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The Bacterially Produced Metabolite Violacein Is Associated with Survival of Amphibians Infected with a Lethal Fungus[▽]

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The disease chytridiomycosis, which is caused by the chytrid fungus *Batrachochytrium dendrobatidis*, is associated with recent declines in amphibian populations. Susceptibility to this disease varies among amphibian populations and species, and resistance appears to be attributable in part to the presence of antifungal microbial species associated with the skin of amphibians. The betaproteobacterium *Janthinobacterium lividum* has been isolated from the skins of several amphibian species and produces the antifungal metabolite violacein, which inhibits *B. dendrobatidis*. In this study, we added *J. lividum* to red-backed salamanders (*Plethodon cinereus*) to obtain an increased range of violacein concentrations on the skin. Adding *J. lividum* to the skin of the salamander increased the concentration of violacein on the skin, which was strongly associated with survival after experimental exposure to *B. dendrobatidis*. As expected from previous work, some individuals that did not receive *J. lividum* and were exposed to *B. dendrobatidis* survived. These individuals had concentrations of bacterially produced violacein on their skins that were predicted to kill *B. dendrobatidis*. Our study suggests that a threshold violacein concentration of about 18 μM on a salamander's skin prevents mortality and morbidity caused by *B. dendrobatidis*. In addition, we show that over one-half of individuals in nature support antifungal bacteria that produce violacein, which suggests that there is a mutualism between violacein-producing bacteria and *P. cinereus* and that adding *J. lividum* is effective for protecting individuals that lack violacein-producing skin bacteria.

The amphibian fungal pathogen *Batrachochytrium dendrobatidis* causes a lethal skin disease that has caused substantial declines in amphibian populations (18). However, some species, such as the bullfrog (*Rana catesbeiana*) and the tiger salamander (*Ambystoma tigrinum*), are relatively asymptomatic when they are infected with this pathogen (4, 5). Variation in survival among species has been attributed to differences in innate immune factors, such as antimicrobial peptides (20) and skin-associated microbial species (8–11), as well as behavior (16). The presence of antifungal microbes is of particular interest because it suggests that these organisms are mutualistic associates of amphibian species. In addition, augmentation of the cutaneous microbial community by adding species of bacteria that inhibit *B. dendrobatidis* has the potential to provide resistance to chytridiomycosis (9).

We have identified a number of bacteria associated with the skin of amphibians that inhibit *B. dendrobatidis* in vitro via secretion of antifungal metabolites (2, 3, 10, 11). The bacterial species used in this study, *Janthinobacterium lividum*, produces the anti-*B. dendrobatidis* metabolites violacein and indole-3-carboxaldehyde (MIC, 1.82 μM and 69 μM, respectively) (3). We have shown that violacein inhibits *B. dendrobatidis* in laboratory assays (3) and is strongly correlated with survival in vivo of the frog species *Rana muscosa* (9). Violacein was also present on three of seven wild-collected red-backed salamanders (*Plethodon cinereus*) at concentrations that inhibit *B. dendrobatidis* in vitro (3), suggesting that this salamander species has a mutualistic community of violacein-producing bacteria on its skin. In this study, we added *J. lividum* to salamander skins to generate a wide range of violacein concentrations in order to determine what concentration is needed to prevent mortality caused by chytridiomycosis in vivo.

MATERIALS AND METHODS

Sixty-two adult red-backed salamanders were collected from the George Washington National Forest in Rockingham County, Virginia, on 16 May 2008. Salamanders were placed in individual sterile plastic containers containing autoclaved filter paper moistened with autoclaved Provosoli medium (artificial pond water) (22) and immediately taken to a laboratory. All salamanders were individually weighed and rinsed twice in sterile Provosoli medium to remove transient bacteria (11). Individuals were formally, randomly assigned to one of the following four treatment groups: control group ($n = 16$) (animals exposed to 5 ml of sterile Provosoli medium), bacterium group ($n = 15$) (animals exposed to 5 ml of Provosoli medium containing 6.7×10^7 *J. lividum* cells isolated from the salamander *Hemidactylium scutatum*), bacterium-*B. dendrobatidis* group ($n = 15$) (animals exposed to 6.7×10^7 *J. lividum* cells 3 days prior to exposure to 6×10^6 *B. dendrobatidis* strain JEL 310 zoospores suspended in 5 ml of Provosoli medium), and *B. dendrobatidis* group ($n = 16$) (animals exposed to Provosoli medium containing 6×10^6 *B. dendrobatidis* zoospores). All individuals were handled to the same extent to control for handling effects. Details of the exposure protocol and culturing of *B. dendrobatidis* have been described previously by Harris et al. (9).

Salamanders were individually weighed and swabbed using the protocol described by Harris et al. (9) prior to experimental treatment and 13 days after exposure to *B. dendrobatidis*. Individuals who died prior to day 13 were swabbed immediately after they died. DNA was extracted from swabs using a DNeasy blood and tissue kit (Qiagen, Germantown, MD) by following the manufacturer's protocol. DNA obtained from the swabs was amplified in triplicate using real-time PCR with the *J. lividum*-specific primers described by Harris et al. (9).

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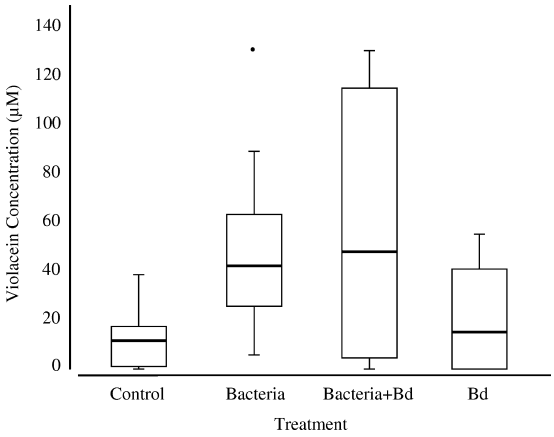

FIG. 1. Concentrations of violacein on the skins of salamanders in the control ($n = 16$), bacterium ($n = 15$), bacterium-*B. dendrobatidis* ($n = 15$), and *B. dendrobatidis* ($n = 16$) treatment groups. Salamanders treated with *J. lividum* prior to exposure to *B. dendrobatidis* had significantly higher violacein concentrations on their skins than nontreated individuals (df = 3, $\chi^2 = 16.381$, and $P = 0.001$, Kruskal-Wallis test). Bd, *B. dendrobatidis*. The interquartile ranges are indicated by the boxes, the medians are indicated by the horizontal lines in the boxes, the bars indicate the highest and lowest values in 1.5 interquartile ranges, and an outlier is indicated by a point.

Twenty-five-microliter PCR mixtures contained 5 μl of DNA template, 0.2 μM of each primer, and 12.5 μl of 2× SYBR green PCR master mixture (Applied Biosystems, Warrington, United Kingdom). Amplification of each sample was completed using a DNA Engine Opticon 2 system (MJ Research, Waltham, MA). The amplification conditions were as follows: an initial cycle consisting of 10 min at 94°C, followed by 40 cycles of 30 s at 94°C, 20 s at 58°C, and 30 s at 72°C and then a final cycle consisting of 10 min at 72°C. DNA was extracted from pure cultures of *J. lividum* with an UltraClean microbial DNA isolation kit (MoBio, Carlsbad, CA) to create standards containing 10^5, 10^4, 10^3, 10^2, and 10 *J. lividum* cell genome equivalents. Standards were amplified along with extracted bacterial swabs. A standard curve was generated for each 96-well plate to estimate the number of *J. lividum* cell equivalents.

The experiment ended 14 days after exposure to *B. dendrobatidis*, when all surviving individuals were euthanized. This day was chosen so that we could accurately analyze violacein concentrations for surviving and dead salamanders when the death rate was high. Immediately following mortality during the experiment or upon euthanasia, a portion of skin between the shoulders and hips of each individual was excised, measured, and extracted with methanol to determine the concentration of violacein on the skin using high-pressure liquid chromatography and comparison to a standard curve for the metabolite. Details of this protocol have been described by Brucker et al. (3).

Data that were not normally distributed were transformed, and if transformation failed, an appropriate nonparametric test was performed to test for differences between treatments or correlation between variables.

RESULTS

J. lividum successfully established on the skins of salamanders. By use of real-time PCR and primers specific for *J. lividum*, we determined that 93% (28/30) of individuals in the bacterium and bacterium-*B. dendrobatidis* treatment groups were negative for the presence of *J. lividum* prior to treatment with *J. lividum* and that all 30 salamanders were positive 2 weeks after treatment.

Treating salamanders with *J. lividum* resulted in a wide range of violacein concentrations (up to 129 μM). The violacein concentrations were higher on salamander skins treated with *J. lividum* than on salamander skins not treated with *J. lividum* (*B. dendrobatidis* and control groups) (Fig. 1) (df = 3, $\chi^2 = 16.381$, and $P = 0.001$, Kruskal-Wallis test). Surprisingly, 63% (20/32) of the salamanders in the nonaugmented *B. dendrobatidis* and control treatment groups had detectable quantities of violacein on their skins. However, only 10% (2/20) of salamanders with detectable levels of violacein on their skins were positive for *J. lividum*, indicating that other violacein-producing bacteria were present. Overall, there was a significant positive correlation between quantitative PCR estimates of the number of *J. lividum* equivalents and violacein concentrations, suggesting that *J. lividum* was a component of the violacein-producing bacterial community (Fig. 2) (df = 60, $r = 0.332$, and $P = 0.0084$, Spearman correlation analysis).

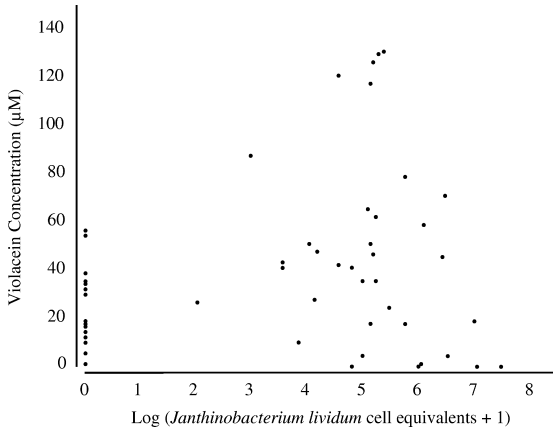

FIG. 2. Correlation between $\log_{10}$ number of *J. lividum* cell equivalents and violacein concentration on the skins of salamanders (df = 60, $r = 0.332$, and $P = 0.0084$, Spearman correlation analysis).

Survival was strongly associated with violacein concentration on the skin (Fig. 3). For the animals exposed to *B. dendrobatidis*, those that lived had significantly higher violacein concentrations than those that died ($Z = -3.908$ and $P < 0.0001$, Wilcoxon two-sample test). Eighty percent (8/10) of the salamanders that died had no detectable violacein concentra-

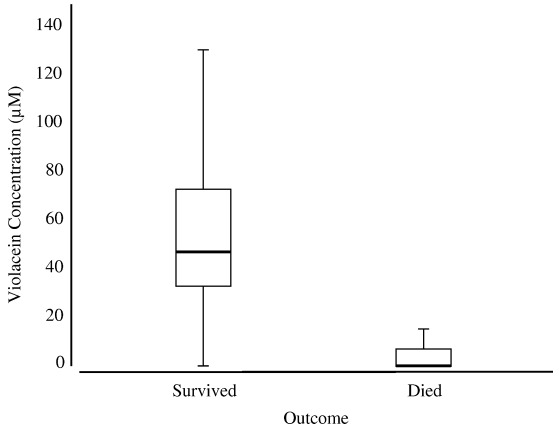

FIG. 3. Violacein concentrations on salamanders that died ($n = 10$) and survived ($n = 21$). Salamanders that died had significantly lower violacein concentrations on their skins ($Z = -3.908$ and $P < 0.0001$, Wilcoxon two-sample test). The interquartile range is indicated by the box, the median is indicated by the horizontal line in the box and the bars indicate the highest and lowest values in 1.5 interquartile ranges.

tion on their skins; the other two salamanders had concentrations less than 18.8 μM on their skins. In contrast, only 14% (3/21) of the salamanders that survived had no detectable concentration of violacein on their skins; the other 18 individuals had violacein concentrations on their skins ranging from 18.3 μM to 129 μM. The proportion of salamanders that survived in the control and bacterium treatment groups was 100% (16/16 and 15/15, respectively). In the bacterium-*B. dendrobatidis* and *B. dendrobatidis* treatment groups, the levels of survival were 73% (11/15) and 56% (9/16), respectively. The salamanders that died did so between days 9 and 13.

DISCUSSION

Adding *J. lividum* to salamanders led to significant increases in the violacein concentrations on their skins. Violacein concentration was strongly associated with whether an individual lived or died when it was exposed to the pathogen *B. dendrobatidis*. Our study suggests that a threshold concentration of violacein of about 18 μM on a salamander's skin prevents mortality and morbidity caused by *B. dendrobatidis*. It is possible that a longer experiment would have led to additional mortality; however, recent results with another amphibian species indicated that violacein concentration is correlated with longer survival (5 months) (9). Violacein was present on the skins of some salamanders that had no detectable *J. lividum*. Since amphibians are not known to produce violacein, the occurrence of violacein on skins of some salamanders without *J. lividum* strongly suggests that another violacein-producing microbial species was present on the skins. The statistically significant relationship between the number of *J. lividum* equivalents and violacein concentration had a fairly low correlation coefficient (Fig. 2), which also suggests that other violacein producers were present on the skins. Violacein is produced by three bacterial species in the betaproteobacterial group closely related to *J. lividum*: *Duganella* sp. (19), *Chromobacterium violaceum*, and *Iodobacter fluviatile* (7, 12). In addition, *Pseudoalteromonas tunicata* and *Pseudoalteromonas luteoviolacea* in the gammaproteobacterial group produce violacein, which protects *P. tunicata* from protozoan grazing and may protect its hosts, such as algae and tunicates, from fouling (13, 15, 23).

The amphibian species used in this study, *P. cinereus*, is not known to be experiencing population declines related to *B. dendrobatidis* infection. The presence of violacein on 63% of the skins of unmanipulated individuals in this study and on 43% (3/7) of individuals straight from the field (3) suggests that their anti-*B. dendrobatidis* microbiotas are a factor that protects *P. cinereus* salamanders from disease symptoms and are an important component of the innate immune system. Amphibians periodically shed their skins, so it is reasonable to expect that some proportion of the population lacks violacein on the skin at any given time. Species that attend their embryos in nests, such as *P. cinereus*, minimize the effects of pathogenic fungi on their embryos (1, 6, 14). We have shown that in another species of salamander, *H. scutatum*, females with culturable antifungal skin bacteria can minimize embryonic mortality due to fungi (1). We hypothesize that species that attend nests have undergone natural selection to support antifungal bacteria as a means to protect embryos from fungi and also protect adults (10).

The implication of our results is that violacein-producing bacteria form a mutualistic association with *P. cinereus*. The salamanders are protected from pathogens such as *B. dendrobatidis* and probably from other species of pathogenic fungi that attack their embryos in the nest. The skin bacteria have a food resource (mucus) and a substrate (the skin). We hypothesize that amphibians' own antimicrobial peptide secretions have selected for a group of microbes with the functional capacity to resist pathogenic fungi (21). A similar mutualism is found in bacteria and corals (17).

Assaying populations of amphibians prior to the arrival of *B. dendrobatidis* for anti-*B. dendrobatidis*, bacterially produced metabolites may provide an indication of which species are at greatest risk, although techniques are needed that allow sampling of small quantities of metabolites from amphibian skins using swab samples. We are developing such an assay using a more selective high-performance liquid chromatography–mass spectrometry protocol. Populations in which a high proportion of individuals have protective metabolites are likely to be at less risk from chytridiomycosis than populations that lack protective metabolites. Many individuals of the salamander species used in this study were protected from *B. dendrobatidis* without bioaugmentation, which can help explain the survival of individuals that were experimentally exposed to *B. dendrobatidis* and not to *J. lividum*. Other amphibian species are likely to be less well protected, and populations that lack anti-*B. dendrobatidis*, bacterially produced metabolites are candidates for a bioaugmentation approach. Adding antifungal skin bacteria to amphibians as a strategy to conserve threatened species could be effective if the added bacteria persist at population densities high enough to limit *B. dendrobatidis* persistence. We predict that bioaugmentation will limit the symptoms of chytridiomycosis, as shown recently in our laboratory study with the frog *Rana muscosa* (9). Other defensive mechanisms, such as antimicrobial peptides, behaviors, skin shedding rates, and adaptive immune responses, are important in patterns of species-specific survival in response to chytridiomycosis. However, these mechanisms are not amenable to manipulation as a management tool in conservation. The effects of bioaugmentation on nontarget species need to be assessed before bioaugmentation can be used in the field. Additional research is needed to understand the ecological interactions on amphibian skin and to lay the groundwork for preventing the devastating effects of *B. dendrobatidis* in nature.

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