Inhibition of Fungal Pathogens across Genotypes and Temperatures by Amphibian Skin Bacteria

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Symbiotic bacteria may dampen the impacts of infectious diseases on hosts by inhibiting pathogen growth. However, our understanding of the generality of pathogen inhibition by different bacterial taxa across pathogen genotypes and environmental conditions is limited. Bacterial inhibitory properties are of particular interest for the amphibian-killing fungal pathogens (Batrachochytrium dendrobatidis and Batrachochytrium salamandrivorans), for which probiotic applications as conservation strategies have been proposed. We quantified the inhibition strength of five putatively B. dendrobatidis-inhibitory bacteria isolated from woodland salamander skin against six Batrachochytrium genotypes at two temperatures (12 and 18°C). We selected six genotypes from across the Batrachochytrium phylogeny: B. salamandrivorans, B. dendrobatidis-Brazil and four genotypes of the B. dendrobatidis Global Panzootic Lineage (GPL1: JEL647, JEL404; GPL2: SRS810, JEL423). We performed 96-well plate challenge assays in a full factorial design. We detected a Batrachochytrium genotype by temperature interaction on bacterial inhibition score for all bacteria, indicating that bacteria vary in ability to inhibit Batrachochytrium depending on pathogen genotype and temperature. Acinetobacter rhizosphaerae moderately inhibited B. salamandrivorans at both temperatures (µ = 46–53%), but not any B. dendrobatidis genotypes. Chryseobacterium sp. inhibited three Batrachochytrium genotypes at both temperatures (µ = 5–71%). Pseudomonas sp. strain 1 inhibited all Batrachochytrium genotypes at 12°C and four Batrachochytrium genotypes at 18°C (µ = 5–100%). Pseudomonas sp. strain 2 and Stenotrophomonas sp. moderately to strongly inhibited all six Batrachochytrium genotypes at both temperatures (µ = 57–100%). All bacteria consistently inhibited B. salamandrivorans. Using cluster analysis of inhibition scores, we found that more closely related Batrachochytrium genotypes grouped together, suggesting that bacterial inhibition strength may be predictable based on Batrachochytrium relatedness. We conclude that bacterial inhibition capabilities change among bacterial strains, Batrachochytrium genotypes and temperatures. A comprehensive understanding of bacterial inhibitory function, across pathogen genotypes and temperatures, is needed to better predict...
the role of bacterial symbionts in amphibian disease ecology. For targeted conservation applications, we recommend using bacterial strains identified as strongly inhibitory as they are most likely to produce broad-spectrum antimicrobial agents at a range of temperatures.

Keywords: symbiont, salamander, Batrachochytrium, antifungal, disease ecology

INTRODUCTION

Interactions between host microbiomes and pathogens influence the severity of host disease. The outcome of microbiome-pathogen interactions can depend on microbiome composition (Chang et al., 2008; Jani and Briggs, 2014; Rovenich et al., 2014), pathogen genotype (Antwis et al., 2015), and environmental context (Duffy and Defago, 1999; Lokmer and Wegner, 2015). In amphibians, the skin microbiome has been implicated in variable host susceptibility to the disease chytridiomycosis (Harris et al., 2009a; Becker and Harris, 2010; Muletz et al., 2012). Chytridiomycosis has been linked to extreme loss of global amphibian biodiversity (Berger et al., 1998; Lips et al., 2006; Martel et al., 2014; Spitzen-van der Suijs et al., 2016; Carvalho et al., 2017; Stegen et al., 2017), and is caused by skin infection by either of two congeneric chytrid fungi, Batrachochytrium dendrobatidis and B. salamandrivorans, hereafter B. dendrobatidis and B. salamandrivorans, respectively. One mechanism by which bacterial symbionts offer protection from Batrachochytrium is through production of inhibitory metabolites that can kill zoospores (Brucker et al., 2008a,b) or cause zoospores to move away from the metabolites (Lam et al., 2011). However, the impacts of amphibian skin microbiomes on fungal disease are difficult to predict because little is known about the ecological and evolutionary factors shaping microbiome functions, such as antifungal properties (e.g., Madison et al., 2017). To better predict the role of bacterial symbionts in amphibian disease ecology it is necessary to quantify antifungal properties across bacterial strains, Batrachochytrium genotypes and temperatures.

Application of antifungal bacteria has been proposed as a preventative strategy and a treatment option for chytridiomycosis in the wild (Muletz et al., 2012; Bletz et al., 2013). Of the approximately 250 bacterial operational taxonomic units (OTUs) identified as B. dendrobatidis-inhibitory (Woodhams et al., 2015; Muletz-Wolz et al., 2017a), nine have been used in bioaugmentation trials (e.g., Harris et al., 2009a,b; Woodhams et al., 2012; Nebergall, 2013; Becker et al., 2015a). These trials have had mixed success in mitigating chytridiomycosis. For instance, three studies found no effect of augmenting Janthinobacterium lividum on amphibian skin in reducing B. dendrobatidis-associated disease symptoms (Becker et al., 2011; Bletz, 2013; Nebergall, 2013), even though J. lividum inhibits B. dendrobatidis growth in vitro (Harris et al., 2006) and can be effective against B. dendrobatidis on amphibian skin (Harris et al., 2009a; Muletz et al., 2012). The variation in impact of bacterial augmentation in vivo suggests that environment and/or pathogen specific factors influence antifungal activity of bacterial symbionts through regulation of metabolite production or pathogen genotype specificity.

Batrachochytrium is a globally distributed genus with a complex evolutionary history within the two described species, B. dendrobatidis and B. salamandrivorans (Farrer et al., 2013, 2017; Rosenblum et al., 2013; Jenkinson et al., 2016). B. dendrobatidis originated at least 30 mya and is comprised of multiple, deeply diverged lineages including a Global Panzootic Lineage (GPL) and four enzootic lineages that are generally confined to their respective regions, B. dendrobatidis-Brazil, B. dendrobatidis-Cape, B. dendrobatidis-CH, and B. dendrobatidis-Korea (Schloegel et al., 2012; Farrer et al., 2013; Rosenblum et al., 2013; Martel et al., 2014; James et al., 2015). Recent mass mortality events and population declines linked to B. dendrobatidis have been primarily associated with B. dendrobatidis-GPL (Fisher et al., 2009; Farrer et al., 2011; James et al., 2015). B. dendrobatidis-GPL is rapidly evolving with genetic differentiation that generally form two clades, B. dendrobatidis-GPL1, which is found primarily in North America, and B. dendrobatidis-GPL2, which is a geographically dispersed group (Schloegel et al., 2012; Rosenblum et al., 2013; James et al., 2015). B. salamandrivorans originated at least 30 mya where it coexisted with an Asian salamander clade (Martel et al., 2014) until its emergence in Western Europe resulted in rapid population declines of European fire salamanders (Martel et al., 2013; Stegen et al., 2017). To date, only B. dendrobatidis-GPL isolates have been tested to identify amphibian skin bacteria with Batrachochytrium-inhibitory traits (Antwis et al., 2015; Woodhams et al., 2015; Madison et al., 2017; Muletz-Wolz et al., 2017a).

Amphibian population declines and mass mortality events linked to B. dendrobatidis often have been more devastating in cooler seasons and higher elevations (Berger et al., 2004; Lips et al., 2006, 2008; Kriger and Hero, 2008; Carvalho et al., 2017). Host and pathogen responses may explain increased virulence at lower temperatures, including changes in (i) B. dendrobatidis fecundity as a life-history tradeoff (Woodhams et al., 2008), (ii) host immune response to infection (Ribas et al., 2009; Longo and Zamudio, 2017), and (iii) antifungal activity by bacterial symbionts (Daskin et al., 2014; Woodhams et al., 2014; Bresciano et al., 2015). For amphibian skin symbionts, temperature influences bacterial growth rate and population size, and high cell density is often needed to produce inhibitory metabolites (Yasumiba et al., 2016). Compared to B. dendrobatidis (Piotrowski et al., 2004; Stevenson et al., 2013), B. salamandrivorans generally has a lower optimal growth temperature (Martel et al., 2013) and B. dendrobatidis-inhibitory bacteria may not be effective against B. salamandrivorans due to temperature-dependent growth.
constraints of pathogen and bacteria, among other reasons [e.g., genetic and phenotypic variation between B. dendrobatidis and B. salamandrivorans (Farrer et al., 2017; Stegen et al., 2017)].

We quantified the inhibition strength of five amphibian skin bacteria cultured from North American woodland salamanders (Plethodon cinereus and P. cylindraceus), previously shown to inhibit B. dendrobatidis (GPL1-JEL404: Muletz-Wolz et al., 2017a) across Batrachochytrium genotypes and temperatures. We had three main objectives, (i) quantify the inhibitory proprieties of putatively anti-B. dendrobatidis bacterial strains against B. salamandrivorans, (ii) quantify the effect of temperature (12 and 18°C), Batrachochytrium genotype and their interaction on bacterial inhibition strength, and (iii) determine if Batrachochytrium relatedness predicts bacterial inhibition strength. Quantifying interactions between pathogen and bacterial symbionts in an environmental and genetic framework strengthens our understanding of disease dynamics and guides conservation measures.

**MATERIALS AND METHODS**

We performed in vitro challenge assays with five bacterial strains and six Batrachochytrium genotypes at two temperatures (12 and 18°C) using 96-well plates in a full factorial experimental design. We selected five bacterial strains to represent a range of inhibition based on their previously quantified inhibition strength against B. dendrobatidis-GPL1-JEL404 at 20°C (Table 1; Muletz-Wolz et al., 2017a). All bacterial strains were isolated from either Plethodon cinereus or P. cylindraceus at Shenandoah National Park, Virginia, in May 2012, and were widespread in these populations (Muletz-Wolz et al., 2017b). We selected six Batrachochytrium isolates from across the Batrachochytrium phylogeny, two GPL1 isolates (JEL647 and JEL404), two GPL2 isolates (SRS810 and JEL423), a Brazilian B. dendrobatidis isolate (JEL649), and B. salamandrivorans (Table 2). Hereafter, we refer to these Batrachochytrium isolates as Batrachochytrium genotypes because genetic analyses have shown that each of these isolates represent distinct genotypes (Schloegel et al., 2012; Martel et al., 2013; James et al., 2015).

**Experimental Set-up**

We performed the experiment using a total of 16 96-well plates. Each plate was assigned to one of four randomly generated configurations of bacterial by Batrachochytrium combinations (Supplementary Figure S1), and housed in one of four incubators (Percival model DR-36VI; two chambers per temperature, Supplementary Figure S2). Each incubator contained a total of four plates, with one plate per configuration.

We set up challenge assays following a protocol based on Muletz-Wolz et al. (2017a), with the following modifications to accommodate the design of the experiment. We passaged cryopreserved bacteria on 1% tryptone plates three times, then inoculated each bacterial strain in 25 mL of 1% tryptone broth and grew for 3 days at room temperature (approximately 21°C) on a shaker at 100 rpm. By 3 days of incubation, the bacterial cultures reached high densities where inhibitory metabolites are produced (Bérdy, 2005). We obtained cell-free supernatants (CFSs) from bacterial monocultures, following the centrifuging and filtering methods outlined in Muletz-Wolz et al. (2017a). By using bacterial CFS, we determined the inhibitory properties of bacterially produced extracellular factors against live Batrachochytrium zoospores, and eliminated the possibility of direct competition or priority effects between bacterial and fungal cultures. As the bacterial strains were

**TABLE 1 |** Five bacteria strains used in the study, including their phylogenetic designation and previously quantified inhibition strength against B. dendrobatidis-GPL1-JEL404 (Muletz-Wolz et al., 2017a).

| Bacteria strain | Strain ID | GenBank accession no. of 16S rRNA gene | Inhibition score for B. dendrobatidis-GPL1-JEL404 at 20°C (%) |
|------------------|-----------|---------------------------------------|-------------------------------------------------------------|
| Acinetobacter rhizosphaerae | THA6-B68 | KU739019 | 32 |
| Pseudomonas sp. strain 1 | RSB5-4 | KU738948 | 99 |
| Pseudomonas sp. strain 2 | SFB8-6 | KU738987 | 82 |
| Chryseobacterium sp. | SFA2-10 | KU738960 | 54 |
| Stenotrophomonas sp. | LSB7-4 | KU738931 | 100 |

**TABLE 2 |** Six Batrachochytrium genotypes used in the study.

| Isolate ID | Phylogenetic lineage | Genotype | Location of isolation | Approximately # passages since isolation |
|------------|----------------------|----------|-----------------------|-----------------------------------------|
| JEL649     | B. dendrobatidis-Brazil | Brazil-JEL649 | Silo Paulo, Brazil | 8 |
| JEL647     | B. dendrobatidis-GPL1 | GPL1-JEL647 | California, United States | 3 |
| SRS810     | B. dendrobatidis-GPL2 | GPL2-SRS810 | Georgia, United States | 6 |
| AMFP13/1   | B. salamandrivorans   | B. salamandrivorans | Zuid-Limburg, Netherlands | 9 |
| JEL423     | B. dendrobatidis-GPL2 | GPL1-JEL423 | El Cope, Panama | 6 |
| JEL404     | B. dendrobatidis-GPL1 | GPL1-JEL404 | Maine, United States | 8 |

Each isolate represented a distinct genotype based on genetic analyses by Schloegel et al. (2012), Martel et al. (2013), and James et al. (2015). We used the combination of Batrachochytrium lineage and isolate to specify the Batrachochytrium genotype.
grown at one temperature prior to the experiment all effects of temperature on inhibition relate to temperature-dependent activity of CFS extracellular factors and/or fungal physiology. For _Batrachochytrium_ genotypes, we passaged cryopreserved isolates (prior passage history ranged between 3 and 9 times; Table 2) on 1% tryptone plates twice, and then grew them for 1 week on multiple 1% tryptone plates at 15°C. We harvested zoospores by flooding the plates with 1% tryptone broth, filtered out the zoosporangia using a sterile coffee filter, and homogenized the zoospore mixture.

To set up the assays, we added 50 µl of approximately 1 × 10^6 zoospores/ml of each _Batrachochytrium_ genotype (counted with a hemocytometer; approximately 50,000 zoospores in each well) to their designated wells in a 96-well plate (Supplementary Figure S1). In sample wells, we added 50 µl of the CFS from each bacterial strain to four wells for each bacterial- _Batrachochytrium_ combination. In total, each bacterial- _Batrachochytrium_ combination was represented in 16 wells distributed over four plates per temperature. In each 96-well assay, we included two positive controls (PCs) and one negative control for each _Batrachochytrium_ genotype using four wells per control. The positive controls were: 50 µl of _Batrachochytrium_ zoospores + 50 µl 1% tryptone broth PC and 50 µl of _Batrachochytrium_ zoospores + 50 µl of water [nutrient-depleted positive control (NDPC)]. The negative control was 50 µl of _Batrachochytrium_ zoospores heat-killed at 60°C for 60 min + 50 µl of 1% tryptone broth (heat-killed _B. dendrobatidis_; HK). We measured optical density (OD_{692 nm}) of each well for 16 96-well plates using a microplate reader every other day starting on day 1, for 27 days.

**_B. dendrobatidis_ Inhibition Score Calculations**

We used R version 3.2.5 for all calculations and statistical analyses (R Core Team, 2016). We visually inspected the optical density (OD) readings for each plate, and excluded data points for 32 wells (2% of wells) with unusually high densities (+0.1 or greater well OD compared to replicate wells on same plate), indicating contamination or error. We corrected for baseline zoospore OD by subtracting the average heat-killed OD of each _Batrachochytrium_ genotype from the corresponding experimental wells in each plate. To achieve a normal distribution, we transformed the corrected OD readings using the following equation, log(OD_{corrected}(1−OD_{corrected})+1). Next, we fit linear regressions to the transformed OD readings over time for each well with the intercept set at zero. We extracted the slope of the linear regression, and interpreted this as _Batrachochytrium_ growth (i.e., Δ optical density/time). We excluded wells in which the linear model had an r² less than 0.20, given the poor fit of the data. After quality filtering, we had slopes for 867 of the 960 sample wells (Supplementary Table S1). Then, we calculated _Batrachochytrium_ growth inhibition, hereafter referred to as inhibition score, by dividing the slope of each sample well by the slope of the average NDPC wells of the corresponding _Batrachochytrium_ genotype on the same plate, and subtracting the subsequent fraction from one, [Inhibition score = 1 – (slope sample well/average slope NDPC)]. We compared the slopes of sample wells to the NDPC wells because this accounts for the issue of nutrient depletion in PC wells and is a more conservative approach in identifying anti- _Batrachochytrium_ bacteria (Bell et al., 2013; Muletz-Wolz et al., 2017a). We created this standardized inhibition scoring system to be able to compare inhibition scores between the two experimental temperatures. We interpreted inhibition scores greater than zero as inhibitory, indicating that the bacterial- _Batrachochytrium_ sample well had less growth than the NDPC wells. Values less than zero we interpreted as non-inhibitory, and to determine if any bacterial symbionts promoted _Batrachochytrium_ growth, we compared the inhibition scores to those of the PC wells (see Statistical Analyses below; Supplementary Figure S3).

**Statistical Analyses**

We quantified the effects of _Batrachochytrium_ genotype, temperature, and their interaction (explanatory variables) on inhibition scores (response variable) using a linear mixed-effects model for each bacterial strain separately, using the `lmer` function in the ‘lme4’ package (Bates et al., 2015). We included plate nested within incubator as a random effect in each model. Next, we used the Anova function in the ‘car’ package with type II sum of squares to determine the significance of each of the fixed-effects (Fox and Weisberg, 2011). Using the ‘lsmeans’ package (Lenth, 2016), we used the lsmeans function to perform post hoc analyses to determine significant difference among _Batrachochytrium_ genotypes and between temperatures. We used the lsmip function to generate _Batrachochytrium_ genotypes by temperature interaction plots for each bacterial strain.

We determined if any bacterial symbiont promoted _Batrachochytrium_ growth by comparing the inhibition score of each bacterial _Batrachochytrium_ combination at each temperature to the inhibition score of the PC well for the corresponding _Batrachochytrium_ genotype (Supplementary Figure S3). We used a linear mixed-effects model for each _Batrachochytrium_ genotype examining the effects of well type (i.e., bacteria or PC), temperature, and their interaction (explanatory variables) on inhibition scores (response variable). We included the same random effects, determined significance and conducted post hoc analyses as described above.

We determined if patterns of bacterial inhibition score reflected _Batrachochytrium_ phylogenetic relatedness by conducting a cluster analysis using the mean inhibition score for each bacteria- _Batrachochytrium_ combination at each temperature. We used two clustering methods to confirm similar clustering patterns: (i) Ward’s hierarchical clustering with Euclidean distances using the pvclust function in the ‘pvclust’ package (Suzuki and Shimodaira, 2006), and (ii) k-means clustering using a plot of within groups sum of squares by number of clusters to determine the appropriate number of clusters (Everitt and Hothorn, 2009). We were unable to perform a full phylogenetic analysis because we could not calculate branch length due to missing genotype data for _B. salamandrivorans_ and GPL1-JEL404 (Schloegel et al., 2012, T. James, pers. comm.).
RESULTS

Batrachochytrium salamandrivoranls was the only Batrachochytrium genotype that was inhibited by all bacterial strains, with moderate to strong inhibition (μ = 43–92%) at both temperatures. Stenotrophomonas sp. and Pseudomonas sp. strain 2 were the most inhibitory against B. salamandrivorans at both temperatures (μ = 91–92%).

We detected a Batrachochytrium genotype by temperature interaction effect on inhibition score for all bacterial strains (post hoc analyses: Supplementary Tables S2, S3), indicating that bacterial inhibition strength is affected by both Batrachochytrium genotype and temperature. No bacterial strain promoted the growth of any Batrachochytrium genotype (Supplementary Figure S3).

Acinetobacter rhizosphaerae only inhibited B. salamandrivorans (Figure 1A), and was moderately inhibitory of B. salamandrivorans at both temperatures (μ = 46–53%). Inhibition scores for A. rhizosphaerae differed among genotypes (X² = 202.7, df = 5, p < 0.001) and this depended on temperature (interaction term: X² = 25.3, df = 5, p < 0.001), but the significant interaction was for scores that were non-inhibitory (Figure 1A).

Two bacterial strains, Chryseobacterium sp. and Pseudomonas sp. strain 1, were weakly to strongly inhibitory of most Batrachochytrium genotypes. Inhibition scores for Chryseobacterium sp. differed among Batrachochytrium genotypes (X² = 932.9, df = 5, p < 0.001) and this depended on temperature (interaction term: X² = 122.0, df = 5, p < 0.001). Chryseobacterium sp. was moderately inhibitory of B. salamandrivorans and Brazil-JEL649 at both temperatures (μ = 38–71%), and was weakly inhibitory of the GPL1 genotypes with GPL1-JEL647 only inhibited at 18°C and GPL1-JEL404 only inhibited at 12°C (Figure 1B). Inhibition scores for Pseudomonas sp. strain 1 differed among genotypes (X² = 479.1, df = 5, p < 0.001), temperatures (X² = 22.8, df = 1, p < 0.001) and showed a significant interaction (X² = 57.4, df = 5, p < 0.001). Pseudomonas sp. strain 1 inhibited all Batrachochytrium genotypes (μ = 5–100%), except for GPL1-JEL404 and GPL2-JEL423 at 18°C (Figure 1C). Pseudomonas sp. strain 1 was significantly more inhibitory of GPL1-JEL404, GPL1-JEL647, GPL2-JEL649 at 12°C compared to 18°C, and was differentially inhibitory among Batrachochytrium genotypes (Supplementary Table S3). For instance, Pseudomonas sp. strain 1 was more inhibitory of Brazil-JEL649 at both temperatures compared to all other Batrachochytrium genotypes.

Two bacterial strains, Pseudomonas sp. strain 2 and Stenotrophomonas sp., were inhibitory of all Batrachochytrium genotypes at both temperatures. Inhibition scores for Pseudomonas sp. strain 2 differed among genotypes (X² = 219.2, df = 5, p < 0.001), temperatures (X² = 10.4, df = 1, p = 0.001) and showed a significant interaction (X² = 101.1, df = 5, p < 0.001). Pseudomonas sp. strain 2 moderately to strongly inhibited all Batrachochytrium genotypes at both temperatures (μ = 57–98%). Inhibition scores for Stenotrophomonas sp. differed among Batrachochytrium genotypes (X² = 40.6, df = 5, p < 0.001), temperatures (X² = 5.5, df = 1, p = 0.02) and showed a significant interaction (X² = 31.9, df = 5, p < 0.001). Stenotrophomonas sp. strongly inhibited all Batrachochytrium genotypes at both temperatures (μ = 70–100%), but was less inhibitory of GPL1-JEL647 and Brazil-JEL649 at 12°C than all other genotypes, except for GPL2-JEL423 (Figure 1D and Supplementary Table S3). Similar to Pseudomonas sp. strain 2, Stenotrophomonas sp. was less inhibitory of GPL1-JEL647 and Brazil-JEL649 at 12°C than all other Batrachochytrium genotypes (Figure 1E and Supplementary Table S3).

Bacterial inhibition strength may be predictable based on Batrachochytrium phylogenetic relatedness. Both clustering methods supported the same two clusters within the data, with B. dendrobatidis-Brazil and B. salamandrivorans clustering together and the B. dendrobatidis-GPL genotypes forming a separate cluster (Figure 2).

DISCUSSION

Our findings suggest that many bacteria identified as Batrachochytrium-inhibitory in vitro are unlikely to be consistently effective in mitigation of chytridiomycosis in vivo. We found that all bacterial strains consistently inhibited B. salamandrivorans growth across temperatures (12 and 18°C), but that three of the five bacterial strains did not consistently inhibit the five B. dendrobatidis genotypes. Similarly, Antwis et al. (2015) found that 3 of 11 previously identified B. dendrobatidis-inhibitory bacterial strains did not consistently inhibit three B. dendrobatidis isolates at 18°C. These results highlight the importance of considering Batrachochytrium genotype and bacterial strain in host-pathogen interactions, and may explain the variation in effectiveness of probiotics in experimental trials (e.g., Harris et al., 2009a; Woodhams et al., 2012; Becker et al., 2015a).

Our results also highlight the importance of considering environmental context in host-pathogen interactions. The effect of temperature on inhibition was not unidirectional, but depended on the context of the interacting Batrachochytrium genotype and bacterial strain. For example, Pseudomonas sp. strain 1 was less inhibitory of GPL1-JEL647 at 12°C compared to 18°C, whereas Pseudomonas sp. strain 2 and Stenotrophomonas sp. were more inhibitory of GPL1-JEL647 at 12°C compared to 18°C. The differences in inhibition between temperatures of the same Batrachochytrium genotype likely related to temperature-dependent changes in bacterial extracellular factor activity (Daskin et al., 2014) and/or Batrachochytrium physiology (Woodhams et al., 2008). Bacterially produced extracellular factors include bacteriocins, siderophores, organic acids, lysozymes, proteases, and secondary metabolites. However, the most likely factors contributing to inhibition are secondary metabolites (reviewed by Verschueren et al., 2006; Raaijmakers et al., 2002), and antimicrobial activity of metabolites can vary among temperatures (Humair et al., 2009; Aguilar and Klotz, 2010). In addition, Batrachochytrium isolates vary in phenotypic traits (e.g., growth rate and zoosporangium size) depending on temperature (Piotrowski et al., 2004; Martel et al., 2013;
Muletz-Wolz et al. Genotype- and Temperature-Dependent Inhibition

**Figure 1** Interaction plot showing the effect of *Batrachochytrium* genotype and temperature on bacterial inhibition score (percent inhibition; note different scale on y-axis). *Acinetobacter rhizosphaerae* (A) was moderately inhibitory of *B. salamandrivorans* at both temperatures, but did not inhibit any *B. dendrobatidis* genotypes. *Chryseobacterium* sp. (B) and *Pseudomonas* sp. strain 1 (C) were weakly to strongly inhibitory of most *Batrachochytrium* genotypes. *Pseudomonas* sp. strain 2 (D) and *Stenotrophomonas* sp. (E) were moderately to strongly inhibitory of all *Batrachochytrium* genotypes at both temperatures, and showed similar inhibition patterns. Percent inhibition is based on the parameter estimates from the linear-mixed effects models.

Stevenson et al., 2013) and this may impact their susceptibility to inhibitory compounds. Investigation into the inhibition strength of *Batrachochytrium*-inhibitory bacterial metabolites (e.g., violacein) at different temperatures would provide insight into whether bacterial factors or *Batrachochytrium* physiology underpin the temperature-dependent differences in inhibition.

Our study supports the hypothesis that similarity of fungal genomes can predict strength of bacterial inhibition. Our results showed separation between two clusters: the two highly
divergent lineages (*B. salamandrivorans* and *B. dendrobatidis*-Brazil) and the GPL genotypes. Generally, *B. salamandrivorans* and *B. dendrobatidis*-Brazil were more likely to be inhibited than the GPL genotypes. The GPL genotypes have increased chromosomal copy numbers (CCNs) and loss of heterozygosity compared to the endemic *B. dendrobatidis* lineages, and these measures are associated with increased virulence (Schloegel et al., 2012; Farrer et al., 2013; Rosenblum et al., 2013; James et al., 2015; Becker et al., 2017). This association may relate to resistance to inhibitory compounds released by host or symbionts. For instance, Farrer et al. (2013) found that *B. dendrobatidis* CCN increased following exposure to a host-produced antimicrobial peptide, which then resulted in reduced inhibition. A greater sampling across *Batrachochytrium* genotypes would be a useful next step to confirm this lineage-based similarity in pathogen response to bacterial inhibition.

While there was a relationship between *Batrachochytrium* relatedness and inhibition patterns, we did not detect a relationship between bacterial relatedness and inhibition patterns. For instance, we found variation within the two *Pseudomonas* sp. strains tested, which were defined as the same OTU (16S rRNA sequence similarity = 99%). *Pseudomonas* sp. strain 1 was generally less inhibitory compared to *Pseudomonas* sp. strain 2, and also non-inhibitory of two pathogen genotypes at 18°C. These findings are similar to other studies that profiled larger numbers of bacterial strains in a phylogenetic framework and found that inhibition strength was not correlated to bacterial phylogeny (Becker et al., 2015b; Muletz-Wolz et al., 2017a). While the majority of genomic information can be highly similar within a bacterial OTU, gene clusters associated with inhibitory metabolite production can differ among strains (Chen et al., 2015), potentially explaining the discrepancy in inhibition strength within an OTU. On the other hand, different OTUs can show similar inhibition patterns as homologous recombination and horizontal gene transfer of genes encoding antifungal compounds can occur between bacterial lineages with levels of DNA divergence as high as 25% (Kinashi et al., 1987; Ravel et al., 2000; Cohan, 2001). For example, we found a strong similarity in the inhibition patterns of *Pseudomonas* sp. strain 2 and *Stenotrophomonas* sp. (16S rRNA sequence similarity = 75%). Documented modes of antibiosis by *Pseudomonas* differ from those of *Stenotrophomonas* spp. (Raaijmakers et al., 2002; Compañ et al., 2005); whole genome sequencing or chemical analyses of these bacterial strains may identify the specific agent(s) underlying this similarity in *Batrachochytrium* inhibition.

For targeted conservation applications, we suggest testing bacterial strains *in vitro* against multiple *Batrachochytrium* genotypes across a range of temperatures to identify probiotics that would be most effective at mitigating
Batrachochytrium infection in situ. If probiotic-based conservation strategies are needed rapidly, previously identified Batrachochytrium-inhibitory bacterial strains that are strongly inhibitory are the most likely to be effective. For instance, we found that two bacterial strains (Pseudomonas sp. strain 2 and Stenotrophomonas sp.) were strongly inhibitory of all Batrachochytrium genotype tested across temperatures. These bacterial strains are good probiotic candidates as they likely produce antifungal compounds at a range of temperatures that inhibit a range of pathogen genotypes (Bletz et al., 2013).

CONCLUSION

We quantified the inhibition of diverse Batrachochytrium genotypes by five bacterial strains that were isolated from woodland salamanders in the eastern United States and previously shown to inhibit an eastern US B. dendrobatidis genotype, GPL1-JEL404 (Muletz-Wolz et al., 2017a). Inhibition capabilities of the symbiotic bacteria changed as a function of bacterial strain, Batrachochytrium genotype and temperature. This has practical implications for understanding host–pathogen dynamics and developing conservation measures. Amphibians and their microbiomes will continue to be exposed to rapidly evolving Batrachochytrium genotypes, and hosts harboring higher numbers of microbial taxa and hence more potentially inhibitory species may provide greater resistance to pathogen invasion than microbiome communities with fewer taxa (Chang et al., 2008; Ling et al., 2015; Lokmer and Wegner, 2015; Longo et al., 2015). Our findings should also be considered in other systems, such as white-nose syndrome in bats and pathogens in agriculture, where probiotic application is used as a disease management strategy (Verschuere et al., 2000; Raaijmakers et al., 2002; Teplitski and Ritchie, 2009; Hoyt et al., 2015; Xue et al., 2015).

DATA ACCESSIBILITY

All experimental data and statistical analyses (R code) will be deposited upon acceptance in figshare (doi: 10.6084/m9.figshare.5297416).

AUTHOR CONTRIBUTIONS

CM-W, GD, KZ, LT, and KL designed the research. AM, FP, KZ, and LT provided the Batrachochytrium isolates. CM-W, JA, and SB conducted the lab work. CM-W and JA analyzed the data with advice from SB and GD. All authors contributed to the interpretation of the data. CM-W wrote the manuscript and all authors provided critical feedback. All authors approved the manuscript's content.

FUNDING

This research was funded by the National Science Foundation through a Research Experience for Undergraduates supplement to grant DEB #1120161 to KL and KZ.

ACKNOWLEDGMENTS

We thank C. Maguire and L. Jackson for help with developing the experiment. We thank T. James for providing some Batrachochytrium isolates and giving advice on the Batrachochytrium phylogeny.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01551/full#supplementary-material

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