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Original Contribution

Towards a Better Understanding of the Use of Probiotics for Preventing Chytridiomycosis in Panamanian Golden Frogs

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Abstract: Populations of native Panamanian golden frogs (*Atelopus zeteki*) have collapsed due to a recent chytridiomycosis epidemic. Reintroduction efforts from captive assurance colonies are unlikely to be successful without the development of methods to control chytridiomycosis in the wild. In an effort to develop a protective treatment regimen, we treated golden frogs with *Janthinobacterium lividum*, a skin bacterium that has been used to experimentally prevent chytridiomycosis in North American amphibians. Although *J. lividum* appeared to colonize *A. zeteki* skin temporarily, it did not prevent or delay mortality in *A. zeteki* exposed to *Batrachochytrium dendrobatidis*, the causative agent of chytridiomycosis. After introduction of *J. lividum*, average bacterial cell counts reached a peak of 1.7×10^6 cells per frog $\sim$2 weeks after treatment but declined steadily after that. When *J. lividum* numbers declined to $\sim 2.8 \times 10^5$ cells per frog, *B. dendrobatidis* infection intensity increased to greater than 13,000 zoospore equivalents per frog. At this point, frogs began to die of chytridiomycosis. Future research will concentrate on isolating and testing antifungal bacterial species from Panama that may be more compatible with *Atelopus* skin.

Keywords: *Batrachochytrium dendrobatidis*, Chytridiomycosis, *Atelopus zeteki*, *Janthinobacterium lividum*, probiotic

INTRODUCTION

Of the 113 harlequin frog species or candidates for description, 30 are presumed extinct and all but 10 species are in decline due to the devastating effects of the amphibian disease chytridiomycosis (La Marca et al. 2005). Panama's harlequin frogs are no exception, and the western most species were severely impacted by the spreading wave of *Batrachochytrium dendrobatidis* (*Bd*), the pathogen responsible for chytridiomycosis (Lips et al. 2008a). *Atelopus chiriquiensis* has not been sighted since 1996 and is feared to be extinct (Lips et al. 2008b). *A. zeteki*, *A. varius*, and *A. limosus*

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have all undergone significant chytridiomycosis-related declines and a similar fate awaits the currently healthy populations of *A. glyphus* and *A. certus* from *Bd*-free habitats in eastern parts of the country (R. Ibanez, pers. comm.). We currently have no tools to control or prevent this disease in the wild, leaving the creation of captive assurance colonies as our only viable tool to conserve some species at this time. Approximately 2,000 *A. zeteki* are now maintained in 50 North American zoos and aquaria and are managed through a Species Survival Program with the ultimate goal of returning these species to their native habitats (Poole 2008; K. Murphy, pers. comm.). Similarly, the Panama Amphibian Rescue Project has established ex-situ assurance populations of all extant *Atelopus* species in Panama, and two in-country facilities now hold founding populations of *A. zeteki*, *A. varius*, *A. limosus*, *A. glyphus*, and *A. certus*. Captive programs have demonstrated some success, but it is unlikely that reintroductions from captive populations will succeed without the development of a way to mitigate the threat of chytridiomycosis, which persists in surviving frog populations that act as a reservoir of the disease in the environment.

Given the unfolding *Bd* crisis in Panama and around the world (Bosch and Martínez-Solano 2006; Lips et al. 2008a; Voyles et al. 2009; Vredenburg et al. 2010) development of tools to mitigate chytridiomycosis in a wild situation is a top priority (Woodhams et al. 2011). Recent studies have identified members of the microbial community colonizing the skin of some amphibian species that have antagonistic effects against *Bd* and other fungal pathogens (Harris et al. 2006; Banning et al. 2008; Lauer et al. 2008; Becker and Harris 2010). In addition, experiments demonstrated that using the inhibitory bacteria *Janthinobacterium lividum*, which produces the anti-*Bd* metabolite violacein, as a probiotic on amphibian skin reduced or prevented chytridiomycosis infections in an anuran *Rana muscosa* (Harris et al. 2009) and a caudate *Plethodon cinereus* (Becker et al. 2009). The use of *J. lividum* to reduce mortality and morbidity in these amphibians suggests a promising tool that could eventually facilitate reintroductions. We conducted an experimental trial to test whether *J. lividum* would be effective at preventing chytridiomycosis in *A. zeteki*.

Material and Methods

Fifty-four surplus juvenile *A. zeteki* individuals were obtained from the Houston Zoo with assistance from the golden frog Species Survival Program. Frogs were housed individually in autoclaved plastic containers with an autoclaved damp paper towel and 225 ml of reverse osmosis (RO) water that was re-constituted with essential minerals and salts. All individuals were randomly assigned a position on metal racks in a climate-controlled room with a temperature of 21°C and a 12:12 h light dark cycle. On a daily basis, frogs were fed fruit flies and crickets ad libitum, misted with RO water, and feces were removed by hand. Containers were changed and cleaned with a 10% bleach solution weekly. Frogs were checked twice daily to monitor behavior and death.

A replicated randomized experiment was conducted with four treatment groups: *Bd* group ($n = 20$), exposure to 3,000 zoospores diluted in RO water; bacteria group ($n = 7$), exposure to 1.1×10^7 *J. lividum* cells diluted in RO water; bacteria + *Bd* group ($n = 20$), exposure to 1.1×10^7 *J. lividum* cells prior to being exposed to 3000 zoospores; control group ($n = 7$), exposure to RO water containing no organisms. The *Bd* strain JEL 310 was used for all pathogen exposures. The strain of *J. lividum* used was isolated from the skin of the salamander *Hemidactylium scutatum* collected in the George Washington National Forest, Rockingham Co., VA, USA. Exposures and culturing of *B. dendrobatidis* and *J. lividum* followed procedures described previously by Harris et al. (2009), except that individuals were exposed to a 1.5% solution of hydrogen peroxide for 25 s one day prior to inoculation with *J. lividum* in order to reduce any pre-existing microbes that may inhibit colonization.

Frogs were swabbed with sterile cotton swabs ten times on the ventral surface, thighs and feet prior to experimental exposures to either *Bd* or *J. lividum*, 13 days after exposure to *Bd*, and approximately every 2 weeks thereafter for 120 days (Harris et al. 2009). Frogs were also swabbed at time of death, or shortly thereafter. Swabs from days 13, 26, 39, 64, 95, and at death were chosen to examine the cell abundances of *J. lividum* and *Bd*. DNA from these swabs was extracted using PrepMan Ultra (Applied Biosystems) and was used to determine the approximate number of *J. lividum* cells present on the skin using real-time PCR procedures described by Becker et al. (2009). The same DNA was amplified using TaqMan real-time PCR to determine the infection intensity of *Bd* on the skin following procedures outlined by Boyle et al. (2004).

The experiment ended 120 days after exposure to *Bd*, when all surviving frogs were euthanized by subcutaneous injection with 1% tricaine methanesulfonate (target dose of 300 mg/kg intracoelomically). Immediately following

euthanasia or death due to *Bd*, the skin between the shoulders and hips of each individual was excised to determine the concentration of violacein on the skin using HPLC (Brucker et al. 2008).

Differences in survival were tested with a Mantel–Cox log-rank test. Cell abundance data for *J. lividum* and *Bd* were not distributed normally and transformations did not result in normality. Tests of normality (Shapiro–Wilk) on the distributions of the data (cell counts and log-transformed cell counts) were significant ($P < 0.05$) for both treatment groups at day 13 and for the *Bd* group at day 26. Therefore, a nonparametric repeated measures procedure using ANOVA-type statistics or ATS (Brunner et al. 1999; Brunner and Puri 2001; Shah and Madden 2004) was used to analyze repeated *J. lividum* cell counts and *Bd* loads. The ATS compares "relative treatment effects" of the groups at the different days. Relative treatment effects range from 0 to 1 with larger (smaller) values indicating a tendency for values in a group on a particular day to be larger (smaller) than values pooled across all groups and days. ATS calculations were done with a SAS macro downloaded from the website of E. Brunner (http://www.ams.med.uni-goettingen.de/amsneu/sasmakr-de.shtml). Differences in *Bd* load at time of death were not normally distributed and were tested with a nonparametric Wilcoxon test.

Results

Control frogs and frogs exposed to *J. lividum* exhibited 100% survival over the length of the experiment. Frogs in *Bd* and bacteria + *Bd* treatment groups displayed a high and equal rate of mortality beginning at day 50 (Fig. 1; log-rank test: χ^2 (1, $n = 40$) = 0.466, $P = 0.495$).

Colonization of *J. lividum* was successful on 19 out of 20 treated frogs reaching an average cell count of 1.7×10^6 cells per frog (SE: 7.1×10^5; range: 0–1.2×10^7) about two weeks after treatment (Fig. 2). Thirty-nine days after treatment, when *J. lividum* cell numbers had declined to less than a mean of 2.8×10^5 cells per frog (SE: 1.8×10^5; range: 0–3.3×10^6), *Bd* cell numbers increased to above 13,000 zoospore equivalents per frog (SE: 5,500; range: 0–98,000). Frogs began to die of chytridiomycosis shortly after this time. *J. lividum* was also detected on 16 out of 20 frogs in the *Bd* treatment group at significantly lower cell numbers than frogs in the bacteria + *Bd* treatment (Fig. 2; ATS: $F(1, 31.558) = 7.899$, $P = 0.008$). *Bd* load in the *Bd* treatment was not significantly different than the load in

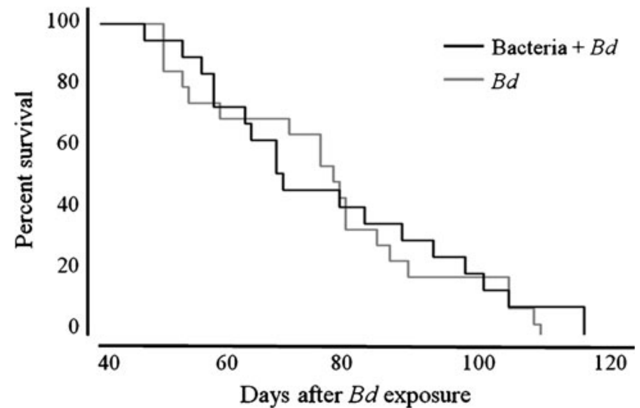

Fig. 1. Survival patterns of frogs treated with *Janthinobacterium lividum* prior to exposure to *Batrachochytrium dendrobatidis* (Bacteria + *Bd*, $n = 20$) and frogs exposed to *B. dendrobatidis* with no prior treatment (*Bd*, $n = 20$) over 118 days after exposure to *B. dendrobatidis*. Log-rank test: χ^2 (1, $n = 40$) = 0.466, $P = 0.495$.

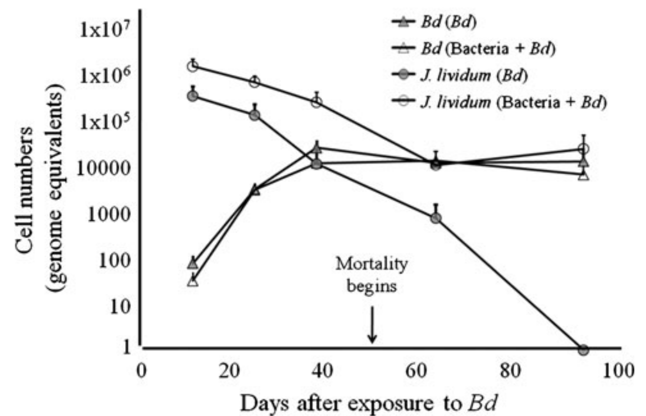

Fig. 2. Number of *Batrachochytrium dendrobatidis* cells (*triangles*) and number of *Janthinobacterium lividum* cells (*circles*) present on the skins of frogs treated with *J. lividum* prior to exposure to *B. dendrobatidis* (bacteria + *Bd*, for days 13, 26, 39, 64, and 95 $n = 20$, 20, 20, 14, and 8, respectively) and frogs exposed to *B. dendrobatidis* with no prior treatment (*Bd*, for days 13, 26, 39, 64, and 95 $n = 20$, 20, 20, 15, and 5, respectively) over time. Shown are means and SE. *B. dendrobatidis* cells, ATS: $F(1, 37.982) = 0.809$, $P = 0.374$. *J. lividum* cells, ATS: $F(1, 31.558) = 7.899$, $P = 0.008$.

the bacteria + *Bd* treatment (Fig. 2; ATS: $F(1, 37.982) = 0.809$, $P = 0.374$). However, the *Bd* load at time of death was significantly lower on frogs treated with *J. lividum* than untreated frogs (Fig. 3; Wilcoxon test: Z ($n = 36$) = -2.282, $P = 0.023$).

Violacein was not detected on any of the frogs when they died or at the end of the experiment. Analytical methods furnished a minimum detection threshold for violacein at ~10 μM, below which no violacein could be identified.

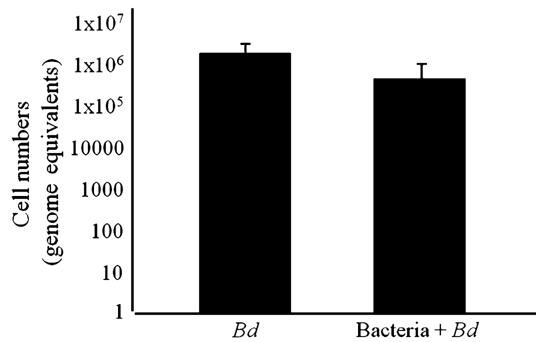

Fig. 3. *Batrachochytrium dendrobatidis* infection intensity (no. of cells) on the skins of frogs treated with *Janthinobacterium lividum* prior to exposure to *B. dendrobatidis* (bacteria + *Bd*, $n = 17$) and frogs exposed to *B. dendrobatidis* with no prior treatment (*Bd*, $n = 19$) at time of death. Wilcoxon test: $Z = -2.282$, $P = 0.023$.

Discussion

Although treating the North American frog *R. muscosa* with *J. lividum* was successful in preventing chytridiomycosis (Harris et al. 2009), we found that treating *A. zeteki* with the anti-*Bd* bacterium was unsuccessful in preventing mortality in *Bd*-infected individuals (Fig. 1). *J. lividum* in the *Bd* treatment group and the bacteria + *Bd* treatment group did not persist and did not protect frogs in either group. When deaths due to *Bd* began to occur, the number of *J. lividum* cells were quite low (Fig. 2). *Bd* burden at time of death was significantly lower on *J. lividum* treated frogs than untreated frogs suggesting that treatment may have helped reduce *Bd* loads, but not enough to prevent mortality (Fig. 3). *Bd* loads increased between day 95, when there was no difference between treatments, and time of death, when *Bd* loads were higher on frogs in the *Bd* treatment, with the increase being much higher in the bacteria + *Bd* treatment (Figs. 2, 3). This pattern can be explained by *J. lividum* cell abundance data on day 95. Swabs from this day indicate that the *Bd* treatment had no detectable *J. lividum* cells, whereas the bacteria + *Bd* treatment had an average of 26,000 cells. The higher density of *J. lividum* in the bacteria + *Bd* treatment limited the growth of *Bd*; however, the limitation was not enough to prevent *Bd* from exceeding a mortality threshold that caused the frogs to die. Alternatively, the differences in *Bd* loads at death between treatments may indicate that treating frogs with *J. lividum* decreased their tolerance to the effects of chytridiomycosis. However, glucocorticoid metabolite concentrations (an indicator of stress) were significantly lower in frogs treated with *J. lividum* and exposed to *Bd* than frogs only exposed to *Bd* (S. Putman, unpublished results). This hypothesis should be interpreted with caution since glucocorticoid metabolite concentrations do not fully measure tolerance to infection.

The unexpected presence of *J. lividum* on frogs that were not treated with the bacterial species, including frogs in the control treatment (data not shown), does not affect the results of this experiment. There was no contamination of *Bd* in the experiment, which suggests that our protocols to minimize contamination were effective. We believe that *J. lividum* came in with the frogs from their original location and was present before the experiment began. Continual transmission from sources in the frogs' original captive environment may explain the high number of cells found on their skins at the beginning of the experiment. Throughout the experiment, frogs were placed in sterile environments, which may explain why *J. lividum* loads quickly decreased. Possible sources of contamination before the experiment began were water in the frog's captive environment and human skin, where *Janthinobacterium* species are commonly found (Saeger and Hale 1993; Grice et al. 2008).

The inability of *J. lividum* to prevent colonization of *Bd*, while it was present, may be a result of the inability of *J. lividum* to produce violacein, a metabolite responsible for *Bd* growth inhibition (Brucker et al. 2008; Becker et al. 2009). In pure cultures of *J. lividum*, violacein production begins during the stationary phase of growth, when population densities are high (Pantanella et al. 2007). In addition, violacein production requires the oxidation of tryptophan and is regulated by the carbon source available to the bacteria in the local habitat (Hoshino 2011; Pantanella et al. 2007). Consequently, if tryptophan was present at low concentrations or if populations of *J. lividum* did not reach a stationary growth phase, violacein production would not have occurred. Evidence of this was the failure to detect violacein on the skin of dead or euthanized frogs.

From this study, it is evident that the skin of tropical *A. zeteki* is an unsuitable habitat for *J. lividum*, explaining the lack of persistence and protection from *Bd*. The failure of *J. lividum* to persist on *A. zeteki* skin may be a direct result of interactions of the anti-*Bd* bacteria with the host's defenses and other microbes present on the skin. *A. zeteki* produces various skin toxins, such as zetekitoxins, that could potentially affect bacterial growth (Yotsu-Yamashita et al. 2004). In this study, zetekitoxins were detected on experimental frogs at low concentrations. However, when tested in vitro, *J. lividum* did not experience growth inhibition when exposed to high concentrations of the toxins

(M.H. Becker, unpublished results). Toxins other than zetekitoxins, such as antimicrobial peptides may have inhibited the persistence of *J. lividum*. Many anuran skin antimicrobial peptides inhibit various bacteria and fungi, including *Bd* (Reviewed in Conlon 2011). However, few or no conventional antimicrobial peptides have been detected on *A. varius*, a closely related species to *A. zeteki* (Woodhams et al. 2006). Even though the initial microbial community was reduced with hydrogen peroxide, interactions with remaining resident skin microbes likely play a large role in the lack of persistence of *J. lividum*. Resistance to invasion may be a direct result of microbial produced toxins and competition for resources and attachment sites (reviewed in Robinson et al. 2010). A recent study that used pyrosequencing has found that cutaneous bacterial communities significantly differed among three species of amphibians inhabiting the same environment, suggesting that interactions between microbes and their host potentially have a large role in determining community composition of the host (McKenzie et al. 2011).

The failure of *J. lividum* as a probiotic in this study contrasts with the successful persistence of *J. lividum* and the inhibition of *Bd* on the frog *R. muscosa* (Harris et al., 2009). This difference may be a result of different evolutionary histories of the host and symbiont (Harris et al. 2009). For example, *J. lividum* has been repeatedly found on *R. muscosa* in nature, suggesting an evolutionary history together (Woodhams et al. 2007; Lam et al. 2010; Vredenburg et al. 2011). *J. lividum* has yet to be detected on frog skins during repeated sampling efforts in Panama (M. H. Becker, unpublished results). Therefore, we recommend prospecting for future anti-*Bd* bacterial candidate species that are strongly anti-*Bd* in vitro and may have some evolutionary history with *A. zeteki*. The clear message that emerges from this study and prior studies is that the success or failure of a disease mitigation strategy that uses probiotics is dependent on finding the proper match between the probiotic species and the amphibian host, e.g., finding a probiotic that inhibits the pathogen and that persists on the host under natural conditions.

Recent survey work in Panama has shown that amphibians persisting in *Bd*-positive areas have high proportions of anti-*Bd* bacteria growing on their skin (J. B. Walke, unpublished results), which lends support to continued exploration of the probiotics approach. The fact that *A. zeteki* is likely to be extinct in the wild, however, makes it impossible to sample wild populations for candidate bacterial species. We, therefore, recommend prospecting for anti-*Bd* candidate bacteria in *Bd*-positive areas in Panama, focusing on persisting populations of *Atelopus* species and Bufonids. Future experiments will focus on screening larger numbers of bacterial candidates for persistence on *Atelopus* skin before exposure trials. We feel that continued work in this area is critical to develop the tools to mitigate the threat of chytridiomycosis in the wild and to allow us one day to reintroduce populations of extinct amphibians back into their native habitats.

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