captivity prior to reintroduction presents many challenges, including the need to preserve genetic diversity and mitigation of disease risks. Recent studies suggest that captivity can alter the suite of symbiotic microbes that play important roles in host health. The Panamanian golden frog (*Atelopus zeteki*) has not been seen in its native habitat in Panamá since 2009. Along with habitat loss and illegal collecting, the lethal disease chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis* (Bd), is responsible for the severe decline of this species. Prior to the spread of Bd into golden frog habitat, conservation organizations collected golden frogs and placed them in captive survival assurance colonies. The skin of amphibians is host to a diverse resident bacterial community, which acts as a defense mechanism in some amphibians to inhibit pathogens. We characterized the cutaneous bacterial community from wild and F1 captive golden frogs originating from the same population with Illumina sequencing to assess how long-term captivity has affected this community. We found that species richness, phylogenetic diversity, and community structure of the skin microbiota was significantly different between wild and captive golden frogs. However, after approximately eight years of living in captivity, the offspring of the original captive golden frogs still shared 70% of their microbial community with wild frogs. These results demonstrate that host-associated microbial communities can be significantly altered by captive management, but most of the community composition can be preserved.

INTRODUCTION

Over the past few decades, we have seen a disturbing rate of species declines and extinctions due to a variety of factors including habitat loss, disease, and climate change (Stuart et al. 2004, Heard et al. 2013). It has even been suggested that we are currently witnessing a sixth mass extinction (Wake and Vredenburg 2008). For many threatened species, captivity is the only tool available to conservation managers to prevent extinction when survival in the organism's native habitat is not possible, as is the case with the Panamanian golden frog, *Atelopus zeteki* (Gagliardo et al. 2008). However, managing species under captive conditions cannot only permanently deteriorate the host's genome (Woodworth et al. 2002), but also alter symbiotic microbial communities associated with these organisms. Symbiotic microbial communities of many wild species including monkeys, bears, seals, grouse, parrots, sponges, and salamanders have been affected while these animals have been kept in captivity (Xenoulis et al. 2010, Schwab et al. 2011, Webster et al. 2011, Nakamura et al. 2011, Wienemann et al. 2011, Nelson et al. 2013, Loudon et al. 2013).

With advancements in molecular and microbial techniques, we are now discovering how vital symbiotic microorganisms are to the health and normal function of the host they inhabit. For example, microbial communities associated with the human gut facilitate metabolic and absorptive processes and stimulate immunity (Bäckhed et al. 2005, Fujimura et al. 2010). In addition, symbiotic microbes in some species, including *Atelopus*, may produce toxins (e.g. tetrodotoxin) that protect the host from predators (Chau et al. 2011). One possible contributing factor to the low historical success rate of reintroductions with endangered species (11 to 53%, Beck et al. 1994, Fischer and Lindenmayer 2000, Wolf et al. 1996) is that captive rearing alters the host's microbial community, decreasing subsequent survival of the animal in the wild (Redford et al. 2012). For example, many attempts to reintroduce the grouse *Tetrao urogallus* have failed (Seiler et al. 2000) likely due to impaired digestion as a result of anatomical changes in the gut (Liukkonen-Anttila et al. 2000) and shifts in the gut microbial community as a result of captive management (Wienemann et al. 2011). While much of this work has focused on birds and mammals, it seems likely that host-associated microbial communities also contribute to the

success of amphibians in their native habitats and could be an important component of successful amphibian reintroduction programs.

Along with most species in the genus *Atelopus,* the Panamanian golden frog is critically endangered (Lips et al. 2010) and it has not been seen in the wild since 2009, despite intensive search efforts (E. Griffith personal communication, La Marca et al. 2005, C.L.R.-Z. unpublished data). The frog's historical range was in a small area of central-western Panamá. Chytridiomycosis, a disease caused by the fungal pathogen *Batrachochytrium dendrobatidis* (Bd), has been spreading through Panamanian amphibian assemblages in a south-easterly direction for almost two decades and has caused severe population declines and extinctions (Lips et al. 2006, Woodhams et al. 2008b, Cheng et al. 2011). In one documented case in Panamá, approximately 50% of the amphibian species and 80% of the individuals disappeared in a few months following the initial detection of Bd (Lips et al. 2006). In response to the declines of related *Atelopus* species (e.g., *A. varius*) and prior to the spread of Bd into the historical range of *A. zeteki*, Project Golden Frog (http://www.projectgoldenfrog.org), in collaboration with multiple zoos in the United States, collected and placed approximately 100 *A. zeteki* in captive survival assurance colonies (Gagliardo et al. 2008). Currently, over 2000 *A. zeteki* are being maintained in North American and Panamanian zoos and aquaria (K. Murphy personal communication, Poole 2008). The ultimate goal of this *ex situ* conservation program is to reintroduce *A. zeteki* back to their native habitat in Panamá. Unfortunately, *A. zeteki* is highly susceptible to chytridiomycosis. Bd still remains in the environment and on less susceptible amphibian species, so it is unlikely that any Bd-free environments exist (Becker et al. 2012). Reintroduction of *A. zeteki* will therefore require Bd mitigation strategies, such as the use of beneficial bacteria (probiotics, Becker et al. 2009, Harris et al. 2009, Becker and Harris 2010, Bletz et al. 2013).

Because cutaneous bacteria have important health-related functions for amphibian hosts and because the use of these bacteria in probiotic-based mitigation strategies is possible, it is important to determine how captivity affects these complex bacterial communities. Many environmental factors, such as humidity, temperature, and pH, affect skin or surface-associated microbial communities in animals (McBride et al. 1977, Webster et al. 2008, Meron et al. 2011). In addition, a lack of natural environmental reservoirs of bacteria can also alter the composition of host-associated microbial communities (Loudon et al. 2013). Therefore, frogs reared in a

captive environment likely have different cutaneous bacterial communities than individuals in wild populations. The aims of this study were to characterize the historical symbiotic bacterial communities associated with the skin of wild *A. zeteki* and to examine the effects of long-term captive management on the structure of these communities.

METHODS

Study species and sites

We characterized and compared the cutaneous microbial community structure from both a wild and a captive population of *A. zeteki*. Samples were collected by swabbing back and forth 3 to 5 times on the surfaces of each the venter, dorsum, thighs, and feet of each frog with a sterile swab, as to sample the entire surface. We changed gloves for the handling of each frog. Wild adult *A. zeteki* (N=27) were sampled from a population located near Río Mata Ahogado in Panamá in 2005 and 2006, and released at the site of capture after swabbing (Richards-Zawacki 2010). These swabs were stored at room temperature in a salt-saturated DMSO solution prior to DNA extraction. Captive adult *A. zeteki* (N=10) were sampled from a population at the Smithsonian National Zoological Park in Washington, D.C. in 2011. These individuals were born in captivity in 2005 from parents that were collected in 2003 from the same population from which wild samples were collected. After laying eggs, parents were removed from enclosures and had no further contact with offspring. We stored swabs from captive frogs at -80 C prior to DNA extraction. DNA preservation technique (other than filter card techniques) and length of time in storage does not significantly affect the assessment of microbial community structure (Dolfing et al. 2004, Lauber et al. 2010, Gray et al. 2013).

Sample preparation and sequencing

We extracted DNA from each swab with PrepMan Ultra (Applied Biosystems, Carlsbad, California) following methods outlined by Hyatt et al. (2007). This DNA extraction method is optimized for the extraction of DNA from Bd, but it is also effective at extracting DNA from prokaryotic cells. The V4 region of the 16s rRNA gene was amplified with PCR and the primers 515F and 806R (Caporaso et al. 2010c). The reverse primers contained a 12 base error-correcting

Golay code (Fierer et al. 2008), which we used to uniquely tag PCR products of each sample. We prepared PCR reactions as described by Costello et al. (2009). Briefly, triplicate reactions of each sample contained 1 µl template DNA, 12 µl DNA-free PCR water (MO-BIO, Carlsbad, California), 10 µl 2.5x HotMasterMix (5 PRIME, Gaithersburg, Maryland), 1 µl of 20 mg/ml bovine serum albumin (Fisher Scientific, Pittsburgh, Pennsylvania), and 0.5 µl of each primer at 10 µM concentration. We ran controls without template for each sample. DNA extracted from a sterile swab was also included as a negative control. We diluted extracted DNA samples that contained PCR inhibitors 1 to 10 in PCR water. The amplification conditions were as follows: an initial cycle for 3 min at 94 C followed by 35 cycles of 34 s at 94 C, 60 s at 50 C, and 90 s at 72 C, with a final cycle for 10 min at 72 C. Amplification conditions for five samples with low DNA concentrations were altered to include 38 cycles. Triplicate reactions of each sample were pooled, visualized on a 1% agarose gel, and quantified with a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, California). We purified PCR products with the Qiagen QIAquik PCR Purification Kit (Qiagen, Valencia, California) using the manufacturer's protocol. An equimolar mixture of all the samples was then sequenced on an Illumina MiSeq instrument (San Diego, California) with a 250 bp paired-end strategy at the Dana-Farber Cancer Institute, following methods similar to those in Caporaso et al. (2012). To compensate for the low base diversity of the amplicon pool, the sample was run with a 10% PhiX control. Version 1.18.42 of the MiSeq Real-Time Analysis software (Illumina) was used to perform base calling and quality scoring.

Sequence data processing

Sequence data were assembled with Fastq-join with default parameters and processed with the Quantitative Insights Into Microbial Ecology pipeline (QIIME v. 1.7.0, Caporaso et al. 2010a). We clustered quality-filtered sequences into distinct bacterial OTUs (operational taxonomic units) at a sequence similarity threshold of 97% and assigned taxonomy with RDP classifier and the Greengenes database. All samples were rarefied to 19 500 sequences to standardize sampling effort. Details of the bioinformatics methods are in the Appendix.

Statistical analysis

Unless noted, all dependent variables were normally distributed and variances were equal among specific comparisons. We computed measures of alpha diversity (within-sample

diversity), including OTU richness, phylogenetic diversity, and Shannon diversity index, with QIIME. We used Student's t-tests to test for significant differences in alpha diversity measures between the wild and captive populations. To compare the microbial community structure between samples, a Bray-Curtis distance matrix (Bray and Curtis 1957) was built on square-root transformed data with the software package Primer 6 (version 6.1.15). We completed all further community composition comparisons with Primer 6 and Permanova+ (version 1.0.5). From the distance matrices, differences in community composition between the wild and captive populations were statistically analyzed with Analysis of Similarity (ANOSIM) and visualized with principal coordinates analysis (PCO). Relative abundances of phyla, genera, and individual OTUs were not normally distributed; therefore, we statistically analyzed differences between populations with Wilcoxon rank-sum tests. We corrected all multiple comparisons with the false discovery rate procedure (FDR, Benjamini & Hochberg 1995). The core microbiota was defined as OTUs that were present on 90% or more of individuals in each population. A phylogenetic tree was built to visualize the distribution of OTUs among dominant phyla that were shared and unique to each population. The tree was constructed with MUSCLE aligned sequences (Edgar 2004) using FastTree (Price et al. 2009) and visualized with the Interactive Tree of Life (Letunic and Bork 2007). The phylogenetic tree is not meant to portray specific evolutionary relationships among individual OTUs.

RESULTS

Alpha diversity (within-sample diversity)

There was a large amount of variation in the diversity of skin communities among individual *A. zeteki* in both wild and captive populations (Fig. 1). For example, the number of OTUs (OTU richness) on wild frogs ranged from 136 to 1451 OTUs per frog (Fig. 1a). Despite the large individual variation, OTU richness and phylogenetic diversity were significantly higher in captive *A. zeteki* than in wild frogs (Fig. 1a, t-test, $P < 0.01$ and Fig. 1b, t-test, $P < 0.01$, respectively). However, wild *A. zeteki* had a significantly higher Shannon diversity index (a measure of evenness) than captive individuals (Fig. 1c, t-test, $P = 0.02$).

Community composition differences and shared microbiota

Although there was a considerable amount of variation in community composition among the microbial communities of frogs within each population, the variation between captive and wild populations was strikingly different (ANOSIM, Global R = 0.443, P = 0.001) and formed two distinct clusters on a PCO plot (Fig. 2). However, the offspring of individuals that were placed in captive assurance colonies in 2003 still shared 2137 OTUs with wild *A. zeteki* (Fig. 3a). When considering only shared OTUs, community structure between the captive and wild populations was still significantly different (ANOSIM, Global R = 0.416, P = 0.001) because the relative abundances of these shared OTUs differed between populations.

Shared OTUs were dominant members in the communities on both wild and captive *A. zeteki* when compared to OTUs only found in only one population (Fig. 3b). For example, the mean relative abundances of the most abundant shared OTU in wild and captive populations (10 and 21%, respectively) was two orders of magnitude higher than the most abundant OTU present only in the wild or captive population (0.37 and 0.30%, respectively). The core microbiota (OTUs present on ≥90% of individuals) of the wild population consisted of 11 OTUs (Table A1), and these were also shared with the captive population. Ten out of the 11 were present on 100% of the captive frogs, with the remaining OTU present on 80% of the captive frogs.

Microbiota unique to each population

Although the wild and captive populations shared many OTUs, there were 2856 OTUs unique to the wild population and 915 unique to the captive population (Fig. 3a). A majority of these OTUs were at low prevalence (proportion of individuals that have a particular OTU) in both populations. Of the OTUs unique to wild frogs, 98% (2663/2856) had a prevalence ≤ 30% (Table A2), while of the OTUs unique to captive frogs, 77% (707/915) had a prevalence ≤ 30%. OTUs unique to each population were distributed throughout all the dominant bacterial phyla present on wild *A. zeteki* (Fig. 3d).

Dominant bacterial phyla and families

The most dominant phyla (mean relative abundance > 0.05% in either population) in the populations were Acidobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes,

Planctomycetes, Proteobacteria, and Verrucomicrobia (Fig. 3c). These eight phyla were present on > 90% of the frogs in both populations. Only three phyla had mean relative abundances that were significantly different between the wild and captive populations (Wilcoxon rank-sum test, FDR-corrected $P < 0.05$). The phylum Bacteroidetes had a mean relative abundance of 27% and 43% on wild and captive *A. zeteki*, respectively. An individual OTU in the genus *Pedobacter* (Family: Sphingobacteriaceae) accounted for 97.6% of this difference. This OTU was present on all captive and wild individuals in the study and had the highest mean relative abundance across all samples. The phyla Planctomycetes and Verrucomicrobia were significantly more abundant on wild frogs. There were 35 dominant bacterial families (mean relative abundance > 0.05%) on *A. zeteki*, which are shown in Figure A1 with their respective relative abundances.

Dominant operational taxonomic units (OTUs, ~bacterial "species")

Many dominant members of the microbial community (OTUs with a mean relative abundance > 0.5%) were also members of the core microbiota (present on ≥90% of individuals) in each population (Fig. 4). Dominant OTUs were all classified in the phyla Bacteroidetes, Actinobacteria, or Proteobacteria. There were 15 dominant OTUs present on wild *A. zeteki* and seven of these OTUs were core members of the microbiota. The relative abundances of nine of these 15 dominant wild frog OTUs were significantly different in the captive population (Wilcoxon rank-sum test, FDR-corrected $P < 0.05$). Six of them were lower in abundance on captive individuals and three were higher in abundance on captive individuals. Those that had lower abundances on captive frogs were drastically lower. For instance, an OTU classified as belonging to the family Actinomycetales had a mean relative abundance of 3.9% on wild frogs and only 0.03% on captive individuals, despite having a prevalence of 100% in both populations. The most dominant OTU in both wild and captive frogs (*Pedobacter*, discussed in 3.4) doubled in relative abundance on captive frogs, thus skewing the OTU relative abundance distribution in these frogs. This likely caused the captive population to have a significantly lower Shannon diversity index (= less even community) than the wild population (Fig. 1c). There were also four OTUs that had low abundance on wild frogs, but were dominant on captive frogs (Wilcoxon rank-sum test, FDR-corrected $P < 0.05$). These OTUs also increased in prevalence in the captive population.

DISCUSSION

From 2001 to 2005, *A. zeteki* from Panamá were collected from their native habitats and placed in captive assurance colonies prior to the invasion of Bd (Gagliardo et al. 2008). Our results indicate that the skin microbiota of F1 captive *A. zeteki* was significantly different than wild frogs, in terms of species richness, evenness, phylogenetic diversity, and community composition. This same pattern has been seen in other animals managed under long-term captive conditions (Isaacs et al. 2009, Xenoulis et al. 2010, Schwab et al. 2011, Nakamura et al. 2011, Wienemann et al. 2011, Nelson et al. 2013). For example, OTU richness was much higher in the surface-associated microbiota of captive sponges (Mohamed et al. 2008) and in the gut microbiota of captive seals (Nelson et al. 2013) and parrots (Xenoulis et al. 2010) than in their wild counterparts, which was also observed for golden frogs in the present study. However, other studies have shown the converse (higher diversity in wild animals, Isaacs et al. 2009, Nakamura et al. 2011).

Some changes in *A. zeteki* microbiota during captive management are likely due to environmental factors such as humidity, temperature, and pH (McBride et al. 1977, Webster et al. 2008, Meron et al. 2011). Captive *A. zeteki* were kept under conditions that resemble their natural habitat. However, it is impossible to simulate in captivity the variety of microhabitat conditions that these frogs experienced in the wild. In addition, the potential for transmission of bacteria from other sources is increased in captivity (Nelson et al. 2013) and may explain why captive frogs had higher richness and phylogenetic diversity than wild frogs. This could happen by co-habitation of several *A. zeteki* in the same enclosure, by interaction with the microbiota of zookeepers, and by exposure to the microbiota of other frog species (through environmental transmission) and microbes living on environmental substrates in enclosures (plants, rocks, soil, and water).

There is also a concern that long-term managed species, with multiple generations born in captivity, are likely to experience permanent microbiota changes due to host factors if genetic variation cannot be preserved. For instance, mutations in genes associated with the immune system can result in changes to the structure of gut-associated microbial communities of mice and humans (Spor et al. 2011). Minimizing time managed under captive conditions may reduce

changes to the microbial community. Sponges placed in captive conditions for short periods of time (< 6 months) had very similar surface microbial communities to wild-caught sponges (Gerçe et al. 2009, Webster et al. 2011). However, after 12 months in captivity, sponges had a very different symbiont community structure than wild-caught sponges with many wild-associated microbes lost and new or rare members becoming dominant (Webster et al. 2011).

Although there were significant differences between the microbial communities of wild and captive golden frog populations, 70% of the OTUs on captive frogs were shared with wild frogs. In addition, all but one core bacterial species of wild *A. zeteki* were also core members of the microbial community of captive frogs. This suggests that even in captivity, the primary symbionts may be maintained over generations. However, the relative abundances of most shared OTUs were drastically different between populations where, with a few exceptions, OTUs abundant on wild frogs were rare on captive frogs and vice versa. So even though the species in the microbial communities were largely shared, the community structure differed significantly between the two populations with a more even community in the wild population and a community dominated by fewer taxa in the captive population.

The fact that a majority of the microbes were retained in captivity suggests that either these microbes are transmitted by vertical or pseudo-vertical transmission or that they are abundant in a broad range of environments. Vertical transmission occurs when microorganisms are transferred from parent to offspring (Bright and Bulgheresi 2010). This seems unlikely since *A. zeteki* parents had no contact with offspring after laying eggs. However, a study of the glass frog *Hyalinobatrachium colymbiphyllum* suggests that skin bacteria can be vertically transmitted from amphibian parents to embryos in some species (Walke et al. 2011). We hypothesize pseudo-vertical transmission, in which microorganisms are transmitted from parent to offspring through an intermediate environmental source, is the more likely mode of transmission for captive *A. zeteki*. For instance, bacteria from the skin of parents could be transmitted to the water within the enclosure. These bacteria could remain in the enclosure after the parents are removed and colonize the skin of offspring.

At the phylum level, there were many similarities in the relative abundances of the dominant phyla present on wild and captive golden frogs, with the exception of three phyla

(Bacteroidetes, Planctomycetes, and Verrucomicrobia). Bacteroidetes was significantly more abundant on captive frogs due to the increase in relative abundance of a single *Pedobacter* species (Family: Sphingobacteriaceae). The increase in relative abundance of this single OTU also likely drove the decrease in evenness in the captive population, despite the greater richness and phylogenetic diversity in the captive population. This *Pedobacter* sp. was the most abundant OTU in both populations and its mean relative abundance more than doubled on captive frogs, resulting in a more skewed distribution. This increase may be due to the ability of this organism to more successfully grow and compete than other bacteria in the microhabitat created by captive conditions. Species of *Pedobacter* are commonly found on the skin of amphibians (Harris et al. 2006, Lauer et al. 2008, Lam et al. 2010). The phyla Planctomycetes and Verrucomicrobia were more abundant on wild frogs. Phylogenetic analysis suggests these two phyla are closely related (Hou et al. 2008) and are commonly found in a variety of aquatic habitats and in association with animals. Verrucomicrobia are also commonly found in soils (Wagner and Horn 2006). Therefore, the decrease in abundance of these phyla on captive individuals may be explained by the lack of transmission from native environmental sources. It is difficult to determine the proportion of amphibian resident bacteria that are derived from the environment, but recent studies have reported that 16 – 90% of the cutaneous bacteria are shared with the amphibian's surrounding environment and may be species-dependent (Kueneman et al. 2013, Loudon et al. 2013, J. Walke unpublished data).

Although there were many similarities between wild and captive frogs at the phylum level, the relative abundances of many bacterial families were strikingly different between populations (Fig. A1). Interestingly, the bacterial families that had higher relative abundances on captive frogs (Cellulomonadaceae, Flavobacteriaceae, Moraxellaceae, Neisseriaceae, Nocardiaceae, Pseudomonadaceae, Sanguibacteraceae, and Sphingobacteriaceae) have also been commonly found in abundance on North American amphibians (McKenzie et al. 2012, J. Walke unpublished data). These results suggest that either environmental conditions of captive *A. zeteki* favored the growth and reproduction of these families, or that OTUs in these families were indirectly transmitted from other amphibians or environmental sources while in captivity in North America.

Overall, the results of our study demonstrate that captive management can significantly alter the structure of the microbial community on *A. zeteki*. Important next steps in this line of research include investigating how the reintroduction of golden frogs to their native habitat will likely affect their skin-associated microbial community. If golden frog microbiota are obtained through environmental sources and mediated through environmental factors, then it is possible that the pre-captivity microbial community composition and structure will be recovered once they are returned to Panamá. However, if golden frogs rely on vertical or pseudo-vertical transmission then bacterial species lost in captivity may never recover. As noted earlier, host-associated microbial communities provide many vital functions to the host and changes to this microbiota may have severe consequences for reintroduction efforts of *A. zeteki*, such as increased susceptibility to endemic or recently emerged pathogens (Schommer and Gallo 2013). Therefore, it may be important to conserve the microbial diversity of captive species, as well as the genetic diversity, if the goal of captive management is reintroduction.

One approach to prevent alterations to host-associated microbiota in species that have environmentally derived microbiota is to provide native environmental sources in their captive enclosures, although care must be taken not to introduce pathogens with these items. For example, a recent study demonstrated that captive management of the red-backed salamander (*Plethodon cinereus*) with native soils present in their enclosures reduced changes to their microbial community when compared to more sterile rearing conditions (Loudon et al. 2013). If microbial communities are largely derived through vertical transmission, then cohabitation of parents and offspring would largely reduce changes of the microbiota. In captive populations, it may also be critical to limit the use of antibiotics, which can have long-lasting and possibly permanent effects to the microbiota (Lozupone et al. 2012). Although our study is limited to the bacterial community associated with amphibians, it is likely that captive management affects other symbiotic microbiota, such as fungi and viruses. For instance, the use of antifungal treatments, which are important for treating and preventing Bd in captive amphibians (Georoff et al. 2013), could also affect symbiotic fungi and/or alter microbial interactions in these complex communities.

When the microbial community of a host is viewed as an extension of the host's genetic makeup and ability to adapt (Rosenberg et al. 2007), it becomes clearer that preserving the

diversity of host-associated microbiota may be important for the success of future reintroduction efforts and the long-term persistence of species, including the Panamanian golden frog. Studies investigating how changes in host-associated microbial communities due to captive management affect host function and disease resistance may be critical when developing a successful reintroduction program for endangered species.

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LITERATURE CITED

Bäckhed, F., R. E. Ley, J. L. Sonnenburg, D. A. Peterson, and J. I. Gordon. 2005. Host-bacterial mutualism in the human intestine. Science 307:1915–1920.

Beck, B. B., L. G. Rapaport, M. R. Stanley Price, and A. C. Wilson. 1994. Reintroduction of captive-born animals. Pages 264–386 *in* P. J. S. Olney, G. M. Mace, and A. T. C. Feistner, editors. Creative Conservation: Interactive Management of Wild and Captive Animals. Chapman and Hall, London.

Becker, M. H., R. M. Brucker, C. R. Schwantes, R. N. Harris, and K. P. C. Minbiole. 2009. The bacterially produced metabolite violacein is associated with survival of amphibians infected with a lethal fungus. Applied and Environmental Microbiology 75:6635–6638.

Becker, M. H., and R. N. Harris. 2010. Cutaneous bacteria of the redback salamander prevent morbidity associated with a lethal disease. PLoS One 5:e10957.

Becker, M. H., R. N. Harris, K. P. C. Minbiole, C. R. Schwantes, L. A. Rollins-Smith, L. K. Reinert, R. M. Brucker, R. J. Domangue, and B. Gratwicke. 2012. Towards a better understanding of the use of probiotics for preventing chytridiomycosis in Panamanian golden frogs. EcoHealth 8:501–506.

Benjamini, Y., and Y. Hochberg. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society B 57:289–300.

Bletz, M. C., A. H. Loudon, M. H. Becker, S. C. Bell, D. C. Woodhams, K. P. C. Minbiole, and R. N. Harris. 2013. Mitigating amphibian chytridiomycosis with bioaugmentation: characteristics of effective probiotics and strategies for their selection and use. Ecology Letters 16:807–820.

Bray, J. R., and J. T. Curtis. 1957. An ordination of the upland forest communities of southern Wisconsin. Ecological Monographs 27:325–349.

Bright, M., and S. Bulgheresi. 2010. A complex journey: transmission of microbial symbionts. Nature Reviews Microbiology 8:218–230.

Caporaso, J. G., J. Kuczynski, J. Stombaugh, K. Bittinger, F. D. Bushman, E. K. Costello, N. Fierer, A. G. Peña, J. K. Goodrich, J. I. Gordon, G. A. Huttley, S. T. Kelley, D. Knights, J. E. Koenig, R. E. Ley, C. A. Lozupone, D. McDonald, B. D. Muegge, M. Pirrung, J. Reeder, J. R. Sevinsky, P. J. Turnbaugh, W. A. Walters, J. Widmann, T. Yatsunenko, J. Zaneveld, and R. Knight. 2010a. QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7:335–336.

Caporaso, J. G., C. L. Lauber, W. A. Walters, D. Berg-Lyons, J. Huntley, N. Fierer, S. M. Owens, J. Betley, L. Fraser, M. Bauer, N. Gormley, J. A. Gilbert, G. Smith, and R. Knight. 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME Journal 6:1621–1624.

Caporaso, J. G., C. L. Lauber, W. A. Walters, D. Berg-Lyons, C. A. Lozupone, P. J. Turnbaugh, N. Fierer, and R. Knight. 2010b. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proceedings of the National Academy of Sciences 108 Suppl:4516–4522.

Chau, R., J. A. Kalaitzis, and B. A. Neilan. 2011. On the origins and biosynthesis of tetrodotoxin. Aquatic Toxicology 104:61–72.

Cheng, T. L., S. M. Rovito, D. B. Wake, and V. T. Vredenburg. 2011. Coincident mass extirpation of Neotropical amphibians with the emergence of the infectious fungal pathogen *Batrachochytrium dendrobatidis*. Proceedings of the National Academy of Sciences 108:9502–9507.

Costello, E. K., C. L. Lauber, M. Hamady, N. Fierer, J. I. Gordon, and R. Knight. 2009. Bacterial community variation in human body habitats across space and time. Science 326:1694–1697.

Dolfing, J., A. Vos, J. Bloem, P. Ehlert, N. Naumova, and P. Kuikman. 2004. Microbial diversity in archived soils. Science 306:813.

Edgar, R. C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 32:1792–1797.

Fierer, N., M. Hamady, C. L. Lauber, and R. Knight. 2008. The influence of sex, handedness, and washing on the diversity of hand surface bacteria. Proceedings of the National Academy of Sciences 105:17994–17999.

Fischer, J., and D. . Lindenmayer. 2000. An assessment of the published results of animal relocations. Biological Conservation 96:1–11.

Fujimura, K., N. Slusher, M. Cabana, and S. Lynch. 2010. Role of the gut microbiota in defining human health. Expert Review of Anti-infective Therapy 8:435–454.

Gagliardo, R., P. Crump, E. Griffith, J. Mendelson, H. Ross, and K. Zippel. 2008. The principles of rapid response for amphibian conservation, using the programmes in Panama as an example. International Zoo Yearbook 42:125–135.

Georoff, T. A., R. P. Moore, C. Rodriguez, A. P. Pessier, A. L. Newton, D. McAloose, and P. P. Calle. 2013. Efficacy of treatment and long-term follow-up of *Batrachochytrium dendrobatidis* PCR-positive anurans following itraconazole bath treatment. Journal of Zoo and Wildlife Medicine 44:395–403.

Gerçe, B., T. Schwartz, M. Voigt, S. Rühle, S. Kirchen, A. Putz, P. Proksch, U. Obst, C. Syldatk, and R. Hausmann. 2009. Morphological, bacterial, and secondary metabolite changes of *Aplysina aerophoba* upon long-term maintenance under artificial conditions. Microbial Ecology 58:865–878.

Gray, M. A., Z. A. Pratte, and C. A. Kellogg. 2013. Comparison of DNA preservation methods for environmental bacterial community samples. FEMS Microbiology Ecology 83:468–477.

Harris, R. N., R. M. Brucker, J. B. Walke, M. H. Becker, C. R. Schwantes, D. C. Flaherty, B. A. Lam, D. C. Woodhams, C. J. Briggs, V. T. Vredenburg, and K. P. C. Minbiole. 2009. Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. ISME Journal 3:818–824.

Harris, R. N., T. Y. James, A. Lauer, M. A. Simon, and A. Patel. 2006. Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. EcoHealth 3:53–56.

Heard, M. J., K. F. Smith, K. J. Ripp, M. Berger, J. Chen, J. Dittmeier, M. Goter, S. T. McGarvey, and E. Ryan. 2013. The threat of disease increases as species move toward extinction. Conservation Biology 27:1378–1388.

Hou, S., K. S. Makarova, J. H. W. Saw, P. Senin, B. V Ly, Z. Zhou, Y. Ren, J. Wang, M. Y. Galperin, M. V Omelchenko, Y. I. Wolf, N. Yutin, E. V Koonin, M. B. Stott, B. W. Mountain, M. A. Crowe, A. V Smirnova, P. F. Dunfield, L. Feng, L. Wang, and M. Alam. 2008. Complete genome sequence of the extremely acidophilic methanotroph isolate V4, *Methylacidiphilum infernorum*, a representative of the bacterial phylum Verrucomicrobia. Biology Direct 3.

Hyatt, A. D., D. G. Boyle, V. Olsen, D. B. Boyle, L. Berger, D. Obendorf, A. Dalton, K. Kriger, M. Heros, H. Hines, R. Phillott, R. Campbell, G. Marantelli, F. Gleason, and A. Coiling. 2007. Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. Diseases of Aquatic Organisms 73:175–192.

Isaacs, L. T., J. Kan, L. Nguyen, P. Videau, M. A. Anderson, T. L. Wright, and R. T. Hill. 2009. Comparison of the bacterial communities of wild and captive sponge *Clathria prolifera* from the Chesapeake Bay. Marine Biotechnology 11:758–770.

Kueneman, J. G., L. Wegener Parfrey, D. C. Woodhams, H. M. Archer, R. Knight, and V. J. McKenzie. 2013. The amphibian skin-associated microbiome across species, space and life history stages. Molecular Ecology:1238–1250.

Lam, B. A., J. B. Walke, V. T. Vredenburg, and R. N. Harris. 2010. Proportion of individuals with anti-*Batrachochytrium dendrobatidis* skin bacteria is associated with population persistence in the frog *Rana muscosa*. Biological Conservation 143:529–531.

Lauber, C. L., N. Zhou, J. I. Gordon, R. Knight, and N. Fierer. 2010. Effect of storage conditions on the assessment of bacterial community structure in soil and human-associated samples. FEMS Microbiology Letters 307:80–86.

Lauer, A., M. A. Simon, J. L. Banning, B. A. Lam, and R. N. Harris. 2008. Diversity of cutaneous bacteria with antifungal activity isolated from female four-toed salamanders. ISME Journal 2:145–157.

Letunic, I., and P. Bork. 2007. Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. Bioinformatics 23:127–128.

Lips, K. R., F. Brem, R. Brenes, J. D. Reeve, R. A. Alford, J. Voyles, C. Carey, L. Livo, A. P. Pessier, and J. P. Collins. 2006. Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. Proceedings of the National Academy of Sciences 103:3165–3170.

Lips, K., F. Solís, R. Ibáñez, C. Jaramillo, and Q. Fuenmayor. 2010. *Atelopus zeteki*. The IUCN red list of threatened speices. Version 2013.2. www.iucnredlist.org

Liukkonen-Anttila, T., R. Saartoala, and R. Hissa. 2000. Impact of hand-rearing on morphology and physiology of the capercaillie (*Tetrao urogallus*). Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 125:211–221.

Loudon, A. H., D. C. Woodhams, L. W. Parfrey, H. Archer, R. Knight, V. McKenzie, and R. N. Harris. 2014. Microbial community dynamics and effect of environmental microbial reservoirs on red-backed salamanders (*Plethodon cinereus*). ISME Journal 8:830–40.

Lozupone, C. A., J. I. Stombaugh, J. I. Gordon, J. K. Jansson, and R. Knight. 2012. Diversity, stability and resilience of the human gut microbiota. Nature 489:220–230.

La Marca, E., K. R. Lips, S. Lotters, R. Puschendorf, R. Ibanez, J. V. Rueda-Almonacid, R. Schulte, C. Marty, F. Castro, J. Manzanilla-Puppo, J. E. Garcia-Perez, F. Bolanos, G. Chaves, J. A. Pounds, E. Toral, and B. E. Young. 2005. Catastrophic population declines and extinctions in Neotropical harlequin frogs (Bufonidae: *Atelopus*). Biotropica 37:190–201.

McBride, M. E., W. C. Duncan, and J. M. Knox. 1977. The environment and the microbial ecology of human skin. Applied and Environmental Microbiology 33:603–608.

McKenzie, V. J., R. M. Bowers, N. Fierer, R. Knight, and C. L. Lauber. 2012. Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. ISME Journal 6:588–596.

Meron, D., E. Atias, L. Iasur Kruh, H. Elifantz, D. Minz, M. Fine, and E. Banin. 2011. The impact of reduced pH on the microbial community of the coral *Acropora eurystoma*. ISME journal 5:51–60.

Mohamed, N. M., V. Rao, M. T. Hamann, M. Kelly, and R. T. Hill. 2008. Monitoring bacterial diversity of the marine sponge *Ircinia strobilina* upon transfer into aquaculture. Applied and Environmental Microbiology 74:4133–4143.

Nakamura, N., K. R. Amato, P. Garber, A. Estrada, R. I. Mackie, and H. R. Gaskins. 2011. Analysis of the hydrogenotrophic microbiota of wild and captive black howler monkeys (*Alouatta pigra*) in Palenque National Park, Mexico. American Journal of Primatology 73:909–919.

Nelson, T. M., T. L. Rogers, A. R. Carlini, and M. V Brown. 2013. Diet and phylogeny shape the gut microbiota of Antarctic seals: a comparison of wild and captive animals. Environmental Microbiology 15:1132–1145.

Poole, V. 2008. Project golden frog. Endangered Species Bulletin 33:7–10.

Price, M. N., P. S. Dehal, and A. P. Arkin. 2009. FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. Molecular Biology and Evolution 26:1641–1650.

Redford, K. H., J. A. Segre, N. Salafsky, C. Martinez del Rio, and D. McAloose. 2012. Conservation and the microbiome. Conservation Biology 26:195–197.

Richards-Zawacki, C. L. 2010. Thermoregulatory behaviour affects prevalence of chytrid fungal infection in a wild population of Panamanian golden frogs. Proceedings of the Royal Society B 277:519–528.

Rosenberg, E., O. Koren, L. Reshef, R. Efrony, and I. Zilber-Rosenberg. 2007. The role of microorganisms in coral health, disease and evolution. Nature Reviews Microbiology 5:355–362.

Schommer, N. N., and R. L. Gallo. 2013. Structure and function of the human skin microbiome. Trends in Microbiology 21:660–668.

Schwab, C., B. Cristescu, J. M. Northrup, G. B. Stenhouse, and M. Gänzle. 2011. Diet and environment shape fecal bacterial microbiota composition and enteric pathogen load of grizzly bears. PLoS One 6:e27905.

Seiler, C., P. Angelstam, and H. H. Bergmann. 2000. Conservation releases of captive-reared grouse in Europe. What do we know and what do we need? Cahiers d'Ethologie 20:235–252.

Spor, A., O. Koren, and R. Ley. 2011. Unravelling the effects of the environment and host genotype on the gut microbiome. Nature Reviews Microbiology 9:279–290.

Stuart, S. N., J. S. Chanson, N. A. Cox, B. E. Young, A. S. L. Rodrigues, D. L. Fischman, and R. W. Waller. 2004. Status and trends of amphibian declines and extinctions worldwide. Science 306:1783–1786.

Wagner, M., and M. Horn. 2006. The Planctomycetes, Verrucomicrobia, Chlamydiae and sister phyla comprise a superphylum with biotechnological and medical relevance. Current Opinion in Biotechnology 17:241–249.

Wake, D. B., and V. T. Vredenburg. 2008. Are we in the midst of the sixth mass extinction? A view from the world of amphibians. Proceedings of the National Academy of Sciences 105:11466–11473.

Walke, J. B., R. N. Harris, L. K. Reinert, L. A. Rollins-Smith, and D. C. Woodhams. 2011. Social immunity in amphibians: evidence for vertical transmission of innate defenses. Biotropica 43:396–400.

Webster, N. S., R. E. Cobb, and A. P. Negri. 2008. Temperature thresholds for bacterial symbiosis with a sponge. ISME journal 2:830–842.

Webster, N. S., R. E. Cobb, R. Soo, S. L. Anthony, C. N. Battershill, S. Whalan, and E. Evans-Illidge. 2011. Bacterial community dynamics in the marine sponge Rhopaloeides odorabile under in situ and ex situ cultivation. Marine Biotechnology 13:296–304.

Wienemann, T., D. Schmitt-Wagner, K. Meuser, G. Segelbacher, B. Schink, A. Brune, and P. Berthold. 2011. The bacterial microbiota in the ceca of Capercaillie (*Tetrao urogallus*) differs between wild and captive birds. Systematic and Applied Microbiology 34:542–551.

Wolf, C. M., B. Griffith, C. Reed, and S. A. Temple. 1996. Avian and mammalian translocations: update and reanalysis of 1987 survey data. Conservation Biology 10:1142–1154.

Woodhams, D. C., V. L. Kilburn, L. K. Reinert, J. Voyles, D. Medina, R. Ibáñez, A. D. Hyatt, D. G. Boyle, J. D. Pask, D. M. Green, and L. A. Rollins-Smith. 2008. Chytridiomycosis and amphibian population declines continue to spread eastward in Panama. EcoHealth 5:268–274.

Woodworth, L. M., M. E. Montgomery, D. A. Briscoe, and R. Frankham. 2002. Rapid genetic deterioration in captive populations: causes and conservation implications. Conservation Genetics 3:277–288.

Xenoulis, P. G., P. L. Gray, D. Brightsmith, B. Palculict, S. Hoppes, J. M. Steiner, I. Tizard, and J. S. Suchodolski. 2010. Molecular characterization of the cloacal microbiota of wild and captive parrots. Veterinary Microbiology 146:320–325.

FIGURES

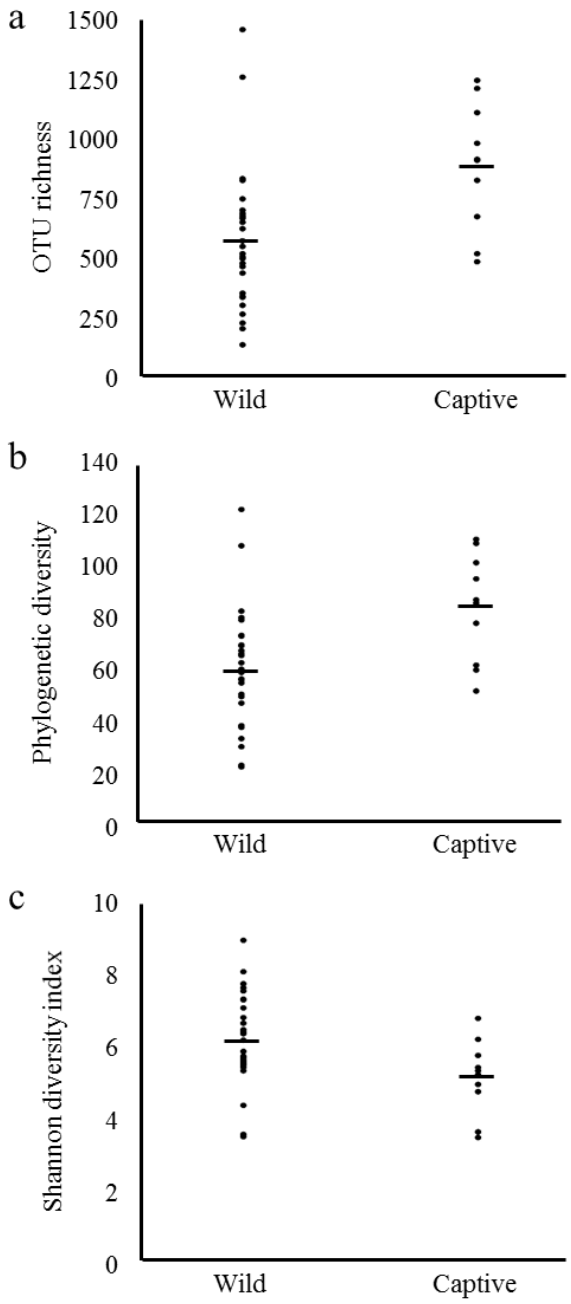

Figure 1. Alpha diversity (within-sample diversity) of skin-associated microbial communities present on captive and wild *Atelopus zeteki*. (A) The number of unique operational taxonomic units (OTUs) at a 97% sequence similarity in each community. (B) Phylogenetic diversity is a measure of the total branch length of a phylogenetic tree covered by a community. (C) Shannon diversity index is a measure of richness and evenness of OTUs in a community. Each point represents a community on an individual frog. Horizontal lines represent sample means.

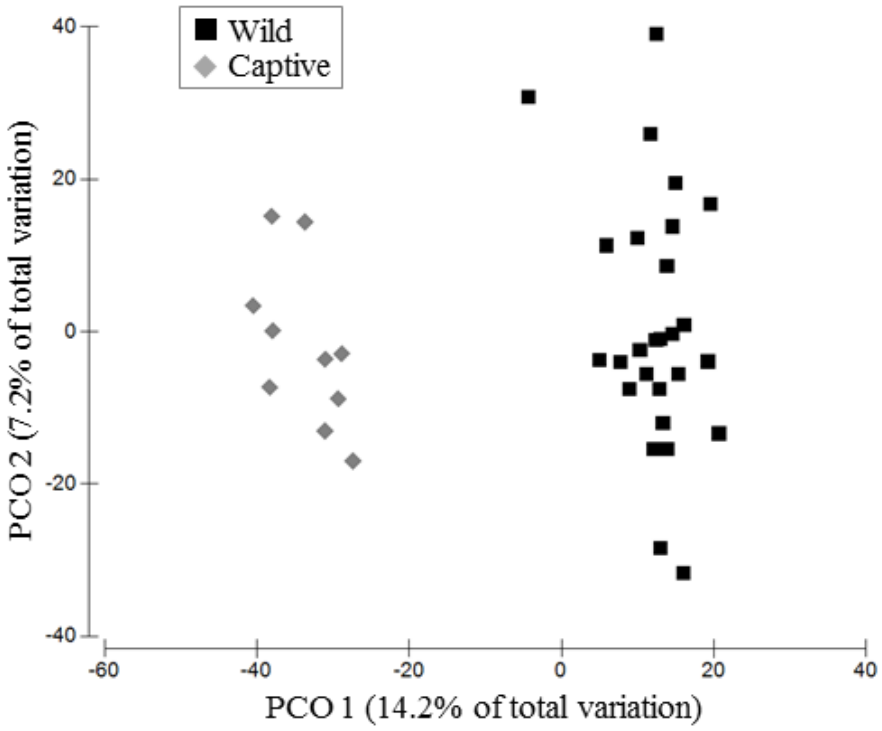

Figure 2. Principal coordinate plot of Bray-Curtis distances between microbial communities present on wild and captive *Atelopus zeteki*. Each point represents a microbial community of an individual frog.

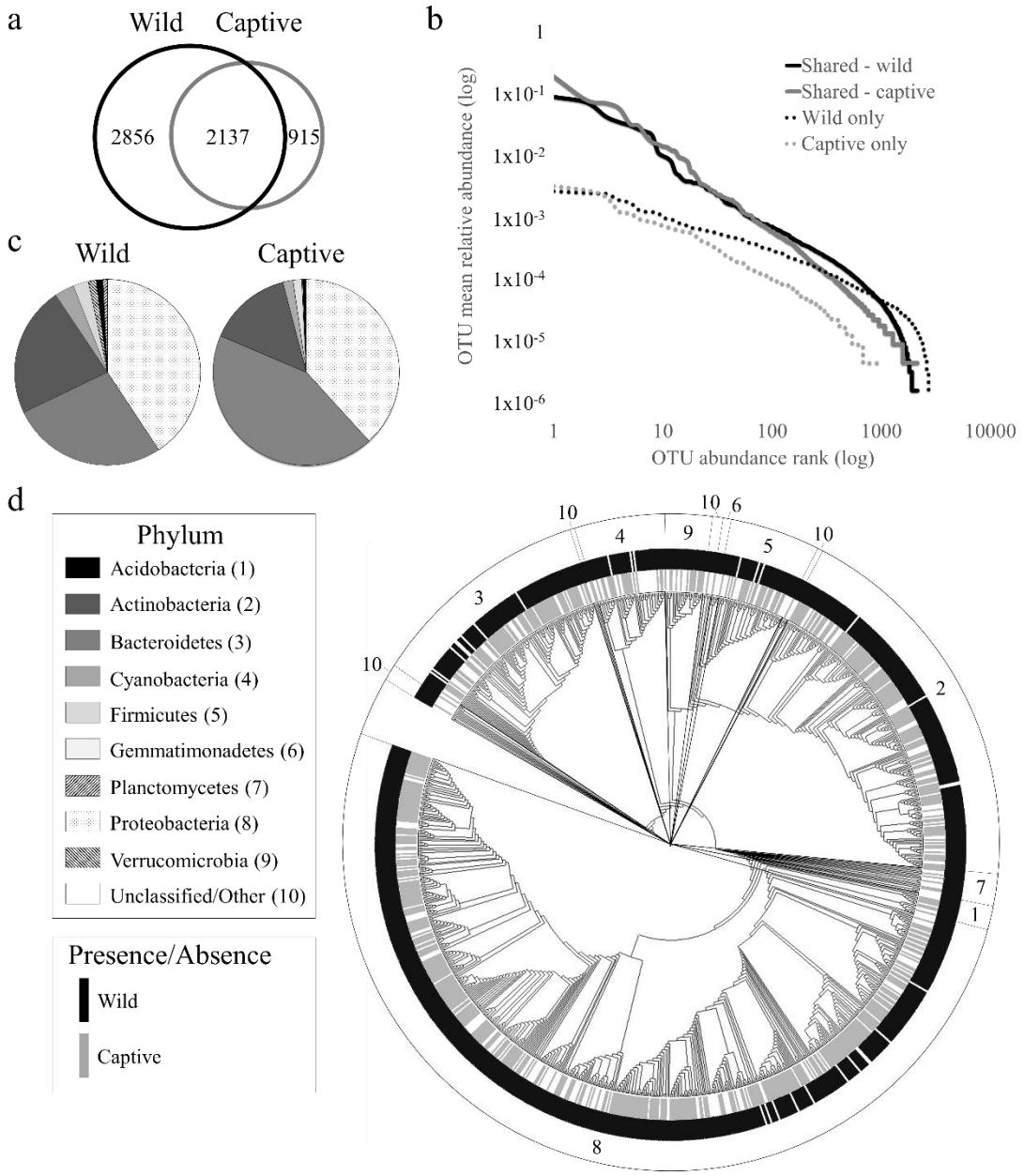

Figure 3. Comparison of shared and unshared operational taxonomic units (OTUs) as well as the taxonomic diversity of the microbial community present on wild and captive *Atelopus zeteki*. (a) Venn diagram displaying the distribution of shared and unshared OTUs present on wild and captive populations of *A. zeteki*. (b) Rank abundance curves of the OTUs in each section of the Venn diagram. (c) Pie charts displaying the mean relative abundance of phyla from each frog population that had a relative abundance greater than 0.05% on either wild or captive *A. zeteki*. (d) Phylogenetic tree constructed from OTUs that had a mean relative abundance greater than 0.01% on wild and captive *A. zeteki*. The tree is divided by bacterial phyla and the numbers on the outermost ring refer to the specific phyla in the corresponding section. The gray and black inner rings refer to the presence of individual OTUs in captive and wild *A. zeteki*, respectively.

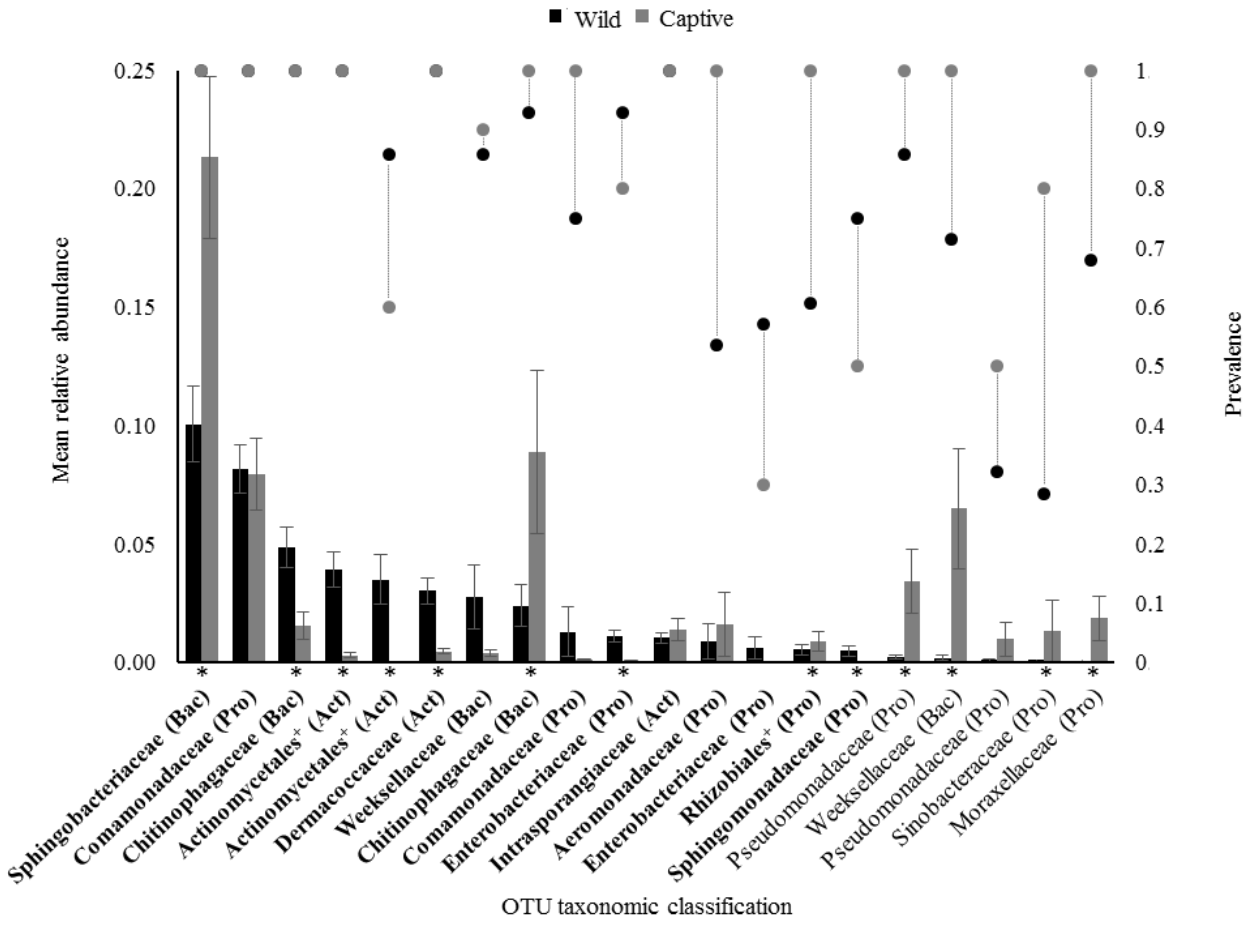

Figure 4. Mean relative abundance (bars) and prevalence (closed circles) of dominant bacteria present on wild and captive *Atelopus zeteki*. Only operational taxonomic units (OTUs) with a mean relative abundance greater than 0.5% in either population are shown (bolded OTU labels represent dominant OTUs of wild frogs). The family (or order when denoted by an addition sign) and phylum (in parentheses) for each OTU are shown on the x-axis. Error bars represent standard error. Wilcoxon rank-sum test FDR-corrected $p < 0.05$ denoted by star. Bac = Bacteroidetes; Pro = Proteobacteria; Act = Actinobacteria.

APPENDIX

16s rRNA gene data processing

Overlapping forward and reverse paired reads were assembled with Fastq-join (https://code.google.com/p/ea-utils/wiki/FastqJoin). The assembled sequences were then processed with the Quantitative Insights Into Microbial Ecology (MacQIIME v. 1.7.0) pipeline (Caporaso et al. 2010b). Sequences were de-multiplexed and quality-filtered following methods similar to those described by Bokulich et al. (2013). Specifically, sequences were discarded if there were any ambiguous base calls, errors in the barcode, less than 75% of read length had consecutive base calls with a phred quality score greater than 20, more than 10 consecutive low-quality base calls, or the read length was not between 252 and 255 bp. After quality filtering, the number of reads retained per sample ranged from 28 907 to 382 901. Quality-filtered sequences were then clustered into OTUs (operational taxonomic units) at a sequence similarity threshold of 97% with the UCLUST method (Edgar 2010) and a minimum cluster size of 0.001% of the total reads (Bokulich et al. 2013). Sequences were first clustered against the Greengenes database (May 2013 release, DeSantis et al. 2006). Sequences that did not match the database were then *de novo* clustered at a 97% sequence similarity threshold. The most abundant sequence for a given cluster was assigned as the representative sequence for that OTU. Taxonomy was assigned for each OTU with RDP classifier (Wang et al. 2007) at a 50% confidence threshold and the Greengenes database, as recommended by Claesson et al. (2009) for the v4 region of the 16s rRNA gene. Representative sequences were aligned to the Greengenes database with PyNAST (Caporaso et al. 2010a) and a phylogenetic tree was constructed with FastTree (Price et al. 2009). All samples were rarefied to 19 500 sequences to standardize sampling effort.

Table A1. List of core operational taxonomic units (OTUs) (≥ 90% prevalence) present on wild *Atelopus zeteki*.

OTU taxonomic classification[a]	Prevalence (%)		Mean relative abundance (%)	
	Wild	Captive	Wild	Captive
Chitinophagaceae (Bac[b])	100	100	4.8	1.5
Actinomycetales (Act[c])	100	100	3.9	0.3
Dermacoccus (Act)	100	100	3.0	0.5
Intrasporangiaceae (Act)	100	100	1.0	1.4
Hylemonella (Pro[d])	100	100	8.2	7.9
Comamonadaceae (Pro)	100	100	0.1	0.2
Pedobacter (Bac)	100	100	10.1	21.3
Hylemonella (Pro)	96	100	0.1	0.1
Chitinophagaceae (Bac)	93	100	2.4	8.9
Comamonadaceae (Pro)	93	100	0.1	0.1
Enterobacteriaceae (Pro)	93	80	1.1	0.05

[a]Lowest taxonomic resolution that could be defined is shown.
[b]Bacteroidetes
[c]Actinobacteria
[d]Proteobacteria

Table A2. Distribution of prevalence for all operational taxonomic units (OTUs) associated with wild *Atelopus zeteki*.

Prevalence of OTUs on wild frogs (%)	**Total # of OTUs**	**# of OTUs absent from captive population**	**Mean prevalence of captive OTUs (min-max) (%)**
90 – 100	11	0	98 (80-100)
80 – 90	12	0	83 (40-100)
70 – 80	13	0	71 (10-100)
60 – 70	30	1	64 (0 – 100)
50 – 60	56	4	48 (0 – 100)
40 – 50	65	6	39 (0 – 100)
30 – 40	159	41	31 (0 – 100)
20 – 30	401	141	22 (0 – 100)
10 – 20	1196	598	13 (0 – 100)
0 – 10	3050	2065	8 (0 – 100)

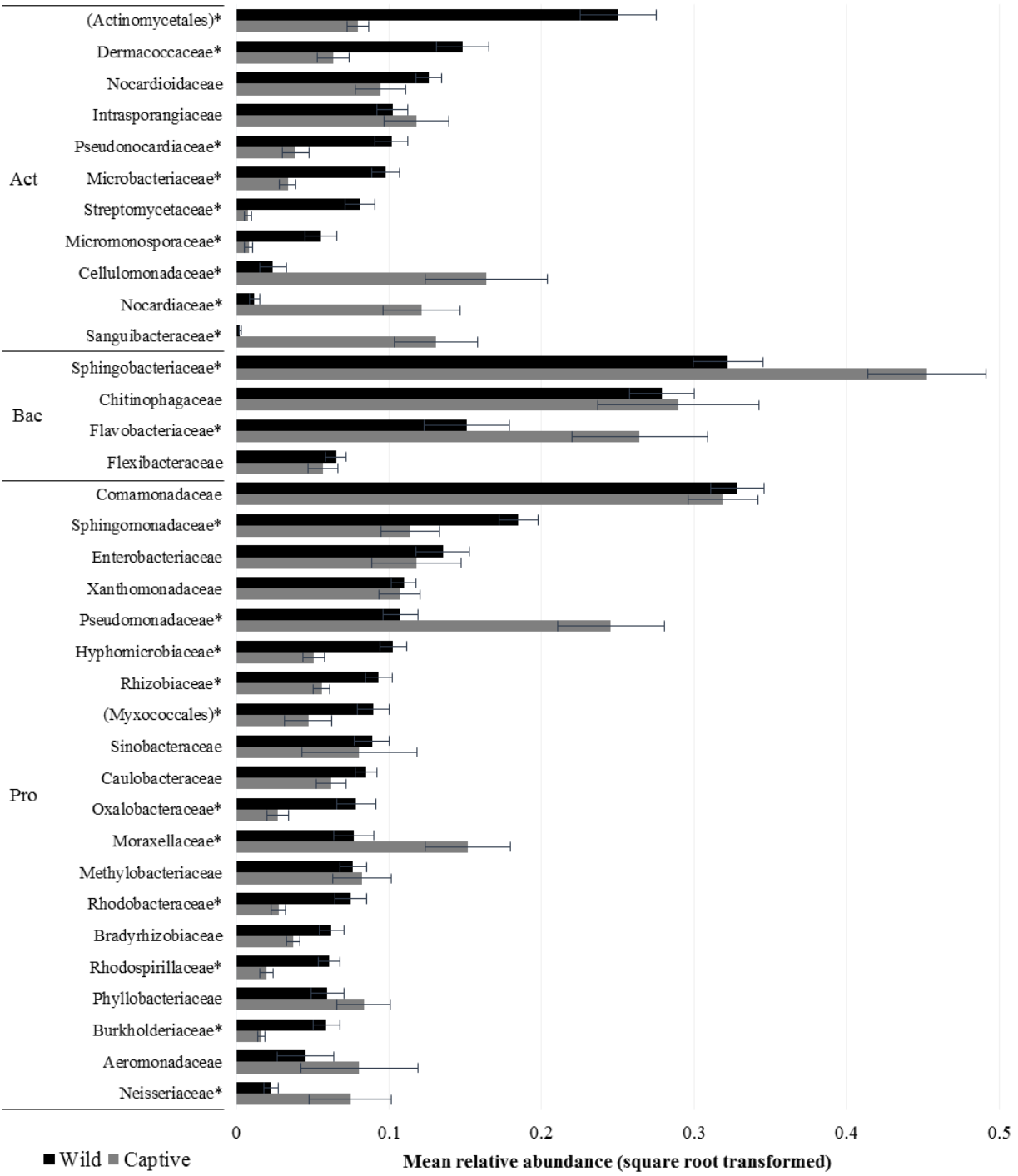

Figure A1. Characterization of the microbial communities of wild and captive populations of *Atelopus zeteki* at the family level. Only taxa with a mean relative abundance greater than 0.05% from either population are shown. Mean relative abundance was square-root transformed to better visualize taxa with low relative abundances. Error bars represent standard error. Taxa in parentheses are orders. Wilcoxon rank-sum test FDR-corrected $p < 0.05$ denoted by a star. Act = Actinobacteria; Bac = Bacteroidetes; Pro = Proteobacteria.

Literature cited

Bokulich, N. A., S. Subramanian, J. J. Faith, D. Gevers, J. I. Gordon, R. Knight, D. A. Mills, and J. G. Caporaso. 2013. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. Nature Methods 10:57–59.

Caporaso, J. G., K. Bittinger, F. D. Bushman, T. Z. DeSantis, G. L. Andersen, and R. Knight. 2010a. PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics 26:266–267.

Caporaso, J. G., J. Kuczynski, J. Stombaugh, K. Bittinger, F. D. Bushman, E. K. Costello, N. Fierer, A. G. Peña, J. K. Goodrich, J. I. Gordon, G. A. Huttley, S. T. Kelley, D. Knights, J. E. Koenig, R. E. Ley, C. A. Lozupone, D. McDonald, B. D. Muegge, M. Pirrung, J. Reeder, J. R. Sevinsky, P. J. Turnbaugh, W. A. Walters, J. Widmann, T. Yatsunenko, J. Zaneveld, and R. Knight. 2010b. QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7:335–336.

Claesson, M. J., O. O'Sullivan, Q. Wang, J. Nikkilä, J. R. Marchesi, H. Smidt, W. M. de Vos, R. P. Ross, and P. W. O'Toole. 2009. Comparative analysis of pyrosequencing and a phylogenetic microarray for exploring microbial community structures in the human distal intestine. PLoS One 4:e6669.

DeSantis, T. Z., P. Hugenholtz, N. Larsen, M. Rojas, E. L. Brodie, K. Keller, T. Huber, D. Dalevi, P. Hu, and G. L. Andersen. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Applied and Environmental Microbiology 72:5069–5072.

Edgar, R. C. 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26:2460–2461.

Price, M. N., P. S. Dehal, and A. P. Arkin. 2009. FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. Molecular Biology and Evolution 26:1641–1650.

Wang, Q., G. M. Garrity, J. M. Tiedje, and J. R. Cole. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Applied and Environmental Microbiology 73:5261–5267.

CHAPTER III. THE PHYLOGENETIC DISTRIBUTION OF THE CAPACITY OF SYMBIOTIC BACTERIA FROM PANAMANIAN AMPHIBIANS TO INHIBIT THE GROWTH OF *BATRACHOCHYTRIUM DENDROBATIDIS*

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ABSTRACT

The introduction of next-generation sequencing has allowed for greater understanding of community composition of symbiotic microbial communities. However, determining the function of individual members of these microbial communities still largely relies on culture-based methods. Here, we present results on the phylogenetic distribution of a defensive functional trait of cultured symbiotic bacteria associated with amphibians. Amphibians are host to a diverse community of cutaneous bacteria and some of these bacteria protect their host from the lethal fungal pathogen *Batrachochytrium dendrobatidis* (Bd) by secreting antifungal metabolites. We cultured over 450 bacterial isolates from the skins of Panamanian amphibian species and tested their interaction with Bd with an *in vitro* challenge assay. For a subset of isolates, we also completed co-culture experiments and found that culturing isolates with Bd had no effect on inhibitory properties of the bacteria, but it significantly decreased metabolite secretion. In challenge assays, approximately 75% of the bacterial isolates inhibited Bd to some extent and these inhibitory isolates were widely distributed among all bacterial phyla. Two genera, *Pseudomonas* and *Stenotrophomonas*, had the highest proportion of inhibitory isolates (73% and 100%, respectively). However, mean inhibition of isolates was not correlated with 16S sequence similarity at the genus and strain-level (≥ 99.5% sequence similarity), and there was not a clear phylogenetic signal of inhibition. Overall, our results demonstrate that antifungal properties are phylogenetically widespread in symbiotic microbial communities of Panamanian

amphibians, functional redundancy occurs in these communities, and Bd inhibition by isolates cannot be inferred simply based on 16S gene sequences.

INTRODUCTION

Determining the phylogenetic distribution of functional traits is critical to understanding how community composition affects ecosystem function (McGill et al. 2006, Devictor et al. 2010). In microbial ecology, determining the composition of a community has been made easier with the introduction of next-generation sequencing techniques (Fierer et al. 2008, Caporaso et al. 2010c). However, determining how community composition and perturbations to communities affect function is much more difficult (Philippot et al. 2010). The first step to understanding this relationship is to link specific functional traits to specific taxa (Martiny et al. 2013, Zimmerman et al. 2013). This can be very difficult since genetic diversity is so high among microbial taxa, even at the species level (Philippot et al. 2010). In addition, bacteria have the ability to take up DNA from the environment and transfer functional genes among even distantly related taxa (lateral gene transfer, Doolittle et al. 1990, Mell & Redfield 2014). Recent evidence suggests that the phylogenetic distribution of functional traits in bacteria is based on the complexity and number of genes involved for a specific function (Martiny et al. 2013). For example, simple functions involving few genes, such as the ability to utilize simple carbon sources, are found throughout bacterial taxa (Martiny et al. 2013). In contrast, very complex functions, such as photosynthesis, are non-randomly distributed (Martiny et al. 2013). Pathogen inhibition is a functional trait of some symbiotic microorganisms and understanding the phylogenetic distribution of traits related to this function is important since many microbes are vital to the health of multicellular organisms including humans, plants, and wildlife (Berg 2009, Fujimura et al. 2010, Krediet et al. 2013, Bletz et al. 2013).

Although research regarding symbiotic microbes has focused mainly on the phylogenetic distribution of virulence (Picard et al. 1999, Jarraud et al. 2002, Johnson et al. 2002, Hwang et al. 2005, Fisher et al. 2009), the few studies investigating the distribution of functions related to pathogen inhibition among symbiotic microorganisms focus on microbial communities

associated with plants (Keel et al. 1996, Ramette et al. 2003, Bakker et al. 2010). Here, we present a unique system to study the relationship between phylogeny and the ability of symbiotic microorganisms to inhibit the growth of a pathogen. Amphibians are host to a diverse array of symbiotic bacteria on their skin (e.g., McKenzie et al. 2012; Kueneman et al. 2013; Walke et al. 2014), some of which have the functional ability to inhibit the cutaneous fungal pathogen *Batrachochytrium dendrobatidis* (Bd, Harris et al. 2006, Lauer et al. 2008, Woodhams et al. 2014). This pathogen is widespread and has been linked to the decline and extinction of many amphibian species (Stuart et al. 2004, Skerratt et al. 2007). However, some amphibian populations that are not susceptible to chytridiomycosis (the disease caused by Bd) possess bacteria on their skin that inhibit Bd by secreting antifungal metabolites (Brucker et al. 2008a, 2008b, Becker et al. 2009, Becker and Harris 2010, Lam et al. 2010). For example, the bacterial species *Janthinobacterium lividum* has been isolated from multiple non-susceptible amphibian species and inhibits Bd by secreting the metabolites indole-3-carboxaldehyde and violacein (Brucker et al. 2008b). Additionally, the bacterial species *Lysobacter gummosus* has been isolated from the redback salamander (*Plethodon cinereus*) and inhibits Bd by secreting 2,4-diacetylphloroglucinol (Brucker et al. 2008a). However, not all amphibian cutaneous bacteria that have been tested inhibit Bd and a few actually seem to facilitate Bd growth, although the mechanism underlying this facilitation is not clear (Bell et al. 2013, Woodhams et al. 2014).

Bacterial isolates have been cultured from amphibians in the United States, Switzerland, Australia, and Colombia to assess how these microbes might influence potential resistance to Bd infection and ultimately, disease outcome for amphibian hosts (Harris et al. 2006, Lam et al. 2010, Flechas et al. 2012, Bell et al. 2013, Roth et al. 2013, Woodhams et al. 2014). Research is focusing on developing some of these isolates into probiotic therapies to prevent chytridiomycosis in susceptible amphibians (Bletz et al. 2013). To date, probiotics have been successfully used to prevent chytridiomycosis in a few North American species and one European frog (Becker et al. 2009, Harris et al. 2009a, 2009b, Woodhams et al. 2014). Success with probiotics largely depends on finding a culturable microbe that can grow on the target host, inhibit Bd under ecologically relevant conditions, and be found on native amphibians or in the local environment (Becker et al. 2012, Bletz et al. 2013). Hence, there is a need for inhibitory bacterial isolates from areas in Central America, including Panama, where amphibian

assemblages have been devastated by the spread of Bd (Lips et al. 2006, Cheng et al. 2011). Unfortunately, there are no published accounts of the ability of symbiotic bacteria of Panamanian amphibian species to inhibit Bd.

In this study, we isolated over 450 bacterial isolates collected from the skins of multiple Panamanian amphibian species and characterized their interaction with Bd using an *in vitro* assay. Our goals were to determine how widespread Bd inhibition is among microbial taxa cultured from tropical amphibians, whether the 16S rRNA gene sequence could provide a reliable estimate of Bd inhibitory ability, and to further aid in the discovery of potential probiotics for Panamanian amphibians susceptible to chytridiomycosis. In addition, we aimed to determine whether the antifungal activity of the bacterial isolates depends on the presence of Bd.

METHODS

Study species and field sites

We isolated cutaneous bacteria from 11 Panamanian frog species (n = 67 individuals) from January to April 2010 (Table 1). We opportunistically sampled adult frogs at five sites: Cerro Bruja (9°28'57.3"N 79°33'54.4"W), El Copé (8˚37’30.4”N 80˚35’05.8”W), Sierra Llorona (9°20'42.0"N 79°48'19.4"W), Soberanía (9°07'00.3"N 79°42'00.0"W), and Torti (8°54'56.4"N 78°23'34.5"W). The coordinates listed are of the nearest city center or geographical landmark and not the exact location of the site because many of the species sampled are threatened with extinction. We captured frogs by hand using sterile nitrile gloves, used sterile swabs to non-lethally sample their skin microbiota, and released them within 24 hours at their point of capture. New gloves were used to capture and sample each frog.

Bacterial isolation and identification

Samples were collected by rinsing each frog twice with 50 mL of sterile water and swabbing back and forth five times on the surfaces of the venter, dorsum, thighs, and hind feet using a sterile rayon swab (MW113, Medical Wire & Equipment Co., Corsham, Wiltshire, UK). Immediately afterward, each swab was streaked onto low nutrient R2A media (Becton,

Dickinson and Co., Sparks, MD). Cultures were incubated at ambient temperature for approximately 48 to 72 h. For each frog, we isolated a pure culture of each morphotype based on colony shape, elevation, margin, surface, and color. To identify each isolate, we extracted the DNA from a pure culture of each isolate with PrepMan Ultra (Applied Biosystems, Carlsbad, California) following the manufacturer's protocol. We amplified bacterial DNA with the primers 8F and 1492R following the methods outlined by Lauer et al. (2008). PCR amplicons were checked by electrophoresis and sequenced with Sanger sequencing at the University of Kentucky Advanced Genetic Technologies Center.

We obtained a consensus sequence for each isolate by aligning the sequences of the forward and reverse amplicons. Taxonomy was assigned to each isolate with the UCLUST consensus taxonomy assigner and the Greengenes 13_8 reference database using QIIME version 1.8.0 (Caporaso et al. 2010b). There were 137 isolates that could not be classified at the genus level with the UCLUST taxonomy assigner. The sequences of these isolates were blasted against the National Center for Biotechnology Information (NCBI) 16S rRNA (Bacteria and Archaea) database (Zhang et al. 2000). With this method, 64 of the 137 unclassified sequences were identified to the genus level. A majority of the remaining unclassified sequences (69/73) were within the family Enterobacteriaceae. Studies have shown that the 16S rRNA gene does not provide enough sequence variation to distinguish some bacterial taxa, including Enterobacteriaceae, at lower taxonomic levels (Mollet et al. 1997, Fukushima et al. 2002). Therefore, the family level was used for all analyses for unclassified Enterobacteriaceae isolates. Sequences from bacterial isolates (≥ 1000 bp) were aligned with PyNAST in QIIME. A 16S gene tree was built based on the neighbor-joining tree build method, the Jukes-Cantor genetic distance model, and a bootstrap analysis (1000 replicates) in Geneious version 6.1.8. The resulting tree was visualized using the Interactive Tree of Life (Letunic and Bork 2007).

Bd challenge assay

We characterized each isolate's interaction with Bd (Panamanian strain JEL 310) in a 96-well challenge assay following methods similar to those outlined by Bell et al. (2013). Each assay was prepared by co-culturing a 1 mL suspension of Bd and a bacterial isolate for approximately 72 h at room temperature (~23 C) on a shaker at 100 RPM. Co-cultures were

made by adding 100 µL of Bd suspended in 1% tryptone broth (taken from a culture that had been incubating for 48 h), 100 µL of a bacterial isolate suspended in 1% trypticase soy yeast extract broth with 20% glycerol (taken from a frozen stock), and 800 µL of 1% tryptone broth. After incubation, cultures were in late log to early stationary phase (based on turbidity). At this time, we centrifuged each co-culture for 5 min at 10,000 RPM and filtered the supernatant through a 0.22 µm filter to obtain the cell-free supernatant (CFS). We assayed the CFS of each isolate with Bd by adding 50 µl of CFS and 50 µL of a Bd suspension ($2x10^6$ zoospore per mL) in each well of a sterile 96-well flat bottom plate (USA Scientific, Ocala, FL). To control for potential variation in Bd zoospore concentrations in the inoculum, we included a positive control on each plate, which contained 50 µL of the Bd zoospore suspension and 50 µL of Bd CFS. Negative controls contained 50 µL of a heat-killed zoospore suspension and 50 µL of Bd CFS. The CFS of each isolate and control was assayed in triplicate. The Bd suspension used in the 96-well assay was prepared by growing a culture of Bd on a 1% tryptone agar plate for 4 d, flooding this plate with 1% tryptone broth, and filtering the suspension through a 20 µm filter. The optical density of each well was measured with a spectrophotometer at 492 nm immediately after the plate setup was complete and 4, 7, and 10 d thereafter. Plates were incubated at 23 C. Optical density readings were transformed using the equation Ln(OD/(1-OD)). Linear regression was used to determine the growth rate of Bd in the presence of CFS from each isolate. To calculate Bd inhibition, the slope of each triplicate was averaged, divided by the average growth rate of the positive control, and subtracted from one.

Co-culture experiment

To determine if co-culturing bacterial isolates with Bd affects bacterial metabolite production and Bd-inhibition, we designed a co-culture experiment with 13 *Acinetobacter* spp. isolates and 13 *Pseudomonas* spp. isolates. We hypothesized that the presence of a competitor (Bd) would stimulate the production and secretion of inhibitory compounds from the isolates. Isolates from these two genera were chosen because they were highly abundant in the sampled amphibian communities and they varied in mean Bd inhibition within each genera. Each of the 26 isolates was grown with and without Bd for 72 h prior to challenging the CFS against Bd in the assay described above. A positive and negative control without Bd CFS was included in the 96-well assay to account for any variation in inhibition due to the absence of Bd secreted

compounds. For seven of the *Acinetobacter* spp. isolates and two of the *Pseudomonas* spp. isolates grown with and without Bd, we assessed metabolite profiles using HPLC-MS following methods in Umile et al. (2014). We included a 1% tryptone-only control and a Bd CFS control to detect compounds within 1% tryptone and those produced by Bd alone, respectively. These compounds were removed from all analyses.

Statistical analysis

Unless noted, data were normally distributed and variances were equal among specific comparisons. To determine if the distribution of isolate mean inhibition was multimodal, we used Hartigan's dip test (Hartigan and Hartigan 1985) with the diptest package (Maechler 2013) in R version 3.0.3 (R Core Team 2014). To calculate the mean of each mode, we fitted the mean inhibition data to a continuous bimodal distribution with the continuous fit function in JMP Pro version 10.0.2 Mean inhibitions of bacterial isolates were not normally distributed and thus differences among dominant bacterial genera (genera with more than four isolates) were tested with a Wilcoxon rank-sum test. To determine if isolates with similar 16S sequences had similar mean Bd inhibition values, a Mantel test (Mantel 1967) was used with 10,000 permutations to compare distance matrices of percent sequence similarity and patristic distance (sum of branch lengths derived from the 16S gene tree) between isolates to a Euclidean distance matrix derived from differences in mean percent inhibition between isolates. Significant differences in mean inhibition of each isolate grown with and without Bd in the co-culture experiment were tested with a Mann-Whitney U test. All multiple comparisons were corrected with the false discovery rate procedure (FDR; Benjamini & Hochberg 1995). Differences in the number of metabolites produced by isolates grown with and without Bd were tested with a paired t-test. To investigate the relationship between number of metabolites produced and mean inhibition of isolates grown with and without Bd, linear regression was used. To compare the metabolite profiles of bacterial isolates grown with and without Bd, a Sørensen distance matrix (based on presence/absence of metabolites) was built and visualized with non-metric multidimensional scaling (NMDS) using the software package Primer 6 version 6.1.15.

RESULTS

We collected 484 bacterial isolates from the skins of the 67 Panamanian frogs (average of 7.2 isolates per frog; Table 1). A majority of the isolates (77.3%) were classified as belonging to the phylum Proteobacteria (Fig 1a). The remaining isolates belonged, in almost equal proportions, to the phyla Actinobacteria (8.3%), Bacteroidetes (7.2%), and Firmicutes (7.2%). Within the phylum Proteobacteria, the genera *Pseudomonas* and *Acinetobacter* were the most dominant (Fig 1b). The dominant genera within the phyla Actinobacteria, Bacteroidetes, and Firmicutes were *Microbacterium*, *Chryseobacterium*, and *Bacillus*, respectively (Fig. 1b).

Among all isolates, mean Bd inhibition ranged from -49.3% to 100% (Fig. 2a). The distribution of mean inhibition was bimodal (Hartigan's dip test, $P = 0.02$) the first mode had a mean of 17.1% and the second mode had a mean of 79.6% (Fig 2b). We considered isolates in the second (higher) mode to be inhibitory; this was 44.2% of the isolates. We considered isolates within the first mode that were significantly higher than the Bd control to be mildly inhibitory; this was 31% of the isolates. We considered isolates that did not significantly differ from the Bd control to have no interaction with Bd; this was 17.2% of the isolates. The final 7.6% of the isolates had an inhibition score that was significantly less than the Bd control, and their interaction with Bd was considered facilitative.

Inhibitory isolates were found in all four phyla documented in this study (Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria). In addition, inhibitory isolates were found across all but one of the 17 dominant genera (all except *Curtobacterium*; Figure 3). Percent inhibition of Bd differed significantly at the genus level (Figure 3; Kruskal-Wallis test, $P < 0.001$). That is, some genera were consistently more inhibitory than others. However, there was a considerable amount of variation in percent inhibition among the individual isolates within each genus. For example, within the genus *Pseudomonas*, isolates had a mean inhibition of 68.1% and a majority of isolates were considered inhibitory (72.8%: 67/92 isolates); however, inhibitions ranged from -36.1% to 100%. Only one genus, *Stenotrophomonas*, comprised isolates ($n = 11$) that were all inhibitory. Inhibitory isolates could be found throughout the phylogeny and percent inhibition varied greatly even among isolates with highly similar 16S sequences (Fig. 4). Mantel tests revealed that isolate relatedness estimated with 16S gene sequences did not correlate with mean

percent inhibition (16S sequence similarity and patritistic distance methods, both $P > 0.05$). In other words, there was no clear clustering of function in the 16S gene tree in terms of Bd inhibition. Additionally, even isolates with 16S sequences that were similar at the strain level (≥ 99.5% sequence similarity) were sometimes found to vary in their interaction with Bd (Fig. 4b).

In the co-culture experiment, growing *Acinetobacter* spp. and *Pseudomonas* spp. isolates with Bd prior to the challenge assay had no effect on Bd inhibition (Fig. 5a; Wilcoxon rank-sum test, FDR-corrected $P > 0.05$). However, co-culturing with Bd significantly decreased the number of metabolites secreted by bacterial isolates (Fig. 5b; Paired t-test, $P = 0.003$). An NMDS ordination of metabolite profiles secreted by each isolate revealed that there was more variation among the different isolates than between the same isolate cultured with and without Bd (Fig. 6).

DISCUSSION

We found that the majority of isolates tested, across four phyla, had some ability to inhibit Bd *in vitro*. This functional redundancy may be indicative of the key role of these skin symbiont communities in host disease resistance, especially in the face of fungal pathogens. However, we also found that phylogenetic relatedness, as determined by 16S sequences, did not correlate with mean Bd inhibition across our group of cultured isolates. This is exemplified at all taxonomic levels. For example, inhibitory isolates were found among all phyla documented in this study (Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria). Isolates belonging to a single genus had a large range of interactions with Bd from facilitating to completely inhibiting Bd growth, with the exception of the *Stenotrophomonas* spp. isolates that were all inhibitory. For instance, within the genus *Pseudomonas*, many isolates completely inhibited Bd growth while others facilitated Bd growth by up to 36% when compared to the Bd positive control. Interactions with Bd were even varied at the strain level (≥ 99.5% 16S sequence similarity). Very closely related strains of *Acinetobacter* ranged from facilitative to inhibitory.

This type of discrepancy between 16S rRNA gene similarity and function has been seen in other functional traits, such as carbon substrate utilization, extracellular enzyme production,

and antifungal activity against plant pathogens (Wolf et al. 2002, Martiny et al. 2013, Zimmerman et al. 2013). For example, a study investigating the antifungal activity of *Stenotrophomonas* spp. isolates cultured from the rhizosphere of several plant species found isolates had very different activity against plant pathogens despite very similar 16S rRNA sequences (Wolf et al. 2002). Additionally, the authors found that DNA-DNA hybridization analysis was a much better predictor of antifungal activity (Wolf et al. 2002). Another factor that could contribute to the functional variation seen, even among strains, is that for some families and genera, the 16S region is not adequate for delineating species and strain level differences (Mollet et al. 1997, Fukushima et al. 2002, Hilario et al. 2004), and that it does not capture the potential of lateral gene transfer of these key symbiont functional traits (Kinashi et al. 1987, Ravel et al. 2000). Lateral gene transfer is very common among a wide diversity of bacteria and often complicates the use of phylogenies, based on housekeeping genes, to predict function even for closely related microorganisms (Ochman et al. 2000). This is important given the increasing reliance on next-generation sequencing of short regions of the 16S rRNA gene to characterize the structure of symbiont communities. Predicting antifungal function for those complex communities based solely on 16S amplicon data will likely be difficult.

The wide phylogenetic distribution of anti-Bd activity among bacteria in this study was expected. Antifungal secondary metabolites can be produced by multiple metabolic pathways and by a wide diversity of bacterial taxa (Kim and Hwang 2007). The few metabolites that are reported to specifically inhibit Bd (2,4-diacetylphloroglucinol, indole- 3-carboxaldehyde and violacein) are produced by distantly related Proteobacteria (*Lysobacter gummosus* and *Janthinobacterium lividum*, Brucker et al. 2008a, 2008b). This wide phylogenetic distribution of antifungal activity may also be explained by the transfer of genes encoding antifungal compounds (Kinashi et al. 1987, Ravel et al. 2000). In addition, recent evidence demonstrates that functions involving few genes are widely distributed among bacterial phylogenies, as opposed to complex functions, such as photosynthesis (Martiny et al. 2013). Functional traits involving the secretion of antifungal metabolites likely require relatively few genes (e.g. violacein and 2,4-diacetylphloroglucinol, Sánchez et al. 2006, Moynihan et al. 2009). Additionally, strong selective pressures, such as competition with fungal species and convergent

evolution, may create wide phylogenetic distribution of a functional trait, as has been seen with the prevalence of antibiotic resistance among diverse microbial taxa (Martinez 2009).

All 17 dominant genera had at least one inhibitory isolate, except for *Curtobacterium*. The genera *Stenotrophomonas* and *Pseudomonas* had the greatest proportion of inhibitory isolates at 100% and 73%, respectively. These two genera are well known for producing antifungal metabolites that inhibit plant pathogens (Walsh et al. 2001, Hayward et al. 2010). Anti-Bd isolates belonging to these two genera have been isolated from a wide range of amphibian hosts (including both frogs and salamanders) and from a wide range of geographic locations, including three *Atelopus* species (family: Bufonidae) in Colombia (Flechas et al. 2012), *Rana muscosa* (family: Ranidae) in California, USA (Woodhams et al. 2007b), *Bufo boreas boreas* (family: Bufonidae) in Wyoming, USA (Park et al. 2014), *Hemidactylium scutatum* (family: Plethodontidae) in Virginia, USA (Harris et al. 2006), *Plethodon cinereus* (family: Plethodontidae) in Virginia, USA (Harris et al. 2006), *Litoria serrata* (family: Hylidae) in Queensland, Australia (Bell et al. 2013), and *Alytes obstetricans* (family: Alytidae) in Switzerland (Woodhams et al. 2014). This suggests that either these bacterial genera are ubiquitous and are adept at colonizing and growing on amphibian skin and/or these bacteria have a long co-evolutionary history with amphibians. Since most amphibians live in moist habitats that are also ideal for fungi, some of which are lethal pathogens, amphibian hosts with cutaneous antifungal bacteria may have been selected over evolutionary history (Green 1999, Densmore and Green 2007).

In the present study, 7.6% of the bacterial isolates facilitated the growth of Bd in our *in vitro* challenge assay. These results are consistent with other studies investigating interactions of amphibian cutaneous bacteria with Bd (Bell et al. 2013, Woodhams et al. 2014). Bell et al. (2013) observed that the number of Bd cells (zoosporangia and zoospores) increased in the presence of the CFS of facilitating bacteria. The facilitation of Bd growth may occur through the secretion of compounds by bacteria that act as nutrients for Bd. This same interaction has been observed between bacteria and mycorrhizal fungi that colonize the roots of plants (Artursson et al. 2006). If facilitative bacteria are dominant members of the cutaneous microbial community, then it may have negative consequences for the amphibian host by increasing Bd infection intensities. However, an *in vivo* experiment where facilitative bacteria are added to the skin of

Bd-infected amphibians, as has been done with inhibitory bacteria (Harris et al. 2009a, 2009b), would be necessary to determine if this facilitative interaction occurs on amphibian skin.

Co-culturing isolates with Bd prior to the challenge assay had no effect on the ability of bacteria to inhibit Bd. This was unexpected, as we hypothesized that the presence of a competitor (Bd) would stimulate the production and secretion of inhibitory compounds. This is based on evidence that demonstrates bacteria can sense other microorganisms via signaling molecules and receptors and can respond to changing biotic conditions by altering gene expression, resulting in secretion of antimicrobial compounds to inhibit competitors (Bassler 1999). Increased production of inhibitory compounds is commonly seen in bacteria when in the presence of competitors and predators (Jousset et al. 2010, Garbeva et al. 2011); in fact, Bd co-culture was utilized to enhance the production of violacein in the bacteria *J. lividum* (Brucker et al. 2008b). Similarly, antiprotozoal activity of the bacteria *Pseudomonas fluorescens* increases when presented with chemical cues of a predatory protozoan, *Acanthamoeba castellanii* (Jousset et al. 2010). Alternatively, co-culturing bacterial isolates with Bd could decrease the inhibitory ability of bacteria by inhibiting the growth and/or the production of antifungal secondary metabolites. Decreased Bd inhibition due to co-culturing with Bd has been demonstrated in a previous study (Bletz et al. 2013). In the present study, although we did not see a decrease in inhibition in response to co-culturing, we did observe a decrease in the number of metabolites secreted by bacteria. This effect may be explained by the release of cellular inhibitory molecules by Bd sporangia, which can induce apoptosis of amphibian lymphocytes (Fites et al. 2013). We speculate that these same toxins could also inhibit bacterial growth and/or gene expression as demonstrated by Woodhams et al. (2014). Additionally, under stress, bacteria may curtail the production of some secondary metabolites, but maintain defensive ones.

Overall, results from the present study demonstrate that Panamanian amphibians are host to a wide diversity of anti-Bd bacteria, and the inhibitory properties of these symbiotic microbial communities cannot be predicted simply through taxonomic community composition obtained with next-generation sequencing of the 16S rRNA gene. Consequently, studies investigating host-associated microbial communities should use a combination of culture-based *in vitro* approaches (as done in this study) and *in vivo* experiments, combined with transcriptomics and community profiling, to fully understand the function of symbiotic bacteria in relation to their

host. Much of Panama's large amphibian diversity is currently being threatened by Bd. We hope that these results will further the development of probiotics to prevent chytridiomycosis outbreaks in areas where Bd is currently not present and to return species that have experienced population-level extinctions, such as the Panamanian golden frog (*Atelopus zeteki*), to their native habitats in areas where Bd epidemics have already occurred.

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LITERATURE CITED

Artursson, V., R. D. Finlay, and J. K. Jansson. 2006. Interactions between arbuscular mycorrhizal fungi and bacteria and their potential for stimulating plant growth. Environmental Microbiology 8:1–10.

Bakker, M. G., J. D. Glover, J. G. Mai, and L. L. Kinkel. 2010. Plant community effects on the diversity and pathogen suppressive activity of soil streptomycetes. Applied Soil Ecology 46:35–42.

Bassler, B. L. 1999. How bacteria talk to each other: regulation of gene expression by quorum sensing. Current Opinion in Microbiology 2:582–587.

Becker, M. H., R. M. Brucker, C. R. Schwantes, R. N. Harris, and K. P. C. Minbiole. 2009. The bacterially produced metabolite violacein is associated with survival of amphibians infected with a lethal fungus. Applied and Environmental Microbiology 75:6635–6638.

Becker, M. H., and R. N. Harris. 2010. Cutaneous bacteria of the redback salamander prevent morbidity associated with a lethal disease. PLoS One 5:e10957.

Becker, M. H., R. N. Harris, K. P. C. Minbiole, C. R. Schwantes, L. A. Rollins-Smith, L. K. Reinert, R. M. Brucker, R. J. Domangue, and B. Gratwicke. 2012. Towards a better understanding of the use of probiotics for preventing chytridiomycosis in Panamanian golden frogs. EcoHealth 8:501–506.

Bell, S. C., R. A. Alford, S. Garland, G. Padilla, and A. D. Thomas. 2013. Screening bacterial metabolites for inhibitory effects against *Batrachochytrium dendrobatidis* using a spectrophotometric assay. Diseases of Aquatic Organisms 103:77–85.

Benjamini, Y., and Y. Hochberg. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society B 57:289–300.

Berg, G. 2009. Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. Applied Microbiology and Biotechnology 84:11–18.

Bletz, M. C., A. H. Loudon, M. H. Becker, S. C. Bell, D. C. Woodhams, K. P. C. Minbiole, and R. N. Harris. 2013. Mitigating amphibian chytridiomycosis with bioaugmentation: characteristics of effective probiotics and strategies for their selection and use. Ecology Letters 16:807–820.

Brucker, R. M., C. M. Baylor, R. L. Walters, A. Lauer, R. N. Harris, and K. P. C. Minbiole. 2008a. The identification of 2,4-diacetylphloroglucinol as an antifungal metabolite produced by cutaneous bacteria of the salamander *Plethodon cinereus*. Journal of Chemical Ecology 34:39–43.

Brucker, R. M., R. N. Harris, C. R. Schwantes, T. N. Gallaher, D. C. Flaherty, B. A. Lam, and K. P. C. Minbiole. 2008b. Amphibian chemical defense: antifungal metabolites of the microsymbiont *Janthinobacterium lividum* on the salamander *Plethodon cinereus*. Journal of Chemical Ecology 34:1422–1429.

Caporaso, J. G., J. Kuczynski, J. Stombaugh, K. Bittinger, F. D. Bushman, E. K. Costello, N. Fierer, A. G. Peña, J. K. Goodrich, J. I. Gordon, G. A. Huttley, S. T. Kelley, D. Knights, J. E. Koenig, R. E. Ley, C. A. Lozupone, D. McDonald, B. D. Muegge, M. Pirrung, J. Reeder, J. R. Sevinsky, P. J. Turnbaugh, W. A. Walters, J. Widmann, T. Yatsunenko, J. Zaneveld, and R. Knight. 2010a. QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7:335–336.

Caporaso, J. G., C. L. Lauber, W. A. Walters, D. Berg-Lyons, C. A. Lozupone, P. J. Turnbaugh, N. Fierer, and R. Knight. 2010b. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proceedings of the National Academy of Sciences 108 Suppl:4516–4522.

Cheng, T. L., S. M. Rovito, D. B. Wake, and V. T. Vredenburg. 2011. Coincident mass extirpation of Neotropical amphibians with the emergence of the infectious fungal pathogen *Batrachochytrium dendrobatidis*. Proceedings of the National Academy of Sciences 108:9502–9507.

Densmore, C. L., and D. E. Green. 2007. Diseases of amphibians. ILAR Journal 48:235–254.

Devictor, V., D. Mouillot, C. Meynard, F. Jiguet, W. Thuiller, and N. Mouquet. 2010. Spatial mismatch and congruence between taxonomic, phylogenetic and functional diversity: the need for integrative conservation strategies in a changing world. Ecology Letters 13:1030–1040.

Doolittle, R. F., D. F. Feng, K. L. Anderson, and M. R. Alberro. 1990. A naturally occurring horizontal gene transfer from a eukaryote to a prokaryote. Journal of Molecular Evolution 31:383–388.

Fierer, N., M. Hamady, C. L. Lauber, and R. Knight. 2008. The influence of sex, handedness, and washing on the diversity of hand surface bacteria. Proceedings of the National Academy of Sciences 105:17994–17999.

Fisher, M. C., J. Bosch, Z. Yin, D. a Stead, J. Walker, L. Selway, A. J. P. Brown, L. a Walker, N. a R. Gow, J. E. Stajich, and T. W. J. Garner. 2009. Proteomic and phenotypic profiling of the amphibian pathogen *Batrachochytrium dendrobatidis* shows that genotype is linked to virulence. Molecular Ecology 18:415–429.

Fites, J. S., J. P. Ramsey, W. M. Holden, S. P. Collier, D. M. Sutherland, L. K. Reinert, A. S. Gayek, T. S. Dermody, T. M. Aune, K. Oswald-Richter, and L. A. Rollins-Smith. 2013. The invasive chytrid fungus of amphibians paralyzes lymphocyte responses. Science 342:366–369.

Flechas, S. V, C. Sarmiento, M. E. Cárdenas, E. M. Medina, S. Restrepo, and A. Amézquita. 2012. Surviving chytridiomycosis: differential anti-*Batrachochytrium dendrobatidis* activity in bacterial isolates from three lowland species of *Atelopus*. PLoS One 7:e44832.

Fujimura, K., N. Slusher, M. Cabana, and S. Lynch. 2010. Role of the gut microbiota in defining human health. Expert Review of Anti-infective Therapy 8:435–454.

Fukushima, M., K. Kakinuma, and R. Kawaguchi. 2002. Phylogenetic analysis of *Salmonella*, *Shigella*, and *Escherichia coli* strains on the basis of the gyrB gene sequence. Journal of Clinical Microbiology 40:2779–2785.

Garbeva, P., M. W. Silby, J. M. Raaijmakers, S. B. Levy, and W. De Boer. 2011. Transcriptional and antagonistic responses of *Pseudomonas fluorescens* Pf0-1 to phylogenetically different bacterial competitors. ISME Journal 5:973–985.

Green, A. 1999. Implications of pathogenic fungi for life-history evolution in amphibians. Functional Ecology 13:573–575.

Harris, R. N., R. M. Brucker, J. B. Walke, M. H. Becker, C. R. Schwantes, D. C. Flaherty, B. A. Lam, D. C. Woodhams, C. J. Briggs, V. T. Vredenburg, and K. P. C. Minbiole. 2009a. Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. ISME Journal 3:818–824.

Harris, R. N., T. Y. James, A. Lauer, M. A. Simon, and A. Patel. 2006. Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. EcoHealth 3:53–56.

Harris, R. N., A. Lauer, M. A. Simon, J. L. Banning, and R. A. Alford. 2009b. Addition of antifungal skin bacteria to salamanders ameliorates the effects of chytridiomycosis. Diseases of Aquatic Organisms 83:11–16.

Hartigan, J., and P. Hartigan. 1985. The dip test of unimodality. The Annals of Statistics 13:70–84.

Hayward, a C., N. Fegan, M. Fegan, and G. R. Stirling. 2010. *Stenotrophomonas* and *Lysobacter*: ubiquitous plant-associated gamma-proteobacteria of developing significance in applied microbiology. Journal of Applied Microbiology 108:756–770.

Hilario, E., T. R. Buckley, and J. M. Young. 2004. Improved resolution on the phylogenetic relationships among *Pseudomonas* by the combined analysis of atp D, car A, rec A and 16S rDNA. Antonie van Leeuwenhoek 86:51–64.

Hwang, M., R. Morgan, S. F. Sarkar, P. W. Wang, and D. S. Guttman. 2005. Phylogenetic characterization of virulence and resistance phenotypes of *Pseudomonas syringae*. Applied and Environmental Microbiology 71:5182–5191.

Jarraud, S., C. Mougel, J. Thioulouse, G. Lina, Helene Meugnier, F. Francoise, X. Nesme, J. Etienne, and F. Vandenesch. 2002. Relationships between *Staphylococcus aureus* genetic background, virulence factors, agr groups (alleles), and human disease. Infection and Immunity 70:631–641.

Johnson, J. R., E. Oswald, T. T. O'Bryan, M. A. Kuskowski, and L. Spanjaard. 2002. Phylogenetic distribution of virulence-associated genes among *Escherichia coli* isolates associated with neonatal bacterial meningitis in the Netherlands. The Journal of Infectious Diseases 185:774–784.

Jousset, A., L. Rochat, S. Scheu, M. Bonkowski, and C. Keel. 2010. Predator-prey chemical warfare determines the expression of biocontrol genes by rhizosphere-associated *Pseudomonas fluorescens*. Applied and Environmental Microbiology 76:5263–5268.

Keel, C., D. Weller, and A. Natsch. 1996. Conservation of the 2, 4-diacetylphloroglucinol biosynthesis locus among fluorescent *Pseudomonas* strains from diverse geographic locations. Applied and Environmental Microbiology 62:552–563.

Kim, B. S., and B. K. Hwang. 2007. Microbial fungicides in the control of plant diseases. Journal of Phytopathology 155:641–653.

Kinashi, H., M. Shimaji, and A. Sakai. 1987. Giant linear plasmids in *Streptomyces* which code for antibiotic biosynthesis genes. Nature 328:454–456.

Krediet, C. J., K. B. Ritchie, V. J. Paul, and M. Teplitski. 2013. Coral-associated micro-organisms and their roles in promoting coral health and thwarting diseases. Proceedings of the Royal Society B 280:20122328.

Kueneman, J. G., L. Wegener Parfrey, D. C. Woodhams, H. M. Archer, R. Knight, and V. J. McKenzie. 2013. The amphibian skin-associated microbiome across species, space and life history stages. Molecular Ecology:1238–1250.

Lam, B. A., J. B. Walke, V. T. Vredenburg, and R. N. Harris. 2010. Proportion of individuals with anti-*Batrachochytrium dendrobatidis* skin bacteria is associated with population persistence in the frog *Rana muscosa*. Biological Conservation 143:529–531.

Lauer, A., M. A. Simon, J. L. Banning, B. A. Lam, and R. N. Harris. 2008. Diversity of cutaneous bacteria with antifungal activity isolated from female four-toed salamanders. ISME Journal 2:145–157.

Letunic, I., and P. Bork. 2007. Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. Bioinformatics 23:127–128.

Lips, K. R., F. Brem, R. Brenes, J. D. Reeve, R. A. Alford, J. Voyles, C. Carey, L. Livo, A. P. Pessier, and J. P. Collins. 2006. Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. Proceedings of the National Academy of Sciences 103:3165–3170.

Maechler, M. 2013. Diptest: Hartigan's dip test statistic for unimodality -corrected code. <http://cran.r-project.org/web/packages/diptest/index.html>

Mantel, N. 1967. The detection of disease clustering and a generalized regression approach. Cancer Research 27:209–220.

Martinez, J. L. 2009. The role of natural environments in the evolution of resistance traits in pathogenic bacteria. Proceedings of the Royal Society B 276:2521–2530.

Martiny, A. C., K. Treseder, and G. Pusch. 2013. Phylogenetic conservatism of functional traits in microorganisms. ISME Journal 7:830–838.

McGill, B. J., B. J. Enquist, E. Weiher, and M. Westoby. 2006. Rebuilding community ecology from functional traits. Trends in Ecology & Evolution 21:178–185.

McKenzie, V. J., R. M. Bowers, N. Fierer, R. Knight, and C. L. Lauber. 2012. Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. ISME Journal 6:588–596.

Mell, J. C., and R. J. Redfield. 2014. Natural competence and the evolution of DNA uptake specificity. Journal of Bacteriology 196:1471–1483.

Mollet, C., M. Drancourt, and D. Raoult. 1997. rpoB sequence analysis as a novel basis for bacterial identification. Molecular Microbiology 26:1005–1011.

Moynihan, J. A., J. P. Morrissey, E. R. Coppoolse, W. J. Stiekema, F. O'Gara, and E. F. Boyd. 2009. Evolutionary history of the phl gene cluster in the plant-associated bacterium *Pseudomonas fluorescens*. Applied and Environmental Microbiology 75:2122–2131.

Ochman, H., J. G. Lawrence, and E. a Groisman. 2000. Lateral gene transfer and the nature of bacterial innovation. Nature 405:299–304.

Park, S., A. Collingwood, S. St-Hilaire, and P. P. Sheridan. 2014. Inhibition of *Batrachochytrium dendrobatidis* caused by bacteria isolated from the skin of boreal toads, *Anaxyrus* (*Bufo*) *boreas boreas*, from Grand Teton National Park, Wyoming, USA. Microbiology Insights 7:1–8.

Philippot, L., S. G. E. Andersson, T. J. Battin, J. I. Prosser, J. P. Schimel, W. B. Whitman, and S. Hallin. 2010. The ecological coherence of high bacterial taxonomic ranks. Nature Reviews Microbiology 8:523–9.

Picard, B., J. S. Garcia, S. Gouriou, P. Duriez, N. Brahimi, E. Bingen, J. Elion, and E. Denamur. 1999. The link between phylogeny and virulence in *Escherichia coli* extraintestinal infection. Infection and Immunity 67:546–553.

R Core Team. 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Ramette, A., M. Frapolli, G. Défago, and Y. Moënne-Loccoz. 2003. Phylogeny of HCN synthase-encoding hcnBC genes in biocontrol fluorescent pseudomonads and its relationship with host plant species and HCN synthesis ability. Molecular Plant-Microbe Interactions 16:525–535.

Ravel, J., E. M. Wellington, and R. T. Hill. 2000. Interspecific transfer of *Streptomyces* giant linear plasmids in sterile amended soil microcosms. Applied and Environmental Microbiology 66:529–534.

Roth, T., J. Foley, J. Worth, J. Piovia-Scott, K. Pope, and S. Lawler. 2013. Bacterial flora on Cascades frogs in the Klamath mountains of California. Comparative Immunology, Microbiology and Infectious Diseases 36:591–598.

Sánchez, C., A. F. Braña, C. Méndez, and J. A. Salas. 2006. Reevaluation of the violacein biosynthetic pathway and its relationship to indolocarbazole biosynthesis. Chembiochem 7:1231–1240.

Skerratt, L. F., L. Berger, R. Speare, S. Cashins, K. R. McDonald, A. D. Phillott, H. B. Hines, and N. Kenyon. 2007. Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. EcoHealth 4:125–134.

Stuart, S. N., J. S. Chanson, N. A. Cox, B. E. Young, A. S. L. Rodrigues, D. L. Fischman, and R. W. Waller. 2004. Status and trends of amphibian declines and extinctions worldwide. Science 306:1783–1786.

Umile, T. P., P. J. McLaughlin, K. R. Johnson, S. Honarvar, A. L. Blackman, E. a. Burzynski, R. W. Davis, T. L. Teotonio, G. W. Hearn, C. a. Hughey, A. F. Lagalante, and K. P. C. Minbiole. 2014. Nonlethal amphibian skin swabbing of cutaneous natural products for HPLC fingerprinting. Analytical Methods DOI:10.1039/c4ay00566j.

Walke, J. B., M. H. Becker, S. C. Loftus, L. L. House, G. Cormier, R. V Jensen, and L. K. Belden. 2014. Amphibian skin may select for rare environmental microbes. ISME Journal doi:10.1038/ismej.2014.77.

Walsh, U. F., J. P. Morrissey, and F. O'Gara. 2001. *Pseudomonas* for biocontrol of phytopathogens: from functional genomics to commercial exploitation. Current Opinion in Biotechnology 12:289–295.

Wolf, A., A. Fritze, M. Hagemann, and G. Berg. 2002. *Stenotrophomonas rhizophila* sp. nov., a novel plant-associated bacterium with antifungal properties. International Journal of Systematic and Evolutionary Microbiology 52:1937–1944.

Woodhams, D. C., H. Brandt, S. Baumgartner, J. Kielgast, E. Küpfer, U. Tobler, L. R. Davis, B. R. Schmidt, C. Bel, S. Hodel, R. Knight, and V. McKenzie. 2014. Interacting symbionts and immunity in the amphibian skin mucosome predict disease risk and probiotic effectiveness. PLoS One 9:e96375.

Woodhams, D. C., V. T. Vredenburg, M.-A. Simon, D. Billheimer, B. Shakhtour, Y. Shyr, C. J. Briggs, L. A. Rollins-Smith, and R. N. Harris. 2007. Symbiotic bacteria contribute to innate immune defenses of the threatened mountain yellow-legged frog, *Rana muscosa*. Biological Conservation 138:390–398.

Zhang, Z., S. Schwartz, L. Wagner, and W. Miller. 2000. A greedy algorithm for aligning DNA sequences. Journal of Computational Biology 7:203–214.

Zimmerman, A. E., A. C. Martiny, and S. D. Allison. 2013. Microdiversity of extracellular enzyme genes among sequenced prokaryotic genomes. ISME Journal 7:1187–1199.

TABLES

Table 1. Number of bacterial isolates collected from the skin of Panamanian frogs.

Site	Frog species (family)	Number of frogs	Number of isolates
Cerro Bruja	*Atelopus limosus* (Bufonidae)	6	30
El Copé	*Craugastor crassidigitus* (Craugastoridae)	6	71
	Silverstoneia flotator (Dendrobatidae)	5	41
	Smilisca sordida (Hylidae)	8	71
Sierra Llorona	*Atelopus limosus* (Bufonidae)	6	50
Soberanía	*Bufo typhonius* (Bufonidae)	6	30
	Silverstoneia flotator (Dendrobatidae)	5	37
Tortí	*Allobates talamancae* (Aromobatidae)	1	12
	Colostethus panamensis (Dendrobatidae)	3	20
	Craugastor crassidigitus (Craugastoridae)	8	42
	Dendrobates auratus (Dendrobatidae)	5	32
	Hyalinobatrachium colymbiphyllum	1	2
	Smilisca sila (Hylidae)	4	14
	Strabomantis bufoniformis (Craugastoridae)	3	32
Total		**67**	**484**

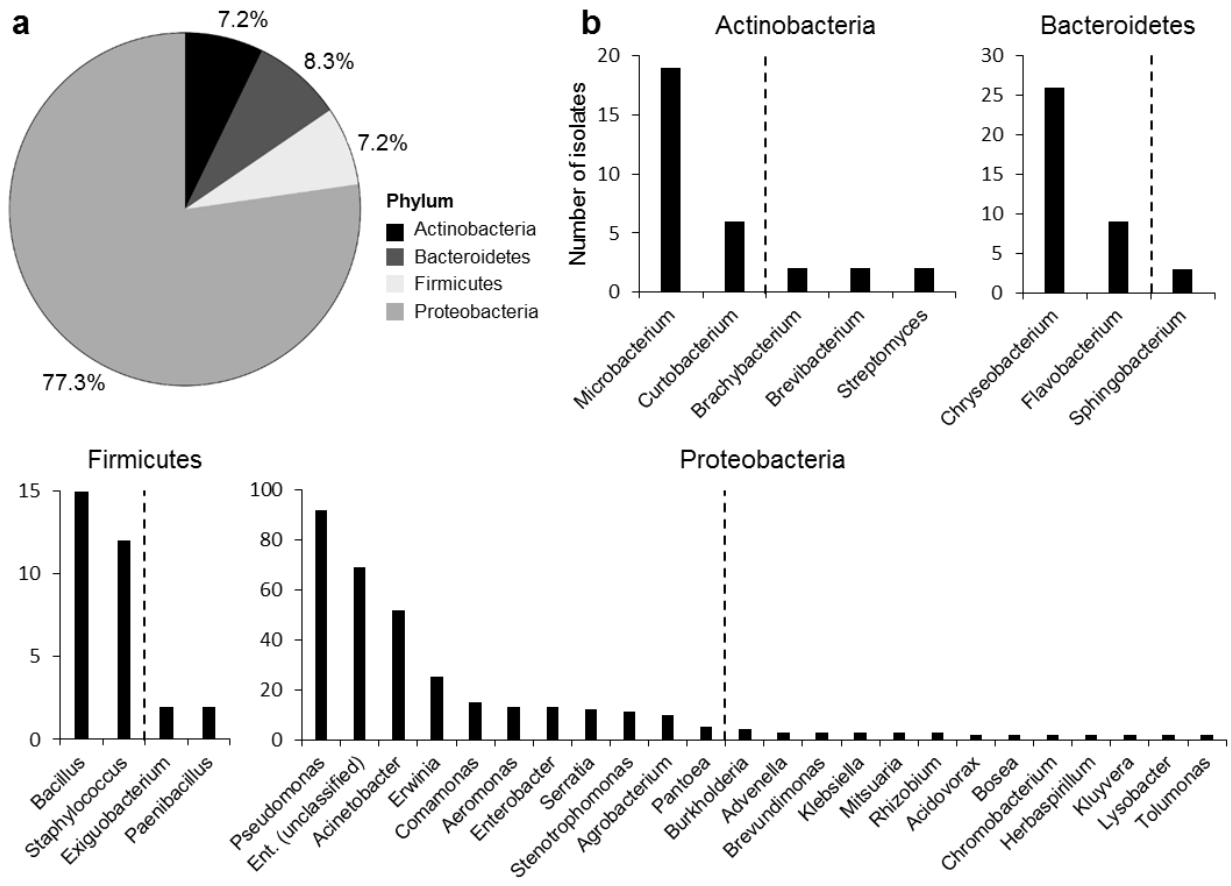

Figure 1. Taxonomic distribution of bacterial isolates at the (a) phylum and (b) genus levels. Only genera consisting of two or more isolates are shown. Many (n = 69) isolates within the family Enterobacteriaceae were unclassified at the genus level and thus are shown at the family level; this is denoted by a star. Genera to the left of the dashed vertical lines were considered dominant (isolates, $n \geq 5$).

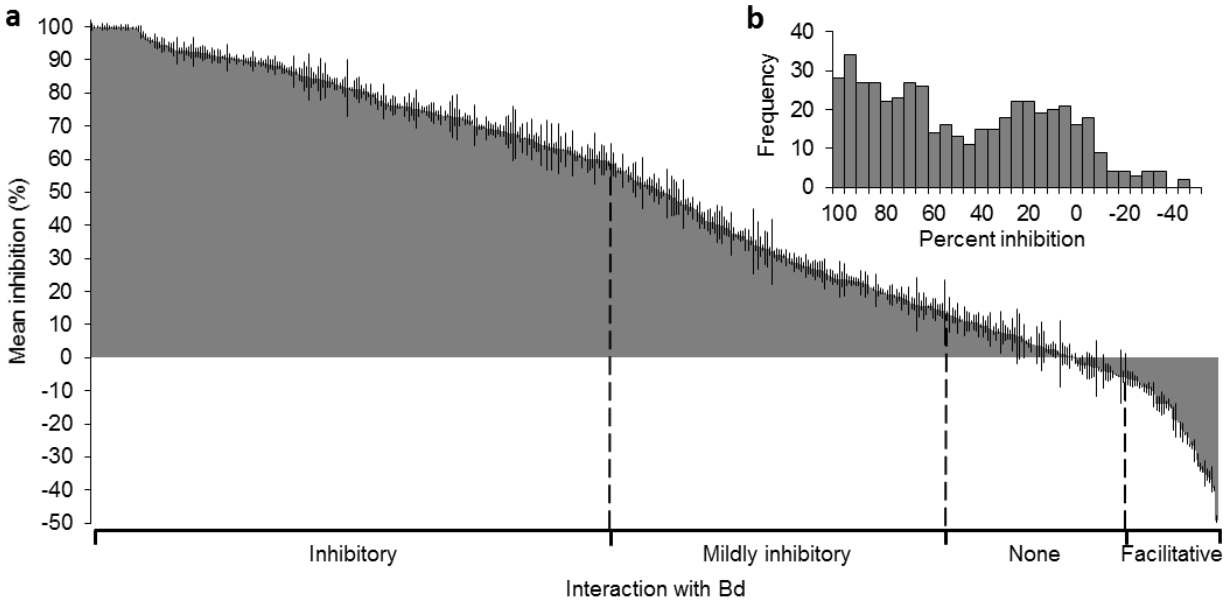

Figure 2. (a) Percent mean Bd inhibition of bacterial isolates and (b) frequency distribution of mean percent inhibition. Error bars represent standard error. Isolates' interactions with Bd were classified based on their mean percent inhibition.

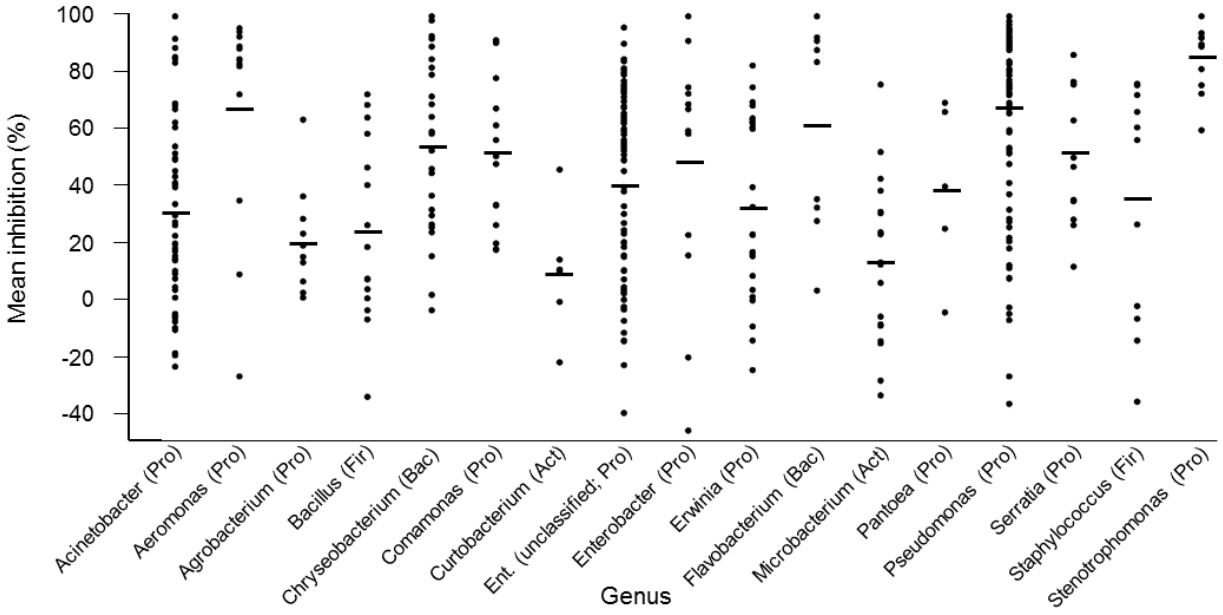

Figure 3. Dot plot of mean percent Bd inhibition of bacterial isolates (n = 404) categorized by genera. Only dominant genera (isolates, $n \geq 5$) are shown. Horizontal bars represent the mean percent inhibition of each genus.

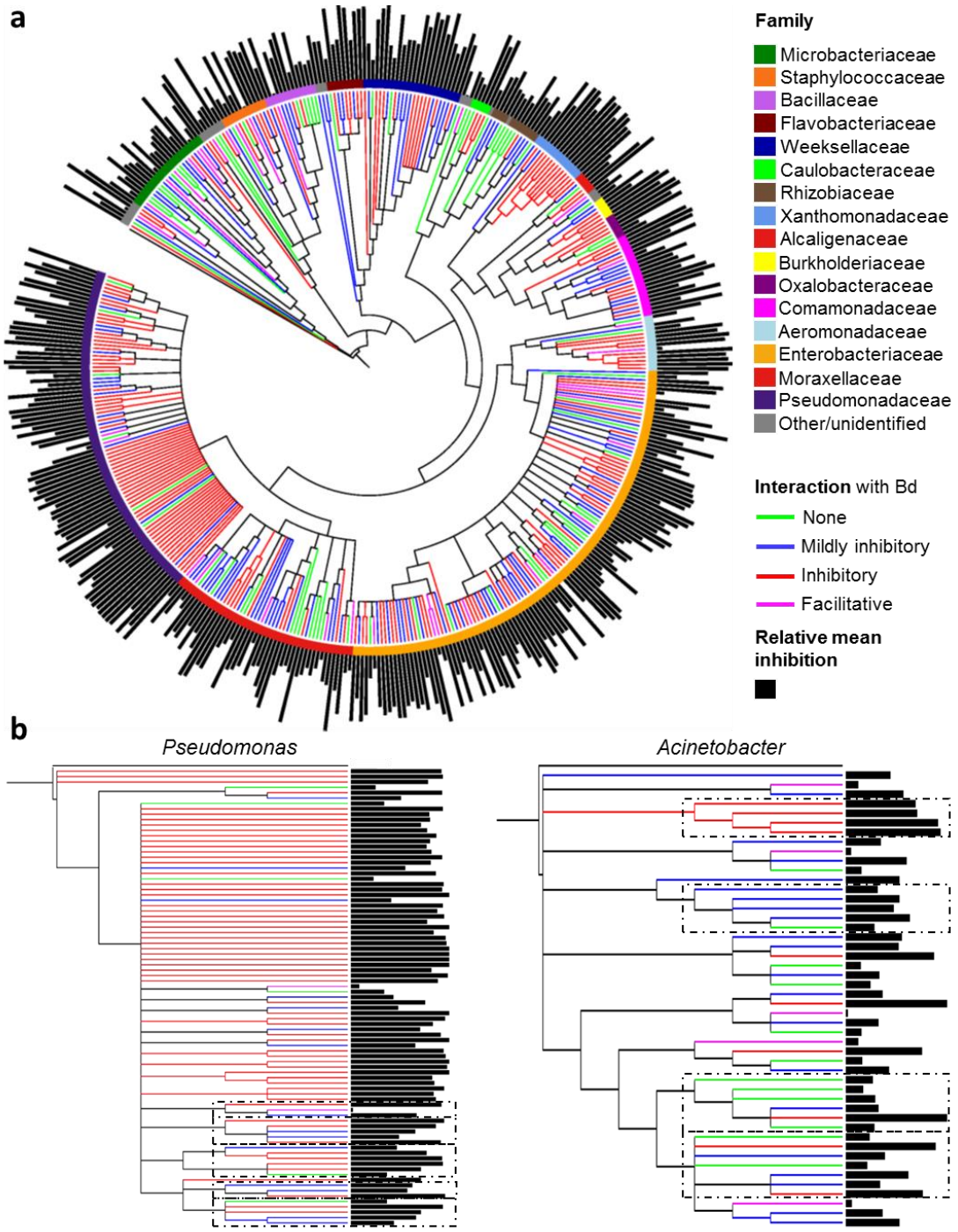

Figure 4. (a) Phylogenetic distribution of functional traits related to a bacterial isolate's interaction with *Batrachochytrium dendrobatidis* (Bd) among all taxa and (b) within the genera *Pseudomonas* and *Acinetobacter*. Each branch represents an individual isolate. The color of the branch corresponds to the isolate's interaction with Bd. In (a), taxonomic classification at the family level of each isolate is indicated by the inner ring. In (b), dashed boxes represent groups of isolates with at least 99.5% sequence similarity. Relative mean percent inhibition is represented by the black bars.

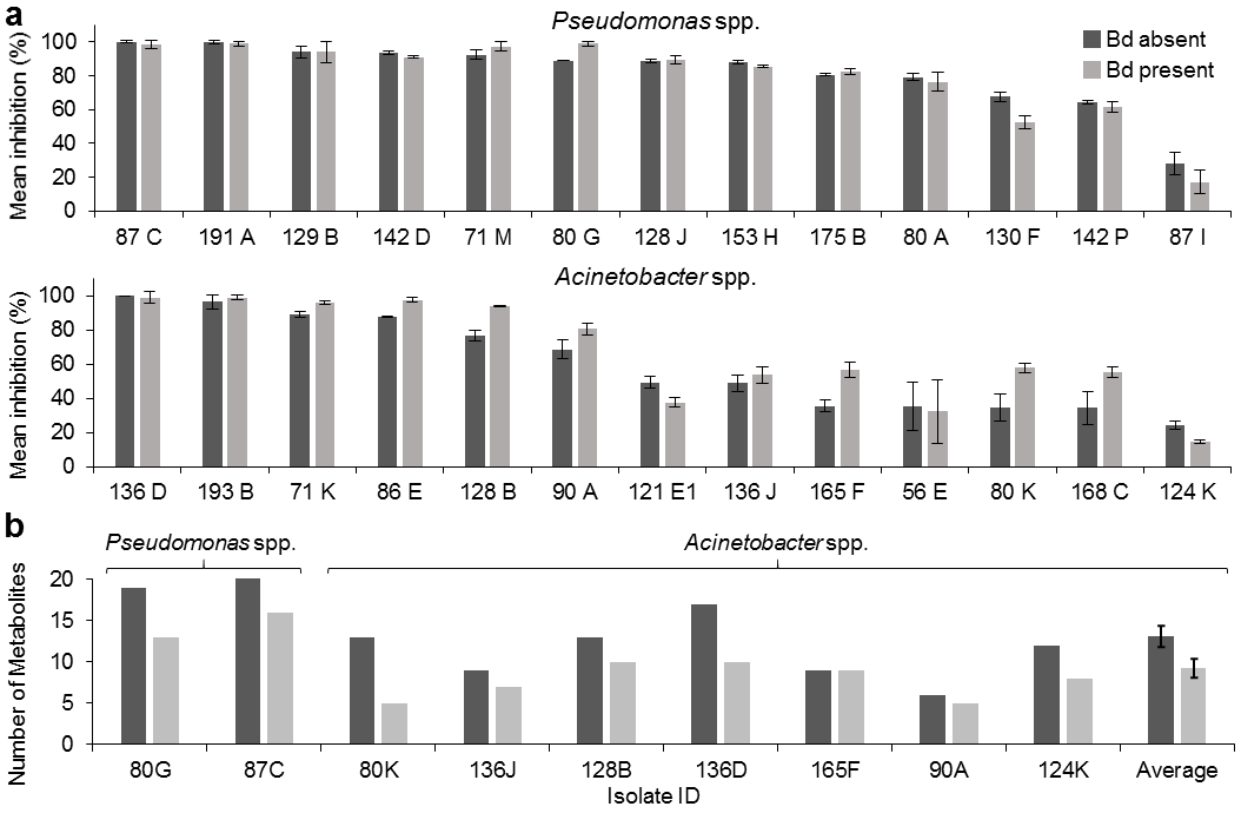

Figure 5. (a) Mean percent inhibition and (b) number of detected metabolites of *Pseudomonas* spp. and *Acinetobacter* spp. isolates grown with and without *Batrachochytrium dendrobatidis* prior to being assayed. Error bars represent standard error.

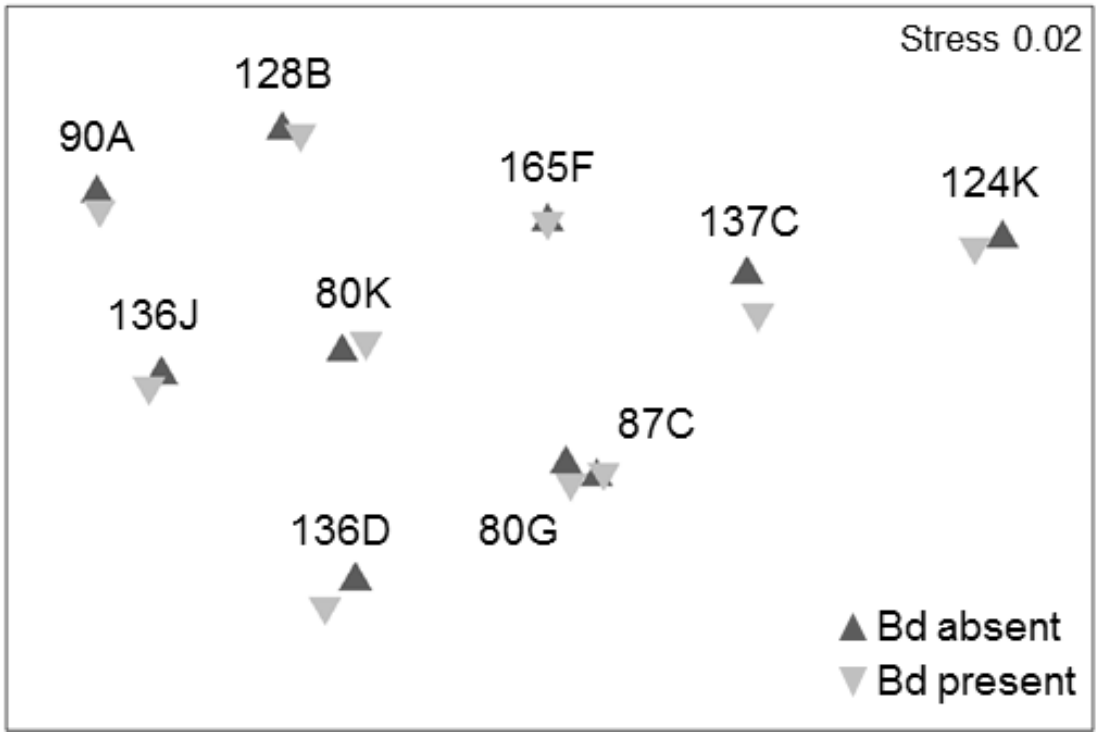

Figure 6. Non-metric Multidimensional Scaling ordination of Sørensen distances between metabolite communities of isolates grown with and without *Batrachochytrium dendrobatidis*. Isolates 80G and 87C are *Pseudomonas* spp. All other isolates shown are *Acinetobacter* spp.

CHAPTER IV. COMPOSITION OF SYMBIOTIC BACTERIA PREDICTS SURVIVAL IN PANAMANIAN GOLDEN FROGS INFECTED WITH A LETHAL FUNGUS

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ABSTRACT

Symbiotic microbes can dramatically impact host health and fitness, and recent research in a diversity of systems suggests that different symbiont community structures may result in distinct outcomes for the host. In amphibians, some symbiotic skin bacteria produce metabolites that inhibit the growth of *Batrachochytrium dendrobatidis* (Bd), a cutaneous fungal pathogen that has caused many amphibian population declines and extinctions. Treatment with beneficial bacteria (probiotics) prevents Bd infection in some amphibian species and creates optimism for conservation of species that are highly susceptible to chytridiomycosis, the disease caused by Bd. In a laboratory experiment, we used Bd-inhibitory bacteria from Bd-tolerant Panamanian amphibians in a probiotic development trial with Panamanian golden frogs, *Atelopus zeteki,* a species currently surviving only in captive assurance colonies. Approximately 30% of infected golden frogs survived Bd exposure by either clearing infection or maintaining low Bd loads, but this was not associated with probiotic treatment. Survival was instead correlated with initial composition of the skin bacterial community and metabolites present on the skin. This suggests a strong link between the structure of these symbiotic microbial communities and amphibian host health in the face of Bd exposure and also suggests a new approach for developing amphibian probiotics.

INTRODUCTION

Animals host a diversity of symbiotic microorganisms, many of which are vital for host fitness. These microbial communities are made of bacteria, fungi, archaea, and viruses that interact with each other and their host along a continuum from mutualistic to parasitic (Taylor et al. 2007, McKenzie et al. 2012, Probst et al. 2013, Sato et al. 2013, Schommer and Gallo 2013). Recent studies suggest that the structure of these symbiotic microbial communities can have direct impacts on their function, and ultimately on host phenotype. For example, in humans, gut communities with high diversity and where Bacteroidetes are relatively abundant appear to contribute to lean phenotypes, while gut communities with low diversity and dominated by Actinobacteria appear to contribute to obesity (Turnbaugh et al. 2009). In marine invertebrates, such as corals and sponges, specific community structures of symbiotic microbes have been linked to healthy and diseased animals (Webster and Taylor 2012). Some symbiotic microbes positively impact host physiology and health by preventing pathogen colonization (Gil-Turnes and Fenical 1992, Krediet et al. 2013) and enhancing immune function (defensive symbiosis, Clay 2014). For example, in mice, immunity against the cutaneous parasite *Leishmania major* is improved through skin microbiota-enhanced effector T-cell responses (Naik et al. 2012). Amphibian skin is also inhabited by diverse bacteria that produce a range of anti-microbial metabolites that defend against cutaneous and embryonic pathogens (Banning et al. 2008, Harris et al. 2009a), including the fungal pathogen *Batrachochytrium dendrobatidis* (Bd, Harris et al. 2006, Brucker et al. 2008b, Becker et al. 2009, Becker and Harris 2010).

Bd causes the disease chytridiomycosis and is responsible for hundreds of amphibian declines and extinctions, including extirpation of the emblematic Panamanian golden frog, *Atelopus zeteki* (Skerratt et al. 2007, Gagliardo et al. 2008, Wake and Vredenburg 2008). This species was historically present in central-western Panamá until Bd arrived. Fortunately, prior to the arrival of Bd, a few *A. zeteki* individuals were captured and placed in survival-assurance colonies (Gagliardo et al. 2008). Currently, successful breeding colonies exist in both Panamá and the United States (Poole 2008). However, Bd still remains on amphibian species that are less susceptible to chytridiomycosis in what was the natural habitat of *A. zeteki* (Rebollar et al. 2014), preventing the reintroduction of highly-susceptible golden frogs.

One promising reintroduction strategy is the use of probiotics (beneficial microbes, Bletz et al. 2013). Probiotic therapies prevent disease, as well as improve growth and survival, in many other systems, including agriculture, aquaculture, livestock, and human medicine (Shu et al. 2001, Berg 2009, Reid et al. 2011, Mohapatra et al. 2013, Nagalingam et al. 2013). For instance, an experimental study demonstrated that the probiotic *Lactobacillus sakei* was effective at protecting its mouse host against *Corynebacterium tuberculostearicum*, a bacterium associated with chronic rhinosinusitis in humans (Abreu et al. 2012). In some amphibian species, bacteria that inhibit Bd *in vitro* prevent chytridiomycosis when incorporated into their existing skin microbiota as probiotics (Harris et al. 2009a, Bletz et al. 2013). *Janthinobacterium lividum* is a bacterium commonly found on the skin of North American amphibians (Lauer et al. 2007, 2008, Brucker et al. 2008b) and has been effective as a probiotic treatment to prevent chytridiomycosis in two North American amphibian species (Becker et al. 2009, Harris et al. 2009a). Unfortunately, probiotic therapy with *J. lividum* did not prevent chytridiomycosis in captive *A. zeteki*, as it failed to persist on the skin of experimental frogs possibly due to host defences or competition from other resident microbes (Becker et al. 2012).

The aims of this study were to investigate the relationship between disease outcome and the structure of the cutaneous bacterial community prior to and after pathogen exposure, and to develop a probiotic treatment method for *A. zeteki*. We hypothesized that antifungal bacterial species collected from Panamanian amphibian species would be better able to colonize and persist on the skin of *A. zeteki* and prevent chytridiomycosis. We isolated four candidate probiotic bacteria from Panamanian amphibians that strongly inhibited Bd growth *in vitro* and tested them experimentally to determine whether they could prevent chytridiomycosis in captive *A. zeteki*.

METHODS

Probiotic candidate selection

Probiotic candidates were chosen from 484 bacteria isolated from skin swabs of 11 Panamanian frog species (67 individuals) sampled from January to April 2010 (M. H. Becker

unpublished data). We tested each isolate's ability to inhibit Bd with a spectrophotometric assay (similar to Bell et al. 2013). We identified each isolate taxonomically by sequencing the 16S rRNA gene (Lauer et al. 2008) and analysing the sequence with the Ribosomal Database Project's Sequence Match tool (http://rdp.cme.msu.edu). We then identified four probiotic candidates that we hypothesized would have the best success at colonizing and persisting on the skin of *A. zeteki* and preventing chytridiomycosis based on the following criteria: (1) high inhibition against Bd *in vitro*, (2) no previous reports of pathogenicity (determined by a literature search), (3) isolation from a host species that is closely related to *A. zeteki* and/or a species that persists in the presence of Bd, and (4) detection on a high proportion of individuals in the host community from which it was collected. The following four bacterial isolates were selected for experimental tests with *A. zeteki* (Table A1): *Chryseobacterium* sp. (hereafter, Chryseo), *Pseudomonas* sp. 1 (hereafter, Pseudo1), *Pseudomonas* sp. 2 (hereafter, Pseudo2), and *Stenotrophomonas* sp. (hereafter, Steno).

Study species and animal care

For our experiment, we obtained 47 adult captive-bred surplus *A. zeteki* frogs from the Maryland Zoo (Baltimore, Maryland, USA), which manages the Association of Zoos and Aquariums Panamanian golden frog species survival program (www.aza.org/species-survival-plan-program). We placed frogs in individual plastic enclosures and followed standard husbandry protocols throughout the experiment as described by Becker et al. (2012). Prior to any treatment, frogs underwent a two week acclimation and observation period.

Experimental design

To initially ensure the probiotic candidates did not have detrimental effects on the frogs, we randomly assigned frogs to five experimental groups, a control group (n = 9) and four probiotic groups (n = 8 each). We then treated all individuals with chlorhexidine (0.05%) every other day for one week to reduce existing cutaneous microbiota that may inhibit colonization of the probiotics. We prepared cultures of each probiotic and treated frogs with 4×10^8 cells of the same probiotic strain (probiotic groups) or sterile water (control group) following procedures in Harris et al. (2009a). Afterwards, each individual was returned to its enclosure. Immediately prior to and every 14 days after probiotic treatment, we weighed each individual to assess body

condition. We monitored frogs twice a day (morning and afternoon) to assess mortality and morbidity associated with probiotic treatment for 84 days.

There were no lethal or sublethal effects in response to any initial treatment with any of the probiotic candidates, thus we proceeded to assess the effectiveness of each probiotic for preventing chytridiomycosis. Frogs were reassigned to six experimental treatment groups. All individuals in each of the four probiotic groups received the identical probiotic as in the initial trial, but were also exposed to Bd (probiotic+Bd; n = 8 each). The nine frogs from the initial trial control group and an additional six frogs were randomly assigned to a control group (no probiotic, no Bd; n = 7) and a Bd only group (no probiotic+Bd; n = 8). We treated frogs within each probiotic+Bd group with the appropriate probiotic strain as in the initial trial. Three days later, we exposed probiotic+Bd and no probiotic+Bd frogs to 3000 zoospores of Bd strain JEL 310 as described by Harris et al. (2009a). This strain was isolated from *Smilisca phaeota* from Fortuna, Panamá and used in a previous *A. zeteki* probiotic experiment (Becker et al. 2012). Control frogs were exposed to sterile water. After these exposures, each frog was returned to its enclosure.

Prior to the second probiotic treatment and approximately every 28 days after Bd exposure, we swabbed each frog with a sterile rayon swab (MW113, Medical Wire & Equipment Co., Corsham, Wiltshire, UK) ten times on the ventral surface, ten times on each thigh, and five times on each hind foot to assess microbial community dynamics and Bd infection intensity. On the same swabbing days, we weighed each individual to assess body condition. We monitored frogs twice a day (morning and afternoon) to assess mortality and morbidity. After death, frogs were preserved by freezing at -20 C.

The experiment was terminated 241 days after *A. zeteki* were exposed to Bd, and at that time 23 frogs had died of chytridiomycosis, nine frogs remained alive with Bd infections, five frogs had cleared infection with no Bd detection via qPCR for three consecutive time-points or in histological examination after euthanasia, and all control frogs remained alive (Table 1). All surviving frogs were swabbed and then euthanized by subcutaneous injection with 1% tricaine methanesulfonate (Becker et al. 2012). We predicted the outcome (clear infection or die) of the nine surviving frogs based on current infection intensity and the change in infection intensity

over time. Six of the nine individuals were predicted to clear infection because they had a low infection intensity at the end of the experiment (< 100 zoospore equivalents) and infection intensity was decreasing on those individuals over the last 45 days of the experiment. The other three individuals were predicted to die because they had a high infection intensity at the end of the experiment (> 100 zoospore equivalents) and infection intensity was increasing on those individuals over the last 45 days of the experiment.

DNA extraction, qPCR, and 16S rRNA amplicon sequencing

We extracted DNA from each swab with a Qiagen DNeasy blood and tissue kit (Valencia, California, USA) following the manufacturer's protocol. To quantify Bd infection intensity, we amplified extracted DNA of all samples in duplicate with TaqMan qPCR following procedures described by Boyle et al. (2004). We used Bd strain JEL 427 to make DNA standards (Boyle et al. 2004). Samples with greater than 0.1 zoospore genomic equivalents (hereafter, zoospore equivalent) were considered positive for Bd infection. If the number of zoospore equivalents from duplicates was inconsistent, the sample was reanalysed and the majority result was retained (e.g., if two of three replicates were positive and one was negative, we considered the individual Bd positive).

We focused on two experimental time-points for assessing the microbial communities: three days prior to Bd exposure and 28 days post-exposure. We chose 28 days after exposure because by then Bd had gone through approximately 6 generations of zoospore production and we should have been able to detect any potential impacts of Bd on the microbial community. At those two time-points, we sequenced the microbial communities of frogs that cleared infection (day -3, n = 5; day 28 n = 5), those that died of chytridiomycosis (day -3, n = 10; day 28, n = 9), and individuals in the control group (day -3, n = 4; day 28, n = 4). We prepared extracted DNA for sequencing by amplifying the V4 region of the 16S rRNA gene following Caporaso et al. (2010c) with the exception that PCR reactions contained 2 µl of template DNA. Controls without template were run for each sample. DNA extracted from a sterile swab was also included as a negative control. We purified PCR products with the Qiagen QIAquik PCR Purification Kit (Qiagen, Valencia, California, USA) using the manufacturer's protocol. An equimolar mixture of all the samples was then sequenced on an Illumina MiSeq instrument (San Diego, California,

USA) with a 250 bp paired-end strategy at the Dana-Farber Cancer Institute, following methods similar to those described in Caporaso et al. (2012). To compensate for the low base diversity of the amplicon pool, the sample was run with a 10% PhiX control.

16S amplicon sequence data were assembled with Fastq-join and processed with the Quantitative Insights Into Microbial Ecology pipeline (QIIME version 1.7.0, Caporaso et al. 2010b) according to methods outlined by Becker et al. (2014). Briefly, we clustered quality-filtered sequences into distinct bacterial OTUs (operational taxonomic units, ~bacterial "species") at a sequence similarity threshold of 97% and assigned taxonomy with RDP classifier and the Greengenes database. All samples were rarefied to 27,000 sequences to standardize sampling effort. Details of the bioinformatics methods are in the Appendix.

Extraction of cutaneous metabolites and LCMS

We excised and extracted the skin of each frog to obtain cutaneous metabolites following procedures described by Brucker et al. (2008b) briefly, excised skins were extracted three times by shaking in HPLC-grade methanol (5 mL each extraction). The combined crude extracts were filtered (0.45 µm PTFE membrane), concentrated, and reconstituted in methanol. Samples were analysed with high performance liquid chromatography-mass spectrometry (LCMS) following procedures described by Umile et al.(2014). To detect inter-sample contamination, we inserted methanol injections into the LCMS queue after every five samples. Tricaine methanesulfonate was detected in the LCMS results and was removed from data analysis. Only frogs that could be processed within eight hours of death were used for metabolite analysis. This was done to minimize the chance of analysing compounds that were produced by microbes after host death. Finally, we removed from all analyses metabolites known to be produced by Bd in culture (T. P. Umile unpublished data).

Statistical analyses

One frog in Pseudo1+Bd and one frog in Pseudo2+Bd did not become infected and one frog in the Chryseo+Bd group died from causes other than chytridiomycosis (qPCR analysis and histological examination showed no sign of Bd infection, but mortality occurred). These individuals were removed from all analyses. Unless noted, all data were normally distributed and

variances were equal among specific comparisons. Differences in survival among treatment groups were tested with a Mantel-Cox log-rank test. Bd infection intensity (number of zoospore equivalents) data were log-transformed to achieve normality. Differences in infection intensity among frogs infected with Bd were tested with ANOVA at two time-points (28 and 196 days after exposure to Bd). The latter time point was chosen because it was the last sampling point in the experiment when sample sizes were large enough for statistical comparison ($n \geq 5$).

Measures of alpha diversity for the bacterial community on each frog (OTU richness, phylogenetic diversity, and Shannon diversity index) were computed with QIIME. We analysed alpha diversity measures of microbiota and the number of metabolites on frogs among probiotic treatment groups with ANOVA. To test for significant differences in alpha diversity measures of microbiota and the number of metabolites on frogs that died of chytridiomycosis and those that cleared Bd infection we used Student's t-tests. Frogs that were predicted to die or clear infection were not included in this and subsequent analyses because we were not positive of their final outcome if the experiment had continued. To compare the microbial community structure, a Bray-Curtis distance matrix (Bray and Curtis 1957) was generated with square-root transformed data using the software package Primer 6 (version 6.1.15). We compared metabolite composition between samples with a Sørensen distance matrix (Sørensen 1948) using Primer 6. The Bray-Curtis distance metric considers relative abundance of individual OTUs within a community, while the Sørensen distance metric uses only presence/absence information. We focused on metabolite presence/absence because the nature of the metabolite LCMS analysis does not allow us to compare relative abundances of different metabolites on the same frog. From the distance matrices, differences in community structure between frogs that died of chytridiomycosis versus those that cleared Bd infection were statistically analysed with permutational multivariate analysis of variance (PERMANOVA) and visualized with principal coordinates analysis (PCO) using the software Permanova+ (version 1.0.5).

To identify whether specific OTUs and metabolites were significantly associated with individuals that died or cleared infection, we used indicator species analysis (Dufrene and Legendre 1997) using the IndVal function in the labdsv package in the program R (version 3.0.1) (R Core Team 2014). We considered an OTU as an indicator species if it had a $P < 0.05$ and an indicator value > 0.7 (Van Rensburg et al. 1999, Castro-luna et al. 2007). These parameters were

also used to identify indicator metabolites. We corrected all multiple comparisons with the false discovery rate procedure (FDR, Benjamini and Hochberg 1995).

RESULTS

Survival and infection intensity

Treating frogs with anti-Bd bacteria prior to Bd exposure did not alter survival rates when compared to frogs without probiotic treatment exposed to Bd (Fig. 1; Log-rank test, P = 0.176). However, five individuals (within the Bd only and Chryseo+Bd treatment groups) became infected with Bd and were able to clear the infection approximately 168 days after exposure (Fig. 2). In contrast, infection intensities on frogs that died of chytridiomycosis (n = 23) increased throughout the experiment or until death. Infection intensity was similar among all survival/infection outcome groups that contained infected individuals (died, n =23; predicted to die, n = 3; cleared, n = 5; predicted to clear, n = 6) at day 28 (ANOVA, F = 0.980, P > 0.05). By day 196, infection intensities among survival/infection outcome groups that still contained infected individuals (died, n = 5; predicted to die, n = 3; predicted to clear, n = 6) significantly differed (Fig. 2; ANOVA, F = 3.942, P = 0.05).

Cutaneous symbiotic microbiota

We sequenced the cutaneous bacterial communities of 37 golden frogs and detected an average of 758 OTUs (range = 542 – 900) on an individual frog. The microbial community structure three days prior to Bd exposure was significantly different on individuals that eventually cleared infection compared to those that died (PERMANOVA, Pseudo-F = 1.586, P = 0.042; Fig. 3*a*). The community structures also varied among these two groups 28 days after exposure to Bd (PERMANOVA, Pseudo-F = 1.671, P = 0.014; Fig. 3*a*). There were no differences in species richness, evenness (Shannon diversity index), and phylogenetic diversity three days prior to Bd exposure or 28 days after Bd exposure among probiotic treatment groups (ANOVAs, P > 0.05) or between frogs that cleared infection and those that died (t-tests, P > 0.05).

Indicator species analysis revealed a diverse array of OTUs that contributed to the dissimilarity between the microbial community composition on frogs that cleared infection and those that died (Fig. 4 and Fig. A1). Three days prior to Bd exposure, six OTUs were associated with individuals that eventually cleared infection and 17 OTUs were associated with frogs that eventually died (Fig. 4). These indicator OTUs accounted for an average of 2.3% of the total sequences per frog. Three days prior to Bd exposure, indicator OTUs on individuals that eventually cleared infection belong to the families Flavobacteriaceae, Sphingomonadaceae, Comamonadaceae, and Rhodocyclaceae. OTUs on individuals that died belong to the families Micrococcineae, Rhizobiaceae, Rhodobacteraceae, Sphingomonadaceae, and Moraxellaceae. Twenty-eight days after Bd exposure, 52 OTUs were associated with frogs that eventually cleared infection and 22 were associated with frogs that died (Fig. A1). Day 28 indicator OTUs accounted for an average of 7.6% of the total sequences per frog, however many of the OTUs were rare members of the community (< 0.1%). Two OTUs, one belonging to the family Micrococcineae and the other to the family Sphingomonadaceae, were significant indicator taxa at both time-points (Fig. 4 and Fig. A1).

Day of sampling had no effect on microbial community structure (3 days prior to Bd exposure vs. 28 days afterward; PERMANOVA, Pseudo-F = 0.902, P > 0.05). In addition, treating frogs with probiotics had no effect on the cutaneous microbial community structure 31 days after treatment (PERMANOVA, Pseudo-F = 0.999, P > 0.05). Comparing the probiotic candidate sequences of the *Stenotrophomonas* sp. isolate and *Chryseobacterium* sp. isolate to the Illumina sequences with a 99% similarity threshold revealed that neither probiotic could be detected 28 days after exposure to Bd (31 days after probiotic treatment). We were unable to look for the two *Pseudomonas* sp. probiotic candidates because the 16S gene is a poor region for distinguishing *Pseudomonas* species and strains (Hilario et al. 2004).

Cutaneous metabolites

We analysed the skin of 28 golden frogs and detected 554 unique metabolites across all individuals. There was an average of 67 (range = 51 – 81) metabolites present on an individual frog. The number of metabolites per frog was similar among all probiotic treatment groups (ANOVA, F = 1.380, P > 0.05) and between frogs that cleared infection and those that died of

chytridiomycosis (t-test, $t = 1.031$, $P > 0.05$). However, the metabolite profiles were significantly different between frogs that cleared infection and died (Fig. 3*b*; PERMANOVA, Pseudo-$F = 2.187$, $P = 0.002$). Indicator species analysis detected three metabolites that were associated with frogs that cleared infection (INDVAL, IV > 0.7, FDR-corrected $P < 0.05$). These metabolites showed retention times of 8.44, 8.91, and 9.78 min. We have not yet been able to identify chemical structures of these metabolites, largely due to their poor absorbance of UV-Visible light. There was also a single metabolite associated with individuals that died, eluting at 10.32 min and showing maximum absorbance at 295 nm.

DISCUSSION

We found that treatment of captive *A. zeteki* with bacteria that are highly inhibitory against Bd *in vitro* was not successful in preventing the colonization and growth of Bd *in vivo*. However, although there were no statistically significant differences in survival among probiotic treatments, some *A. zeteki* frogs acquired a Bd infection and were able to clear this infection. This was unexpected since *A. zeteki* is highly susceptible to Bd, as demonstrated by three independent studies (Bustamante et al. 2010, Becker et al. 2012, Langhammer et al. 2013). In these prior infection studies, only one out of 228 golden frogs infected with Bd was documented as having acquired and then cleared infection. In the present study, approximately 30% of golden frogs either cleared infection (14%) or were predicted to clear infection (16%).

Susceptibility to Bd can differ among amphibian species and populations (Blaustein et al. 2005), likely due to variation in defence mechanisms of the host (Rollins-Smith 2009, Savage and Zamudio 2011, Bletz et al. 2013), the virulence of Bd (Woodhams et al. 2008a, Langhammer et al. 2013), and properties of the environment where the host and pathogen interact (Richards-Zawacki 2010, Murphy et al. 2011). The environment and Bd strain in the present study were constant across all frogs and were therefore not factors in the variation in Bd susceptibility. Of the known host defence mechanisms [antimicrobial peptides (Rollins-Smith 2009), diversity in major histocompatibility complex genes (Savage and Zamudio 2011), acquired immune response (Voyles et al. 2011), and skin microbial communities (Bletz et al. 2013)], our results suggest that

the community composition of skin bacteria likely plays a role in the ability of golden frogs to clear Bd and survive exposure.

The eventual clearance of Bd was correlated with the cutaneous microbial community structure present on frogs both three days prior to and 28 days after exposure to Bd. Bd clearance was also correlated with the cutaneous metabolite profile. These results suggest that bacteria and metabolites present on the skin of golden frogs may be responsible for the ability of some frogs to clear infection. Additionally, the fact that the bacterial community structure on frogs that eventually cleared infection and those that died of chytridiomycosis was significantly different prior to Bd exposure suggests the community was not responding to Bd and therefore is responsible for the clearance of Bd. This conclusion is consistent with results from other studies that have demonstrated importance of cutaneous symbiotic bacteria and their antifungal secretions in protecting amphibians from fungal pathogens, including Bd (Banning et al. 2008, Becker et al. 2009, Becker and Harris 2010). Predicting disease susceptibility with microbial community data has also been demonstrated with human intestinal pathogens (Stecher et al. 2010, Morrow et al. 2013). With recent technological advances in sequencing, researchers have demonstrated the importance of symbiotic microbes to host health by linking host-associated microbial community structure to healthy and diseased hosts in several systems (Turnbaugh et al. 2009, Webster and Taylor 2012, Berendsen et al. 2012, Abreu et al. 2012).

In our study, the resident microbiota appeared to influence the interactions between Bd and *A. zeteki*. Inhibition of Bd could have been accomplished through direct interaction between particular resident microbes and Bd through competition and the production of antifungal metabolites (Brucker et al. 2008b, Becker et al. 2009). In addition, the resident microbiota may have indirectly prevented the growth of Bd by stimulating the host immune system (immunomodulation, Reid et al. 2011). These interactions are commonly seen in others systems (White Jr et al. 2010). For example, commensal bacteria that reside in the human gut demonstrate competitive exclusion of pathogenic bacteria through antibiotic production, preventing attachment to epithelial cells, competing for resources, and stimulating the host's immune system (Guarner and Malagelada 2003, Reid et al. 2011). In the present study, many indicator OTUs that were associated with frogs that cleared infection belong to bacterial families that inhibit Bd *in vitro* and have been isolated from non-susceptible Panamanian amphibians

(Flavobacteriaceae, Comamonadaceae, Pseudomonadaceae; Chapter III). However, it is worth noting that the ability of bacteria to inhibit Bd *in vitro* varies greatly even at the genus or strain level (Chapter III).

Indicator species analysis also revealed OTUs three days prior to Bd exposure and 28 days after exposure that were associated with frogs that died. These OTUs may have facilitated the colonization and growth of Bd or may have been opportunistic pathogens. Facilitation of Bd growth and colonization could occur by the means of symbiotic bacteria reducing the capability of other microbes to inhibit Bd. In a recent study, bacteria isolated from cyclamen and tomato plants, including a *Novosphingobium* sp. and a *Sphingomonas* sp., suppressed production of antimicrobial compounds produced by symbiotic bacteria that inhibit phytopathogens (Someya and Akutsu 2009). Interestingly, in our study, several indicator OTUs in the genera *Novosphingobium* or *Sphingomonas* were associated with dying frogs (3 days prior to Bd, 8/17 OTUs; day 28, 2/10 OTUs), suggesting that the same mechanism could operate in this system. Indicator taxa that were associated with dying frogs at day 28 may also be opportunistic pathogens that caused secondary infections. Opportunistic secondary infections involving bacteria are common in amphibians (Densmore and Green 2007). In particular, secondary or co-infections of the bacteria *Aeromonas hydrophila* and the fungus *Saprolegnia* sp. have been documented in amphibians with chytridiomycosis (Taylor et al. 1999, Bodinof 2010), and these may occur through microbial invasion of the damaged epidermis (Densmore and Green 2007).

The correlation between cutaneous metabolite profile and survival/infection outcome further supports the hypothesis that either microbial or host-produced metabolites influenced infection dynamics. Host-produced antimicrobial compounds, such as antimicrobial peptides, provide defence against pathogens and are common throughout the plant and animal kingdoms (Zasloff 2002). However, research suggests that captive *A. zeteki* do not produce antimicrobial peptides (B. Sheafor personal communication). Previous studies have shown that bacterial-produced metabolites are capable of inhibiting Bd *in vitro* (Brucker et al. 2008a, 2008b) and their concentrations on the skin is correlated with survival in infected salamanders (*Plethodon cinereus*) [14]. In the present study, a majority of the metabolites identified through indicator species analysis (3/4) were associated with, and only occurred on, frogs that cleared infection, and none of them were identified as Bd-derived metabolites (T. P. Umile unpublished data). At

least one compound (8.91 min) was not observed on any frogs in the control group, suggesting that it is bacterially-associated and not produced by the frog.

Although the bacterial isolates used as probiotics in our experiment were highly inhibitory against Bd in *in vitro* assays, they were not successful at preventing infection and subsequent death when applied to the skin of *A. zeteki*. Thirty-one days after treating the frogs with probiotic candidates, we were unable to detect the presence of two probiotic isolates, *Chryseobacterium* sp. and *Stenotrophomonas* sp. that we looked for in the 16S rRNA sequence data. This suggests that the isolates simply may not have been able to colonize and grow on *A. zeteki* skin. The probiotic candidates could have been inhibited by compounds produced by either resident microbiota or the host (Rollins-Smith et al. 2011), or a constant environmental inoculum may be needed for the isolates to persist on the skin (Loudon et al. 2013). In many cases, probiotics designed to prevent and treat human intestinal diseases rely on continual administration to be effective (Bezkorovainy 2001).

We attempted to maximize our chances of choosing a bacterial isolate that would persist on *A. zeteki* by using bacteria found to grow on closely related amphibian species in Panamá. For example, the *Stenotrophomonas* sp. used in this study was isolated from *A. limosus*, which is one of the most closely related species to *A. zeteki*. Alternatively, the probiotic isolates may not be able to produce anti-Bd compounds on *A. zeteki* skin. The lack of an *in vivo* probiotic effect, despite compelling *in vitro* results, has been documented in fish (Gram et al. 2001). The development of a more realistic *in vitro* model to examine persistence and inhibitory ability of potential probiotics on amphibian skin, as has been done for the human gut (Forestier et al. 2001), would be helpful in amphibian probiotic development. In addition, isolating the bacteria that were correlated with clearance of Bd in the present study may contribute to the development of probiotics for *A. zeteki* and other *Atelopus* species. Future success with probiotics will require studies investigating how amphibian cutaneous microbial communities assemble, how microbial populations interact with each other and host immune defences, and how individual microbes are transmitted.

Defensive symbioses are commonly found in many systems including mammals, insects, marine invertebrates, algae, and plants (White Jr et al. 2010, Gallo and Nakatsuji 2011, Clay

2014). For example, some species of fungus-growing ants have a symbiotic relationship with Actinomycetales bacteria that prevents overgrowth of a fungal parasite in their fungal gardens (Fernández-Marín et al. 2013), and some seaweed species are protected from fouling microorganisms through association with epibiotic bacteria (Egan et al. 2013). Our results suggest that members of the amphibian skin microbial community are also important defensive symbionts, potentially through their production of anti-microbial metabolites that prevent pathogen colonization or growth. For the critically-endangered *A. zeteki*, these skin microbes may be an important determinant for survival in Bd infected individuals. Additionally, the ability to detect microbial community composition differences among survival/infection outcome groups prior to infection is important and, in combination with ecological factors (Bielby et al. 2008), may allow conservationists to predict susceptibility in free-living amphibian populations threatened by Bd.

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LITERATURE CITED

Abreu, N. A., N. A. Nagalingam, Y. Song, F. C. Roediger, S. D. Pletcher, A. N. Goldberg, and S. V Lynch. 2012. Sinus microbiome diversity depletion and *Corynebacterium tuberculostearicum* enrichment mediates rhinosinusitis. Science Translational Medicine 4:151ra124.

Banning, J. L., A. L. Weddle, G. W. Wahl, M. A. Simon, A. Lauer, R. L. Walters, and R. N. Harris. 2008. Antifungal skin bacteria, embryonic survival, and communal nesting in four-toed salamanders, *Hemidactylium scutatum*. Oecologia 156:423–429.

Becker, M. H., R. M. Brucker, C. R. Schwantes, R. N. Harris, and K. P. C. Minbiole. 2009. The bacterially produced metabolite violacein is associated with survival of amphibians infected with a lethal fungus. Applied and Environmental Microbiology 75:6635–6638.

Becker, M. H., and R. N. Harris. 2010. Cutaneous bacteria of the redback salamander prevent morbidity associated with a lethal disease. PLoS One 5:e10957.

Becker, M. H., R. N. Harris, K. P. C. Minbiole, C. R. Schwantes, L. A. Rollins-Smith, L. K. Reinert, R. M. Brucker, R. J. Domangue, and B. Gratwicke. 2012. Towards a better understanding of the use of probiotics for preventing chytridiomycosis in Panamanian golden frogs. EcoHealth 8:501–506.

Becker, M. H., C. L. Richards-Zawacki, B. Gratwicke, and L. K. Belden. 2014. The effect of captivity on the cutaneous bacterial community of the critically endangered Panamanian golden frog (*Atelopus zeteki*). Biological Conservation 176:199–206.

Bell, S. C., R. A. Alford, S. Garland, G. Padilla, and A. D. Thomas. 2013. Screening bacterial metabolites for inhibitory effects against *Batrachochytrium dendrobatidis* using a spectrophotometric assay. Diseases of Aquatic Organisms 103:77–85.

Benjamini, Y., and Y. Hochberg. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society B 57:289–300.

Berendsen, R. L., C. M. J. Pieterse, and P. A. H. M. Bakker. 2012. The rhizosphere microbiome and plant health. Trends in Plant Science 17:478–486.

Berg, G. 2009. Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. Applied Microbiology and Biotechnology 84:11–18.

Bezkorovainy, A. 2001. Probiotics: determinants of survival and growth in the gut. The American Journal of Clinical Nutrition 73:399–405.

Bielby, J., N. Cooper, A. A. Cunningham, T. W. J. Garner, and a. Purvis. 2008. Predicting susceptibility to future declines in the world's frogs. Conservation Letters 1:82–90.

Blaustein, A. R., J. M. Romansic, E. A. Scheessele, B. A. Han, A. P. Pessier, and J. E. Longcore. 2005. Interspecific variation in susceptibility of frog tadpoles to the pathogenic fungus *Batrachochytrium dendrobatidis*. Conservation Biology 19:1460–1468.

Bletz, M. C., A. H. Loudon, M. H. Becker, S. C. Bell, D. C. Woodhams, K. P. C. Minbiole, and R. N. Harris. 2013. Mitigating amphibian chytridiomycosis with bioaugmentation: characteristics of effective probiotics and strategies for their selection and use. Ecology Letters 16:807–820.

Bodinof, C. M. 2010. Translocation and conservation of hellbenders (*Cryptobranchus alleganiensis*) in Missouri. University of Missouri.

Boyle, D. G., D. B. Boyle, V. Olsen, J. A. T. Morgan, and A. D. Hyatt. 2004. Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Diseases of Aquatic Organisms 60:141–148.

Bray, J. R., and J. T. Curtis. 1957. An ordination of the upland forest communities of southern Wisconsin. Ecological Monographs 27:325–349.

Brucker, R. M., C. M. Baylor, R. L. Walters, A. Lauer, R. N. Harris, and K. P. C. Minbiole. 2008a. The identification of 2,4-diacetylphloroglucinol as an antifungal metabolite produced by cutaneous bacteria of the salamander *Plethodon cinereus*. Journal of Chemical Ecology 34:39–43.

Brucker, R. M., R. N. Harris, C. R. Schwantes, T. N. Gallaher, D. C. Flaherty, B. A. Lam, and K. P. C. Minbiole. 2008b. Amphibian chemical defense: antifungal metabolites of the microsymbiont *Janthinobacterium lividum* on the salamander *Plethodon cinereus*. Journal of Chemical Ecology 34:1422–1429.

Bustamante, H. M., L. J. Livo, and C. Carey. 2010. Effects of temperature and hydric environment on survival of the Panamanian golden frog infected with a pathogenic chytrid fungus. Integrative Zoology 5:143–153.

Caporaso, J. G., J. Kuczynski, J. Stombaugh, K. Bittinger, F. D. Bushman, E. K. Costello, N. Fierer, A. G. Peña, J. K. Goodrich, J. I. Gordon, G. A. Huttley, S. T. Kelley, D. Knights, J. E. Koenig, R. E. Ley, C. A. Lozupone, D. McDonald, B. D. Muegge, M. Pirrung, J. Reeder, J. R. Sevinsky, P. J. Turnbaugh, W. A. Walters, J. Widmann, T. Yatsunenko, J. Zaneveld, and R. Knight. 2010a. QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7:335–336.

Caporaso, J. G., C. L. Lauber, W. A. Walters, D. Berg-Lyons, J. Huntley, N. Fierer, S. M. Owens, J. Betley, L. Fraser, M. Bauer, N. Gormley, J. A. Gilbert, G. Smith, and R. Knight. 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME Journal 6:1621–1624.

Caporaso, J. G., C. L. Lauber, W. A. Walters, D. Berg-Lyons, C. A. Lozupone, P. J. Turnbaugh, N. Fierer, and R. Knight. 2010b. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proceedings of the National Academy of Sciences 108 Suppl:4516–4522.

Castro-luna, A. A. A., V. J. Sosa, and G. Castillo-Campos. 2007. Quantifying phyllostomid bats at different taxonomic levels as ecological indicators in a disturbed tropical forest ecological indicators in a disturbed tropical forest. Acta Chiropterologica 9:219–228.

Clay, K. 2014. Defensive symbiosis: a microbial perspective. Functional Ecology 28:293–298.

Densmore, C. L., and D. E. Green. 2007. Diseases of amphibians. ILAR Journal 48:235–254.

Dufrene, M., and P. Legendre. 1997. Species assemblages and indicator species: the need for a flexible asymmetrical approach. Ecological Monographs 67:345–366.

Egan, S., T. Harder, C. Burke, P. Steinberg, S. Kjelleberg, and T. Thomas. 2013. The seaweed holobiont: understanding seaweed-bacteria interactions. FEMS microbiology reviews 37:462–476.

Fernández-Marín, H., G. Bruner, E. B. Gomez, D. R. Nash, J. J. Boomsma, and W. T. Wcislo. 2013. Dynamic disease management in Trachymyrmex fungus-growing ants (Attini: Formicidae). The American Naturalist 181:571–582.

Forestier, C., C. De Champs, C. Vatoux, and B. Joly. 2001. Probiotic activities of *Lactobacillus casei rhamnosus*: *in vitro* adherence to intestinal cells and antimicrobial properties. Research in Microbiology 152:167–173.

Gagliardo, R., P. Crump, E. Griffith, J. Mendelson, H. Ross, and K. Zippel. 2008. The principles of rapid response for amphibian conservation, using the programmes in Panama as an example. International Zoo Yearbook 42:125–135.

Gallo, R. L., and T. Nakatsuji. 2011. Microbial symbiosis with the innate immune defense system of the skin. The Journal of Investigative Dermatology 131:1974–1980.

Gil-Turnes, M. S., and W. Fenical. 1992. Embryos of *Homarus americanus* are protected by epibiotic bacteria. Biological Bulletin 182:105–108.

Gram, L., T. Løvold, J. Nielsen, J. Melchiorsen, and B. Spanggaard. 2001. *In vitro* antagonism of the probiont *Pseudomonas fluorescens* strain AH2 against *Aeromonas salmonicida* does not confer protection of salmon against furunculosis. Aquaculture 199:1–11.

Guarner, F., and J.-R. Malagelada. 2003. Gut flora in health and disease. Lancet 361:512–519.

Harris, R. N., R. M. Brucker, J. B. Walke, M. H. Becker, C. R. Schwantes, D. C. Flaherty, B. A. Lam, D. C. Woodhams, C. J. Briggs, V. T. Vredenburg, and K. P. C. Minbiole. 2009. Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. ISME Journal 3:818–824.

Harris, R. N., T. Y. James, A. Lauer, M. A. Simon, and A. Patel. 2006. Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. EcoHealth 3:53–56.

Hilario, E., T. R. Buckley, and J. M. Young. 2004. Improved resolution on the phylogenetic relationships among *Pseudomonas* by the combined analysis of atp D, car A, rec A and 16S rDNA. Antonie van Leeuwenhoek 86:51–64.

Krediet, C. J., K. B. Ritchie, V. J. Paul, and M. Teplitski. 2013. Coral-associated micro-organisms and their roles in promoting coral health and thwarting diseases. Proceedings of the Royal Society B 280:20122328.

Langhammer, P. F., K. R. Lips, P. A. Burrowes, T. Tunstall, C. M. Palmer, and J. P. Collins. 2013. A fungal pathogen of amphibians, *Batrachochytrium dendrobatidis*, attenuates in pathogenicity with *in vitro* passages. PloS One 8:e77630.

Lauer, A., M. A. Simon, J. L. Banning, E. André, K. Duncan, and R. N. Harris. 2007. Common cutaneous bacteria from the eastern red-backed salamander can inhibit pathogenic fungi. Copeia 2007:630–640.

Lauer, A., M. A. Simon, J. L. Banning, B. A. Lam, and R. N. Harris. 2008. Diversity of cutaneous bacteria with antifungal activity isolated from female four-toed salamanders. ISME Journal 2:145–157.

Loudon, A. H., D. C. Woodhams, L. W. Parfrey, H. Archer, R. Knight, V. McKenzie, and R. N. Harris. 2014. Microbial community dynamics and effect of environmental microbial reservoirs on red-backed salamanders (*Plethodon cinereus*). ISME Journal 8:830–840.

McKenzie, V. J., R. M. Bowers, N. Fierer, R. Knight, and C. L. Lauber. 2012. Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. ISME Journal 6:588–596.

Mohapatra, S., T. Chakraborty, V. Kumar, G. DeBoeck, and K. N. Mohanta. 2013. Aquaculture and stress management: a review of probiotic intervention. Journal of Animal Physiology and animal nutrition 97:405–30.

Morrow, A. L., A. J. Lagomarcino, K. R. Schibler, D. H. Taft, Z. Yu, B. Wang, M. Altaye, M. Wagner, D. Gevers, D. V Ward, M. a Kennedy, C. Huttenhower, and D. S. Newburg. 2013.

Early microbial and metabolomic signatures predict later onset of necrotizing enterocolitis in preterm infants. Microbiome 1:13.

Murphy, P. J., S. St-Hilaire, and P. S. Corn. 2011. Temperature, hydric environment, and prior pathogen exposure alter the experimental severity of chytridiomycosis in boreal toads. Diseases of Aquatic Organisms 95:31–42.

Nagalingam, N. A., E. K. Cope, and S. V Lynch. 2013. Sinus microbiome diversity depletion and *Corynebacterium tuberculostearicum* enrichment mediates rhinosinusitis. Trends in Microbiology 21:485–492.

Naik, S., N. Bouladoux, C. Wilhelm, M. J. Molloy, R. Salcedo, W. Kastenmuller, C. Deming, M. Quinones, L. Koo, S. Conlan, S. Spencer, J. A. Hall, A. Dzutsev, H. Kong, D. J. Campbell, G. Trinchieri, J. A. Segre, and Y. Belkaid. 2012. Compartmentalized control of skin immunity by resident commensals. Science 337:1115–1119.

Poole, V. 2008. Project golden frog. Endangered Species Bulletin 33:7–10.

Probst, A. J., A. K. Auerbach, and C. Moissl-Eichinger. 2013. Archaea on human skin. PLoS One 8:e65388.

R Core Team. 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rebollar, E. A., M. C. Hughey, R. N. Harris, R. J. Domangue, D. Medina, R. Ibáñez, and L. K. Belden. 2014. The lethal fungus *Batrachochytrium dendrobatidis* is present in lowland tropical forests of far eastern Panamá. PLoS One 9:e95484.

Reid, G., J. A. Younes, H. C. Van der Mei, G. B. Gloor, R. Knight, and H. J. Busscher. 2011. Microbiota restoration: natural and supplemented recovery of human microbial communities. Nature Reviews Microbiology 9:27–38.

Van Rensburg, B. J., M. A. McGeoch, S. L. Chown, and A. S. Van Jaarsveld. 1999. Conservation of heterogeneity among dung beetles in the Maputaland Centre of Endemism, South Africa. Biological Conservation 88:145–153.

Richards-Zawacki, C. L. 2010. Thermoregulatory behaviour affects prevalence of chytrid fungal infection in a wild population of Panamanian golden frogs. Proceedings of the Royal Society B 277:519–528.

Rollins-Smith, L. A. 2009. The role of amphibian antimicrobial peptides in protection of amphibians from pathogens linked to global amphibian declines. Biochimica et Biophysica Acta 1788:1593–1599.

Rollins-Smith, L. A., J. P. Ramsey, J. D. Pask, L. K. Reinert, and D. C. Woodhams. 2011. Amphibian immune defenses against chytridiomycosis: impacts of changing environments. Integrative and Comparative Biology 51:552–562.

Sato, Y., B. L. Willis, and D. G. Bourne. 2013. Pyrosequencing-based profiling of archaeal and bacterial 16S rRNA genes identifies a novel archaeon associated with black band disease in corals. Environmental Microbiology 15:2994–3007.

Savage, A. E., and K. R. Zamudio. 2011. MHC genotypes associate with resistance to a frog-killing fungus. Proceedings of the National Academy of Sciences 108:16705–16710.

Schommer, N. N., and R. L. Gallo. 2013. Structure and function of the human skin microbiome. Trends in Microbiology 21:660–668.

Shu, Q., F. Qu, and H. S. Gill. 2001. Probiotic treatment using *Bifidobacterium lactis* HN019 reduces weanling diarrhea associated with rotavirus and *Escherichia coli* infection in a piglet model. Journal of Pediatric Gastroenterology and Nutrition 33:171–177.

Skerratt, L. F., L. Berger, R. Speare, S. Cashins, K. R. McDonald, A. D. Phillott, H. B. Hines, and N. Kenyon. 2007. Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. EcoHealth 4:125–134.

Someya, N., and K. Akutsu. 2009. Indigenous bacteria may interfere with the biocontrol of plant diseases. Die Naturwissenschaften 96:743–747.

Sørensen, T. 1948. A method of establishing groups of equal amplitude in plant sociology based on similarity of species and its application to analyses of the vegetation on Danish commons. Biologiske Skrifter 5:1–34.

Stecher, B., S. Chaffron, R. Käppeli, S. Hapfelmeier, S. Freedrich, T. C. Weber, J. Kirundi, M. Suar, K. D. McCoy, C. von Mering, A. J. Macpherson, and W.-D. Hardt. 2010. Like will to like: abundances of closely related species can predict susceptibility to intestinal colonization by pathogenic and commensal bacteria. PLoS Pathogens 6:e1000711.

Taylor, M. W., R. Radax, D. Steger, and M. Wagner. 2007. Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. Microbiology and Molecular Biology Reviews 71:295–347.

Taylor, S. K., E. S. Williams, E. T. Thorne, K. W. Mills, D. I. Withers, and A. C. Pier. 1999. Causes of mortality of the Wyoming toad. Journal of wildlife diseases 35:49–57.

Turnbaugh, P. J., M. Hamady, T. Yatsunenko, B. L. Cantarel, A. Duncan, R. E. Ley, M. L. Sogin, W. J. Jones, B. A. Roe, J. P. Affourtit, M. Egholm, B. Henrissat, A. C. Heath, R. Knight, and J. I. Gordon. 2009. A core gut microbiome in obese and lean twins. Nature 457:480–484.

Umile, T. P., P. J. McLaughlin, K. R. Johnson, S. Honarvar, A. L. Blackman, E. a. Burzynski, R. W. Davis, T. L. Teotonio, G. W. Hearn, C. a. Hughey, A. F. Lagalante, and K. P. C. Minbiole. 2014. Nonlethal amphibian skin swabbing of cutaneous natural products for HPLC fingerprinting. Analytical Methods DOI:10.1039/c4ay00566j.

Voyles, J., E. B. Rosenblum, and L. Berger. 2011. Interactions between *Batrachochytrium dendrobatidis* and its amphibian hosts: a review of pathogenesis and immunity. Microbes and infection / Institut Pasteur 13:25–32.

Wake, D. B., and V. T. Vredenburg. 2008. Are we in the midst of the sixth mass extinction? A view from the world of amphibians. Proceedings of the National Academy of Sciences 105:11466–11473.

Webster, N. S., and M. W. Taylor. 2012. Marine sponges and their microbial symbionts: love and other relationships. Environmental Microbiology 14:335–346.

White Jr, J., F. Torres, and S. Mónica (Eds.). 2010. Defensive mutualism in microbial symbiosis. CRC Press, Boca Raton.

Woodhams, D. C., R. A. Alford, C. J. Briggs, M. Johnson, C. Woodhams, A. Alford, and J. Briggs. 2008. Life-history trade-offs influence disease in changing climates: strategies of an amphibian pathogen. Ecology 89:1627–1639.

Zasloff, M. 2002. Antimicrobial peptides of multicellular organisms. Nature 415:389–395.

TABLES

Table 1. Sample sizes of treatment groups in relation to survival and infection outcome.

		treatment groups (n = x)					
		Bd	Pseudo1 +Bd	Pseudo2 +Bd	Chryseo +Bd	Steno +Bd	total
survival / infection outcome	died	4	5	5	2	7	**23**
	predicted to die	0	1	2	0	0	**3**
	cleared	4	0	0	1	0	**5**
	predicted to clear	0	1	0	4	1	**6**
	total	**8**	**7**	**7**	**7**	**8**	**37**

FIGURES

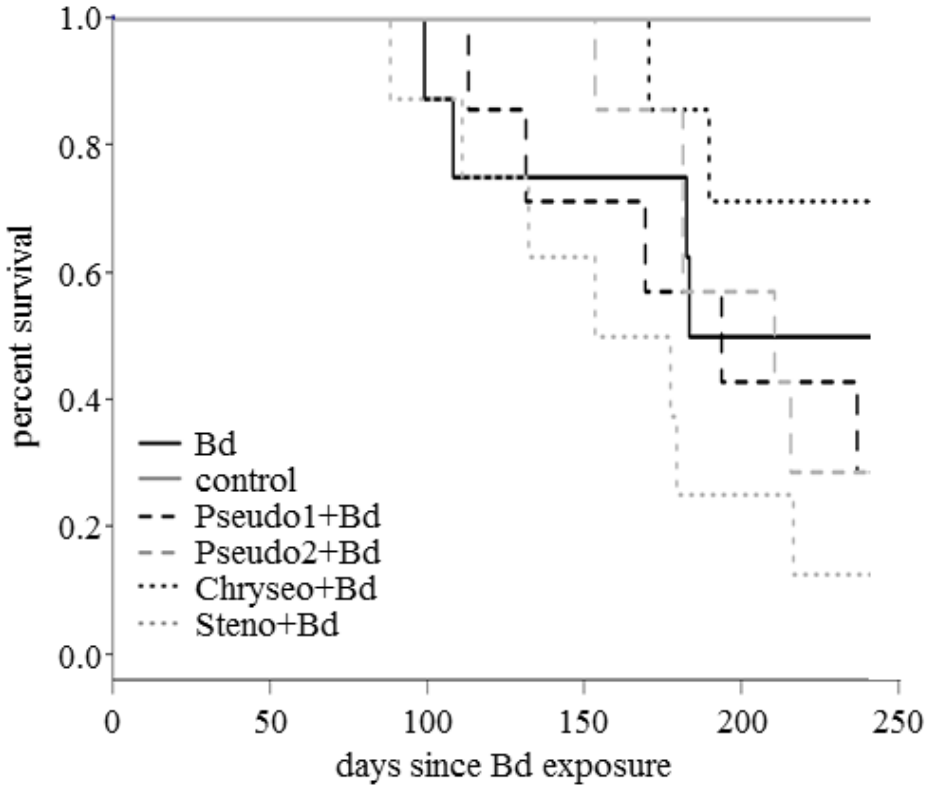

Figure 1. Survival of *Atelopus zeteki* treated with probiotics and exposed to Bd (probiotic+Bd), exposed only to Bd (Bd), and exposed to sterile water (control).

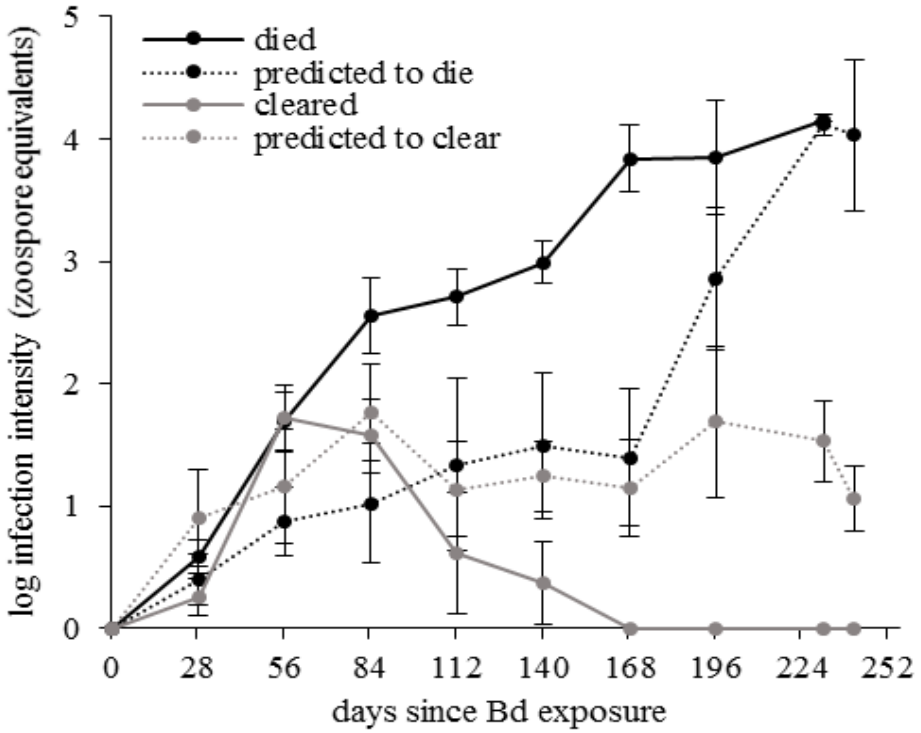

Figure 2. Average infection intensity (zoospore equivalents) of *Atelopus zeteki* that died of chytridiomycosis, were predicted to die of chytridiomycosis, cleared Bd infections, or were predicted to clear Bd infection. Error bars represent SE.

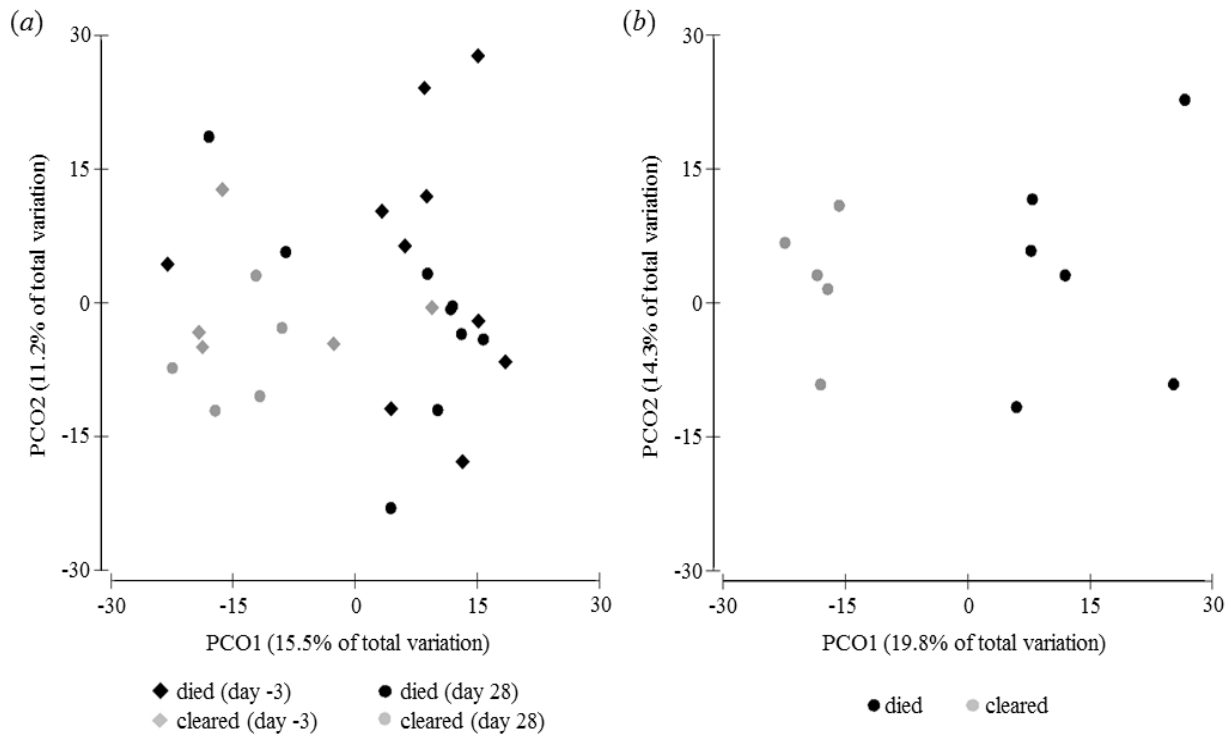

Figure 3. Principal coordinates plots of (a) Bray-Curtis distances between microbial communities present on *Atelopus zeteki* three days prior to Bd exposure and 28 days after exposure that died of chytridiomycosis or cleared Bd infection, and (b) Sørensen distances between metabolites present on frogs that died of chytridiomycosis or cleared Bd infection.

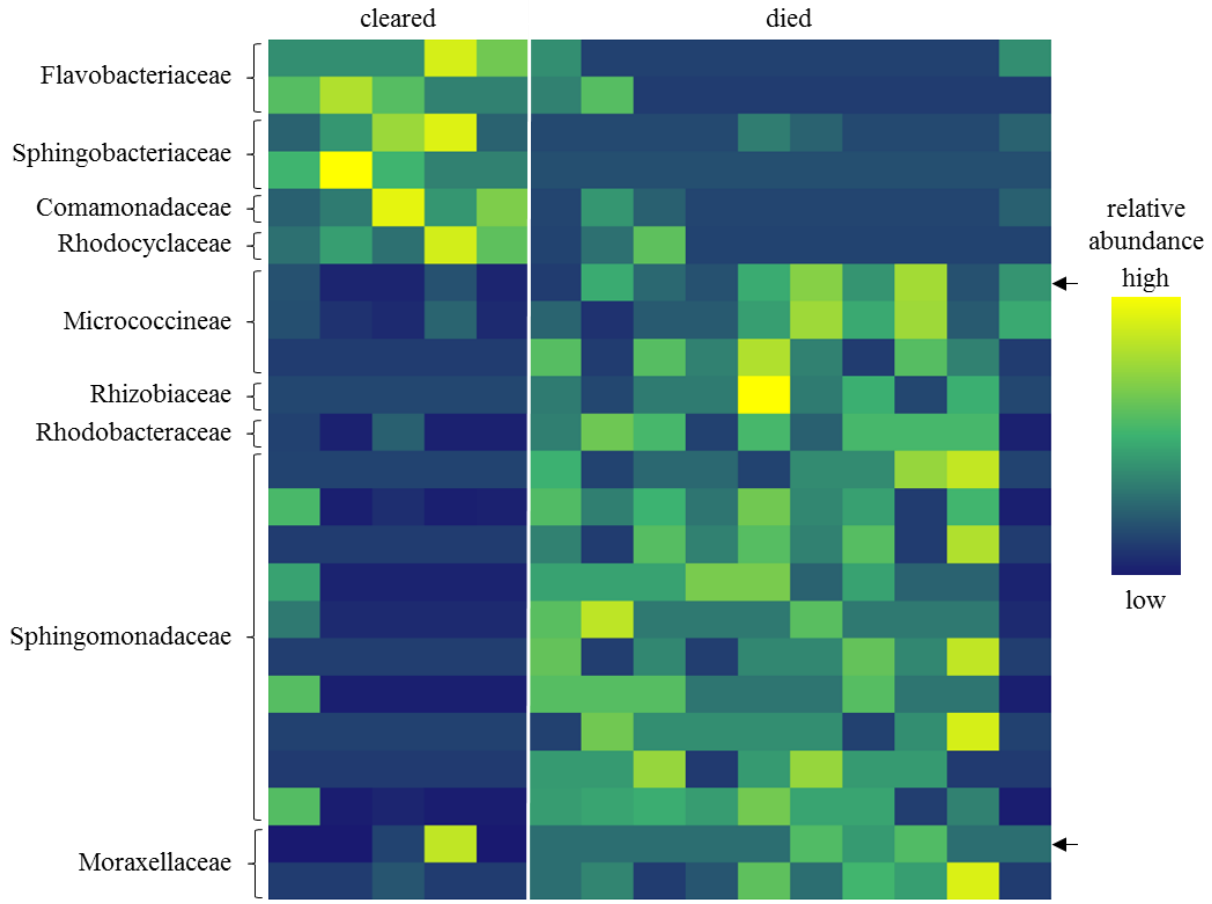

Figure 4. Heat map of the relative abundances of indicator OTUs from indicator species analysis associated with *Atelopus zeteki* three days prior to Bd exposure that cleared Bd infection or died of chytridiomycosis. Rows indicate unique OTUs and columns indicate individual frogs. Family level taxonomic classification is shown for each OTU. Arrows are indicator OTUs that were detected both three days prior to and 28 days after Bd exposure.

APPENDIX

Detailed 16S rRNA gene data processing methods

For the 16S amplicon data, overlapping forward and reverse paired reads were assembled with Fastq-join (https://code.google.com/p/ea-utils/wiki/FastqJoin) using default parameters and then processed with the Quantitative Insights Into Microbial Ecology (QIIME v. 1.7.0) pipeline (Caporaso et al. 2010b). Sequences were de-multiplexed and quality-filtered following methods similar to those in Bokulich et al. (2013). Specifically, sequences were discarded if there were any ambiguous base calls, errors in the barcode, less than 75% of read length had consecutive base calls with a phred quality score greater than 20, more than 10 consecutive low-quality base calls, or the read length was not between 252 and 255 bp. After quality filtering, the number of reads retained per sample ranged from 27,083 to 94,881. Quality-filtered sequences were then clustered into operational taxonomic units (OTUs, ~bacterial "species") at a sequence similarity threshold of 97% with the UCLUST method (Edgar 2010) and a minimum cluster size of 0.001% of the total reads (Bokulich et al. 2013). Sequences were first clustered against the Greengenes database (May 2013 release) (DeSantis et al. 2006). Sequences that did not match the database were then *de novo* clustered at a 97% sequence similarity threshold. The most abundant sequence for a given cluster was assigned as the representative sequence for that OTU. We assigned taxonomy for each OTU with RDP classifier (Wang et al. 2007) at an 80% confidence threshold and the Greengenes database. We aligned representative sequences to the Greengenes database with PyNAST (Caporaso et al. 2010a) and constructed a phylogenetic tree with FastTree (Price et al. 2009). All samples were rarefied to 27,000 sequences to standardize sampling effort. We used a closed reference OTU picking approach to determine if any Illumina sequences matched the sequences of the four candidate probiotics at a 99% sequence similarity.

Table A1. Bacterial isolates chosen for probiotic treatments.

Isolate ID	Classification	Bd inhibition[a]	Frog species[b]	Prevalence[c]	# of species[d]
Pseudo1	*Pseudomonas sp.*	100	*Bufo typhonius*	20/67	7/11
Pseudo2	*Pseudomonas sp.*	100	*Craugastor crassidigitus*	10/67	5/11
Chryseo	*Chryseobacterium sp.*	100	*Craugastor crassidigitus*	2/67	2/11
Steno	*Stenotrophomonas sp.*	98	*Atelopus limosus*	3/67	3/11

[a]Percent inhibition calculated based on growth of Bd in the presence of the bacterial isolate's cell-free supernatant compared to Bd grown without cell-free supernatant (Bell et al. 2013).
[b]Species from which the bacterial isolate was collected.
[c]Prevalence of bacterial isolate among sampled amphibians.
[d]Number of amphibian species from which the isolate was collected.

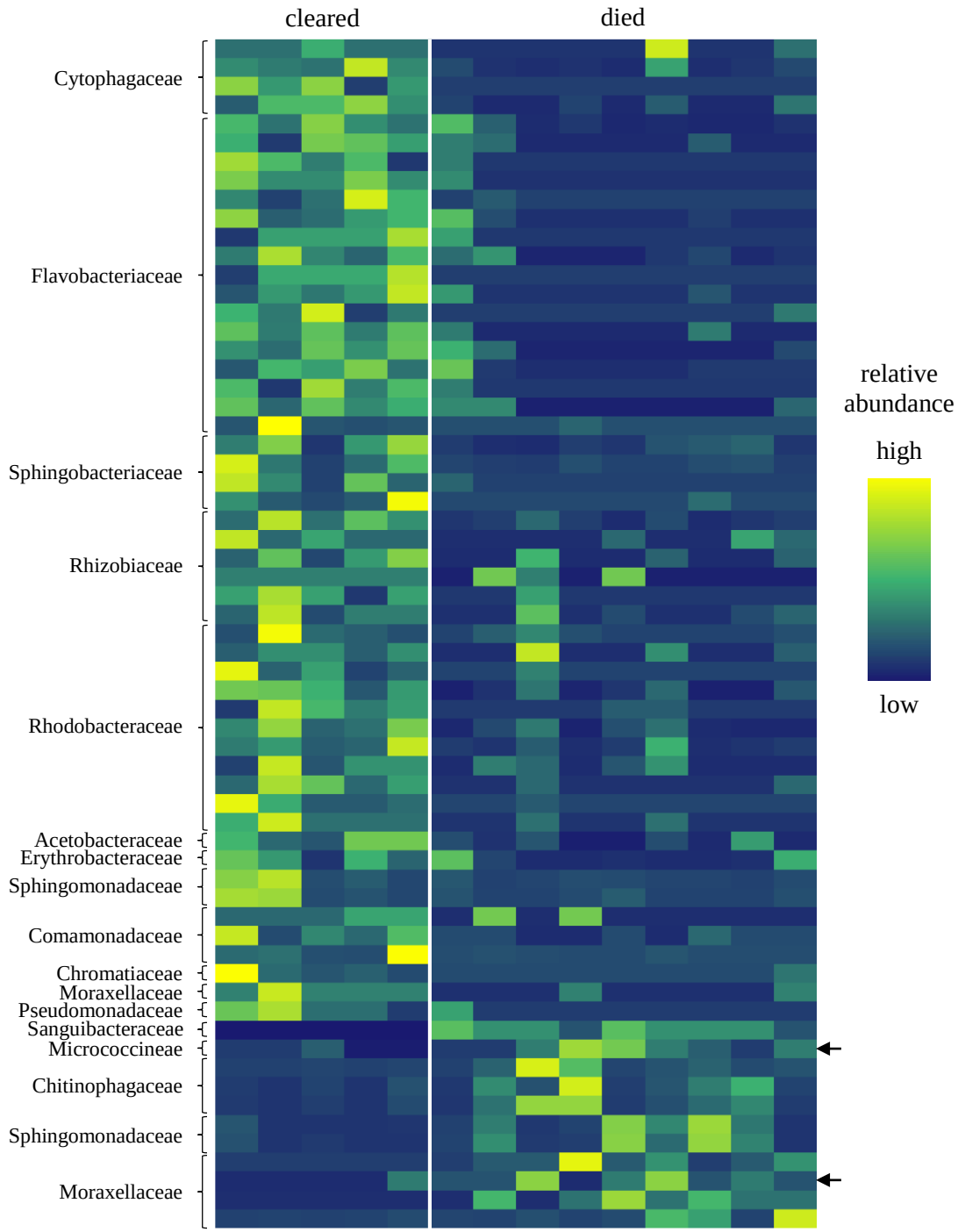

Figure A1. Heat map of the relative abundances of indicator OTUs from indicator species analysis associated with *Atelopus zeteki* 28 days after Bd exposure that cleared Bd infection or died of chytridiomycosis. Rows indicate unique OTUs and columns indicate individual frogs. Family level taxonomic classification is shown for each OTU. Arrows are indicator OTUs that were detected both three days prior to and 28 days after Bd exposure.

Literature cited

Bokulich, N. A., S. Subramanian, J. J. Faith, D. Gevers, J. I. Gordon, R. Knight, D. A. Mills, and J. G. Caporaso. 2013. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. Nature Methods 10:57–59.

Caporaso, J. G., K. Bittinger, F. D. Bushman, T. Z. DeSantis, G. L. Andersen, and R. Knight. 2010a. PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics 26:266–267.

Caporaso, J. G., J. Kuczynski, J. Stombaugh, K. Bittinger, F. D. Bushman, E. K. Costello, N. Fierer, A. G. Peña, J. K. Goodrich, J. I. Gordon, G. A. Huttley, S. T. Kelley, D. Knights, J. E. Koenig, R. E. Ley, C. A. Lozupone, D. McDonald, B. D. Muegge, M. Pirrung, J. Reeder, J. R. Sevinsky, P. J. Turnbaugh, W. A. Walters, J. Widmann, T. Yatsunenko, J. Zaneveld, and R. Knight. 2010b. QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7:335–336.

DeSantis, T. Z., P. Hugenholtz, N. Larsen, M. Rojas, E. L. Brodie, K. Keller, T. Huber, D. Dalevi, P. Hu, and G. L. Andersen. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Applied and Environmental Microbiology 72:5069–5072.

Edgar, R. C. 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26:2460–2461.

Price, M. N., P. S. Dehal, and A. P. Arkin. 2009. FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. Molecular Biology and Evolution 26:1641–1650.

Wang, Q., G. M. Garrity, J. M. Tiedje, and J. R. Cole. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Applied and Environmental Microbiology 73:5261–5267.

CHAPTER V. CONCLUSIONS

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Approximately 40% of all known amphibian populations are rapidly declining around the world (Stuart et al. 2004). These unprecedented declines have illustrated how ill-equipped the conservation community is to manage severe threats to biodiversity. Since the discovery of Bd in the late 1990's, conservation organizations have struggled to assemble the necessary resources to mitigate the threats imposed by Bd. The only action conservationists have been able take to prevent the extinction of a few species affected by chytridiomycosis is to place highly susceptible species into captive survival assurance colonies. However, non-profit organizations and zoos do not have the resources to take care of the vast number of species affected by Bd, and such a response lacks a positive long-term re-introduction outcome at this point, as Bd continues to remain in native frog habitats. Conservation actions are needed that will help us mitigate Bd and sustain amphibian biodiversity in the field, as well as helping to begin reintroductions of species in captive colonies.

Building on previous studies that have shown the importance of cutaneous microbes to the health of amphibians, my dissertation research presents an approach to investigating the role amphibian symbiotic microbes have in protecting their host from chytridiomycosis. I also developed a framework for the use of beneficial bacteria as probiotics for highly susceptible species, such as the Panamanian golden frog (*Atelopus zeteki*). To accomplish this, I developed the following strategy: First, determine the variety of bacterial taxa that can persist on the skin of *A. zeteki* under natural conditions (Chapter II). Second, target amphibian species that are persisting with Bd in the historical geographical range of *A. zeteki* and isolate bacteria from their skin (Chapter III). Third, test the effectiveness of these bacteria to inhibit Bd with *in vitro* assays (Chapter III). Fourth, choose highly inhibitory bacteria that have a history of being able to grow on *A. zeteki* skin (Chapter II) and experimentally test their effectiveness to prevent chytridiomycosis in *A. zeteki* (Chapter IV).

In Chapter II, using the latest sequencing technology, I characterized the microbiota that were naturally associated with wild golden frogs to determine the types of bacterial taxa that

might be successful as probiotics to prevent chytridiomycosis in this frog species. I found that the microbial community composition of dominant microbiota on *A. zeteki* is very similar to that of North American amphibians at higher taxonomic levels, such as phylum and class; however, at lower taxonomic levels there are large structural and compositional differences (McKenzie et al. 2012, Kueneman et al. 2013, Walke et al. 2014). One important difference is the absence of the anti-Bd bacteria *Janthinobacterium lividum*, which is important if defending some North American species from chytridiomycosis (Becker et al. 2009, Harris et al. 2009a). This may also explain why *J. lividum* was unsuccessful at persisting and preventing chytridiomycosis in a previous probiotic experiment with *A. zeteki* (Becker et al. 2012).

In Chapter II, I also found that captive management can change the structure of *A. zeteki* skin microbiota, but the composition of these communities largely remains the same. However, it remains unclear how the original microbiota from wild populations persists after a generation in captivity. Future work in this area should focus on how the microbiota of other species responds to captive conditions and how amphibians obtain their cutaneous microbiota. The latter will shed light on ways to minimize changes to symbiotic microbial communities in captivity. In addition, we do not know how the microbial community of *A. zeteki* will respond when frogs are reintroduced into native habitats. It may be possible that their microbial community will return to their preexisting structure even following multiple generations in captivity. Results from this work could have important implications for how amphibians are housed in captivity. Additionally, reducing shifts in the microbial community of amphibians while in captivity could mitigate vulnerability to other pathogens that are inhibited by the natural microbiota.

In Chapter III, I demonstrated that amphibians persisting with Bd in Panama have a taxonomically diverse set of anti-Bd bacteria. I also found that this functional trait is seen throughout the phylogeny of bacteria observed in this study and differs even at the strain level. Since taxonomic identity has very little power to predict ability to inhibit Bd, this has important implications for studies relying solely on 16s rRNA gene sequencing to correlate microbial community structure to disease outcome of the host. Therefore, future research in this area should focus on determining the differences in the functional ability of microbiota to inhibit Bd among amphibian species that differ in their susceptibility to chytridiomycosis. Unfortunately, my study was limited in sample sizes to make any conclusions on this. In addition, this research

question would greatly benefit by using culture-independent techniques, such as Illumina sequencing, to investigate functional genes of cutaneous microbiota. In this chapter, I also assembled a library of anti-Bd bacteria and a framework for probiotic discovery that could be used for other amphibian species affected by chytridiomycosis. However, since we are limited to using culturable bacteria for probiotics, future research should also focus on determining the percent of bacteria we are able to culture using our current methods. In other systems, the percent of bacteria actually cultured is extremely low (0.001-15%, Amann et al. 1995, Schleifer 2004).

In Chapter IV, I synthesize the results from Chapter II and Chapter III to provide a framework for selecting an effective probiotic for *A. zeteki*. I also present results that demonstrate that *in vitro* bacterial activity against Bd (Chapter III) does not correlate to *in vivo* prevention of chytridiomycosis. This may have been due to the inability of the selected probiotics to persist on the skin of *A. zeteki*. This was surprising given that bacterial species closely related to the probiotics used were found previously on wild and captive *A. zeteki* (Chapter II). It is likely that probiotics were unable to persist on the skin due to interactions with the host's immune defenses and with microbes already present on the skin because of the methods used to treat *A. zeteki* with the probiotics. For example, in the experiment, I treated frogs with a concentrated solution of probiotic bacteria for a short period of time; such a large dose may have stimulated the immune system and thus prevented colonization. Therefore, future research in this area should focus on effective ways to treat amphibians with probiotics. Providing a continual source of the probiotic within the environment of the amphibian has been successful with the salamander *Plethodon cinereus* (Muletz et al. 2012). However, this may not be practical in a field setting. I believe that the success of the framework for finding and selecting a probiotic to prevent chytridiomycosis presented in this dissertation relies on establishing a successful treatment method.

In Chapter IV, I also present results that correlate survival with the microbial communities and metabolites initially present on *A. zeteki*. The survival of infected golden frogs is surprising because in prior laboratory experiments this species rarely survives chytridiomycosis (1 out of 228 infected frogs, Becker et al. 2012, Bustamante et al. 2010, Langhammer et al. 2013). These results have important implications for strategies to be used in future studies investigating a way to mitigate the threat of Bd in *A. zeteki*. For instance, studies

are needed to confirm the effect of microbial community structure on Bd-resistance. This could be accomplished by infecting *A. zeteki* that have microbial communities both similar and different to those presented in Chapter IV and monitoring their survival. If individuals survive infection, they could be re-infected to determine if the trait to prevent chytridiomycosis is stable over time. Ultimately, identifying stable, heritable defense mechanisms of Bd resistance may give conservationists a tool to establish colonies of Bd-resistant amphibians that could be reintroduced into the wild.

New techniques provided by Woodhams et al. (2014) could also have important implications on how research in amphibian conservation continues. In this study, the authors developed an assay to test the effectiveness of the amphibian mucosome against Bd and correlate mucosome function against Bd with susceptibility to chytridiomycosis. The authors' define the mucosome as the "micro-ecosystem of the mucus" and it contains host cells and secretions, such as antimicrobial peptides and mucosal antibodies, as well as microbial secretions, such as antifungal metabolites. This assay could aid in the selection of probiotics for *A. zeteki* by allowing us to test if the mucosome of golden frogs is able to inhibit potential probiotic bacteria, thereby allowing us to disregard probiotics that are incompatible with the host or the host's microbiota. Then we could examine whether probiotic therapy with compatible anti-Bd bacteria increase the mucosome function against Bd. This new approach will improve the framework for probiotic development presented in this dissertation.

This new framework is as follows: First, identify target amphibian species for probiotic therapy. Second, isolate bacteria from wild amphibians persisting with Bd originating from the site chosen for reintroductions. Third, test the effectiveness of the isolated bacteria to inhibit Bd with the *in vitro* assay presented in Chapter III. Fourth, test the compatibility of the resulting anti-Bd isolates with the mucosome of the target frog species (Woodhams et al. 2014). Fifth, treat the target frog species with anti-Bd bacteria that are compatible with the target frog species to determine if mucosome function against Bd increases. Lastly, infect frogs treated with anti-Bd bacteria that increase mucosome function with Bd. If long-term prevention of chytridiomycosis is achieved then mesocosm and field trials could be conducted.

Overall the results presented here demonstrate that the structure of symbiotic microbial communities of *A. zeteki* is important to host health, and building on this might provide the best hope for reintroducing this iconic species back to its native habitat.

LITERATURE CITED

Amann, R. I., W. Ludwig, K. H. Schleifer, R. I. Amann, and W. Ludwig. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiological Reviews 59:143–169.

Becker, M. H., R. M. Brucker, C. R. Schwantes, R. N. Harris, and K. P. C. Minbiole. 2009. The bacterially produced metabolite violacein is associated with survival of amphibians infected with a lethal fungus. Applied and Environmental Microbiology 75:6635–6638.

Becker, M. H., R. N. Harris, K. P. C. Minbiole, C. R. Schwantes, L. A. Rollins-Smith, L. K. Reinert, R. M. Brucker, R. J. Domangue, and B. Gratwicke. 2012. Towards a better understanding of the use of probiotics for preventing chytridiomycosis in Panamanian golden frogs. EcoHealth 8:501–506.

Bustamante, H. M., L. J. Livo, and C. Carey. 2010. Effects of temperature and hydric environment on survival of the Panamanian golden frog infected with a pathogenic chytrid fungus. Integrative Zoology 5:143–153.

Harris, R. N., R. M. Brucker, J. B. Walke, M. H. Becker, C. R. Schwantes, D. C. Flaherty, B. A. Lam, D. C. Woodhams, C. J. Briggs, V. T. Vredenburg, and K. P. C. Minbiole. 2009. Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. ISME Journal 3:818–824.

Kueneman, J. G., L. Wegener Parfrey, D. C. Woodhams, H. M. Archer, R. Knight, and V. J. McKenzie. 2013. The amphibian skin-associated microbiome across species, space and life history stages. Molecular Ecology:1238–1250.

Langhammer, P. F., K. R. Lips, P. A. Burrowes, T. Tunstall, C. M. Palmer, and J. P. Collins. 2013. A fungal pathogen of amphibians, *Batrachochytrium dendrobatidis*, attenuates in pathogenicity with *in vitro* passages. PloS One 8:e77630.

McKenzie, V. J., R. M. Bowers, N. Fierer, R. Knight, and C. L. Lauber. 2012. Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. ISME Journal 6:588–596.

Muletz, C. R., J. M. Myers, R. J. Domangue, J. B. Herrick, and R. N. Harris. 2012. Soil bioaugmentation with amphibian cutaneous bacteria protects amphibian hosts from infection by *Batrachochytrium dendrobatidis*. Biological Conservation 152:119–126.

Schleifer, K.-H. 2004. Microbial diversity: facts, problems and prospects. Systematic and Applied Microbiology 27:3–9.

Stuart, S. N., J. S. Chanson, N. A. Cox, B. E. Young, A. S. L. Rodrigues, D. L. Fischman, and R. W. Waller. 2004. Status and trends of amphibian declines and extinctions worldwide. Science 306:1783–1786.

Walke, J. B., M. H. Becker, S. C. Loftus, L. L. House, G. Cormier, R. V Jensen, and L. K. Belden. 2014. Amphibian skin may select for rare environmental microbes. ISME Journal:doi:10.1038/ismej.2014.77.

Woodhams, D. C., H. Brandt, S. Baumgartner, J. Kielgast, E. Küpfer, U. Tobler, L. R. Davis, B. R. Schmidt, C. Bel, S. Hodel, R. Knight, and V. McKenzie. 2014. Interacting symbionts and immunity in the amphibian skin mucosome predict disease risk and probiotic effectiveness. PLoS One 9:e96375.