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Amphibian Symbiotic Bacteria Do Not Show a Universal Ability To Inhibit Growth of the Global Panzootic Lineage of *Batrachochytrium dendrobatidis*

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Microbiomes associated with multicellular organisms influence the disease susceptibility of hosts. The potential exists for such bacteria to protect wildlife from infectious diseases, particularly in the case of the globally distributed and highly virulent fungal pathogen *Batrachochytrium dendrobatidis* of the global panzootic lineage (*B. dendrobatidis* GPL), responsible for mass extinctions and population declines of amphibians. *B. dendrobatidis* GPL exhibits wide genotypic and virulence variation, and the ability of candidate probiotics to restrict growth across *B. dendrobatidis* isolates has not previously been considered. Here we show that only a small proportion of candidate probiotics exhibited broad-spectrum inhibition across *B. dendrobatidis* GPL isolates. Moreover, some bacterial genera showed significantly greater inhibition than others, but overall, genus and species were not particularly reliable predictors of inhibitory capabilities. These findings indicate that bacterial consortia are likely to offer a more stable and effective approach to probiotics, particularly if related bacteria are selected from genera with greater antimicrobial capabilities. Together these results highlight a complex interaction between pathogens and host-associated symbiotic bacteria that will require consideration in the development of bacterial probiotics for wildlife conservation. Future efforts to construct protective microbiomes should incorporate bacteria that exhibit broad-spectrum inhibition of *B. dendrobatidis* GPL isolates.

The ability to withstand or mitigate pathogenic infection is partly determined by the host immune response. This has traditionally been examined in the context of immunity intrinsic to the host phenotype or genotype. However, all multicellular organisms coexist with a plethora of microbial organisms that are influential for host growth, development, and health (1). Although some of these microbes may be detrimental to the host, the importance of this microbiome in maintaining and improving host health is increasingly being recognized. The most obvious example of this is the gut community of humans: gut bacteria are essential for the digestion of food, but recent research has indicated that a healthy gut microbiome may also contribute to the prevention or moderation of liver, heart, and mental disease (reviewed in reference 2). The benefits to humans of a diverse microbiome are mirrored in other animal species, where the presence and composition of gut, buccal, and skin microbial communities have been linked to the occurrence and severity of both chronic and infectious disease (1).

Conservation practitioners are increasingly interested in manipulating host microbiomes as a management tool to combat infectious diseases that pose threats and welfare issues to wild animals. The use of host-associated bacteria to act as probiotics for disease mitigation is already common practice in agriculture and human health (e.g., see the reviews in references 3 and 4). The fundamental strategy is to identify bacterial genotypes that inhibit pathogens *in vitro* and apply these to susceptible hosts. Amphibians provide a particularly interesting example of this. This class of vertebrates is currently undergoing major population declines and extinctions in the wild, with 31% of species being listed as threatened by the International Union for Conservation of Nature (5, 6). This is in part due to pathogenic chytridiomycete fungi and the resulting chytridiomycosis disease (7, 8), which is arguably the most devastating infectious disease confronting wildlife today. Two chytridiomycete fungal species have been identified, *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans*, and both of these species infect the skin of amphibian hosts and cause disease in an extraordinary range of species (8–11). Current methods to mitigate the disease (e.g., antifungals, heat treatment of hosts) cannot be practically used for wild populations, but one that holds some promise and has been the subject of significant scrutiny and research investment is the application of so-called probiotic bacteria (reviewed in reference 12). Several bacteria that reside on amphibian skin have been shown to inhibit the growth and survival of *B. dendrobatidis in vitro*. The presence of such bacteria on some host species or the application of such bacteria to some host species has proven to reduce the likelihood of infection and disease significantly (13–17). However, *B. dendrobatidis* is a rapidly evolving pathogen composed of multiple, deeply diverged lineages (18, 19). Studies of potential probiotics

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have not yet explored how reliable these bacteria are when confronted with the shifting targets that amphibian-associated chytridiomycete fungi present. The globally distributed and hypervirulent *B. dendrobatidis* of the global panzootic lineage (*B. dendrobatidis* GPL) is the genetic lineage of *B. dendrobatidis* that has been associated with mass mortalities and rapid population declines of amphibians (11, 19, 20). Isolates within this lineage exhibit enormous and unpredictable genetic variation (18) and significant variation in virulence, even within a single host species exposed under laboratory conditions (19). To date, single bacterial species have been used in the majority of amphibian probiotic studies, and although they have proven effective in inhibiting the growth of single *B. dendrobatidis* GPL isolates and can be effective at limiting disease when applied as supplements to augment amphibian microbiomes (e.g., see references 13, 14, and 16), it is not clear if this ability is universal across isolates of the *B. dendrobatidis* GPL. This would be essential because amphibian communities may be host to multiple *B. dendrobatidis* GPL genotypes, all of which may cause mortality in susceptible hosts (19).

In the study described here, we used a quantitative *in vitro* assessment to determine whether potentially probiotic bacteria exhibit differences in inhibitory capabilities across isolates of *B. dendrobatidis*, focusing on isolates typed to the global panzootic lineage. All bacteria used in this study are amphibian associated and therefore have the potential to act as probiotics in the event that they inhibit *B. dendrobatidis* growth and reproduction. Our objectives were 2-fold: first, to determine if candidate bacterial isolates could inhibit any of the three *B. dendrobatidis* isolates that made up our panel of pathogens and, second, to ascertain if bacterial taxonomy, characterized using 16S rRNA typing, could be used to infer inhibitory capacity. This second objective is important for developing a strategy for mining amphibian microbiomes for target probiotics.

MATERIALS AND METHODS

Ethics statement. Before it was started, this study was approved by the University of Manchester Research Ethics Committee. Bacteria were collected from wild populations of *Agalychnis moreletii* and *Agalychnis callidryas* frogs and exported with the permission of the Belize Forestry Department (research and export permit number CD/60/3/12) and imported into the United Kingdom by permission of the United Kingdom Department for Environment, Food & Rural Affairs (authorization number TARP/2012/224).

Bacterial sampling from *Agalychnis* frogs. Eight *A. moreletii* frogs and eight *A. callidryas* frogs (four males and four females of each species) were collected from Elegans Pond at the Las Cuevas Research Station, Chiquibul Rainforest, Belize (16°43′N, 88°59′W), placed individually in sterile plastic bags, and returned to the research station (distance, ~200 m). Sterile gloves were worn throughout handling and changed between frogs to minimize cross-contamination. Frogs were rinsed twice on their dorsal and ventral surfaces using sterile water to remove any transient bacteria from their skin and then swabbed all over their skin using sterile Eurotubo collection swabs (Deltalab, Spain), after which the swabs were placed into 1.5-ml sterile screw-top tubes containing 1 ml of 1 M $NaCl_2$ solution. Care was taken to ensure that the frogs were not harmed during this process, and the frogs were released back at the pond the same evening they were collected. Tubes containing swabs were shaken vigorously for 30 s, and the contents were poured onto R2A agar plates [14], which were covered in Parafilm and inverted, and the bacteria were left to grow at ambient temperature (~25°C) for 8 days. Morphologically distinct bacterial colonies were collected using individual sterile swabs and placed into screw-top tubes containing 1 ml R2A broth medium. The tubes were then shipped to the United Kingdom, where the contents were poured onto fresh R2A agar plates and incubated at 25°C until bacteria grew (~3 days). These were then restreaked to ensure that a pure culture was obtained. In total, 56 strains of bacteria were isolated and sequenced as described previously (21).

***In vitro B. dendrobatidis* challenges.** We initially tested the anti-*B. dendrobatidis* capabilities of all 56 bacterial isolates using *in vitro* agar plate challenges against two *B. dendrobatidis* isolates (*B. dendrobatidis* GPL SFBC 014 and *B. dendrobatidis* GPL AUL 1.2) as described previously (21). Briefly, *B. dendrobatidis* cultures were grown in 1% tryptone gelatin hydrolysate lactose (TGhL) liquid medium at 18°C until the zoospore density and activity reached ~10,000 zoospores/ml (at about 3 days postpassage). Three milliliters of active *B. dendrobatidis* zoospores was spread across the surface of 1% tryptone, 1% agar plates and left to dry in a sterile hood. Two bacterial pure cultures were then streaked onto opposing sides of each plate, which were inverted and incubated at 18°C for 10 days. Bacterial streaks were scored for the presence or absence of a zone of inhibition, characterized by dead *B. dendrobatidis* zoosporangia and zoospores and no evidence of continuing *B. dendrobatidis* growth and reproduction. If both bacterial streaks on one plate exhibited inhibition, the *in vitro* challenge was repeated for both bacterial isolates separately using a noninhibitory bacterial isolate as a control.

Based on the results of the initial screening, we selected four bacterial isolates that inhibited the growth of *B. dendrobatidis* GPL SFBC 014, four bacterial isolates that inhibited the growth of *B. dendrobatidis* GPL AUL 1.2, three bacterial isolates that inhibited the growth of both *B. dendrobatidis* isolates, and four bacterial isolates that had not shown any inhibition of *B. dendrobatidis in vitro* ($n = 15$ bacterial isolates). Three previously unassessed *B. dendrobatidis* isolates (*B. dendrobatidis* GPL CORN 3.2, isolated from a *Mesotriton alpestris* newt in the United Kingdom; *B. dendrobatidis* GPL JEL 423, isolated from a *Agalychnis lemur* frog in Panama; and *B. dendrobatidis* GPL VA05, isolated from a *Alytes obstetricans* toad in Spain) were cultured, and *in vitro* inhibition assays were conducted using the methods described above, with each bacterial isolate being replicated on three different plates and never being paired with the same bacterial isolate twice. These *B. dendrobatidis* isolates were chosen because their zoospores exhibited good growth on 1% tryptone, 1% agar plates, and one (JEL 423) originated from within the natural range of *A. callidryas* frogs from which some of the bacteria were isolated. *Batrachochytrium dendrobatidis* plate challenges were conducted as described above, again using 3 ml of *B. dendrobatidis* cultures containing ~10,000 zoospores/ml. Care was taken to ensure that similarly sized colonies were picked for each streak for the three repeats of a given bacterial strain, as well as across bacterial strains, for all the inhibition assays.

Inhibition scores. Each plate was photographed, and the areas of the zone of *B. dendrobatidis* inhibition around each bacterial streak along with the areas of the bacterial streaks were measured with ImageJ software (http://imagej.nih.gov/ij/). The inhibitory capabilities of each bacterium were quantified by dividing the area of the zone of inhibition by the area of the bacterial streak, and the result was rounded to the nearest integer to give an inhibition score. The inclusion of the size of the bacterial streak in this data conversion step ensured that bacterial density was controlled for in the analyses. An alternative method of quantifying *B. dendrobatidis* inhibition using 96-well plates may be more accurate and quantifiable than plate challenges, but it does not allow consideration of the direct competition (e.g., for space and resources) that may occur between *B. dendrobatidis* and bacteria and that may also occur on the skin of amphibians (22).

Statistical analyses. The effects of *B. dendrobatidis* isolate, bacterial isolate, and their interaction on inhibition scores were analyzed using a generalized linear model with a Poisson error structure and log link. To control for the phylogenetic structure in the data, models initially contained bacterial isolate nested within genus as random effects, but this model structure was too complex, given the data, and the models would not converge, and so generalized linear models were used. In addition,

individual generalized linear models with a Poisson error structure and log link were run for each bacterial strain separately to determine differences in inhibition between *B. dendrobatidis* isolates.

Multiple bacterial isolates of four genera (*Acinetobacter*, *Chryseobacterium*, *Enterobacter*, and *Serratia*) were tested, and so differences in the overall propensity of a given genus to inhibit *B. dendrobatidis* GPL isolates were analyzed using a generalized linear mixed model with a Poisson error structure and log link. Genus, *B. dendrobatidis* isolate, and their interaction were fitted as fixed effects, and bacterial isolate nested within genus was fitted as a random effect to control for the phylogenetic structure in the data. Statistical significance was determined by stepwise removal of terms from the maximal model (*B. dendrobatidis* × genus) and performing likelihood ratio tests between nested models. Where appropriate, *post hoc* tests were performed on the models by collapsing factor levels within an explanatory variable (e.g., by combining multiple *B. dendrobatidis* isolates into one factor level) and testing the simplified model against the original model with a likelihood ratio test. A nonsignificant result suggests that the combined factor levels all have a similar influence on the response variable and that the simpler model explains the data equally well.

Poisson models make distributional assumptions about the data, including the assumption that the variance is equal to the fitted mean. To test the robustness of the distributional assumptions of the models, analyses were rerun using ordinal models and the package MCMCglmm (23). Five competing models were fitted, and all had the same random effects structure described above (genus/bacterium). The most complex model contained *B. dendrobatidis* GPL, bacterial genus, and their interaction as fixed effects. All four nested models were also fitted: *B. dendrobatidis* GPL and bacterial isolate as main effects without their interaction, the *B. dendrobatidis* GPL isolate only, bacterial genus only, and an intercept-only model. All five models were compared using the deviance information criterion (DIC). All models were run for 100,000 iterations following a burn-in of 20,000 iterations, with a thinning interval of 100 being used. Uninformative priors were used for the random effects (*G*) structure, specifying shape parameters *V* and nu to be equal to 1 and 0.002, respectively. As the residual variance is not identifiable for ordinal models, it was fixed at 1.

Nucleotide sequence accession numbers. The GenBank accession numbers for the 56 strains of bacteria collected from the frogs are KC853168 to KC853184, KC853186 to KC853194, KC853196 to KC853224, and KF444793.

RESULTS

Fifty-six bacterial strains isolated from wild *Agalychnis callidryas* and *Agalychnis moreletii* frogs were initially screened for their antifungal capabilities against two *B. dendrobatidis* GPL isolates. Of these, six inhibited isolate AUL 1.2, six inhibited isolate SFBC 014, and three inhibited both isolates (see Table S1 in the supplemental material). Because these challenges were not replicated, no statistical analyses were performed. Four bacterial isolates that inhibited the growth of SFBC 014, four bacterial isolates that inhibited AUL 1.2, three bacterial isolates that inhibited both *B. dendrobatidis* isolates, and four bacterial isolates that had not previously shown any inhibition of *B. dendrobatidis in vitro* ($n = 15$ bacterial isolates) were then used for a quantitative assessment of anti-*B. dendrobatidis* capabilities using three previously unassessed *B. dendrobatidis* GPL isolates (CORN 3.2, VA05, and JEL 423). Inhibition scores were significantly predicted by bacterial strain ($\chi^2 = 53.442$, degrees of freedom [df] = 14, $P < 0.001$), *B. dendrobatidis* isolate ($\chi^2 = 20.270$, df = 2, $P > 0.001$), and the interaction between bacterial strain and *B. dendrobatidis* isolate ($\chi^2 = 68.173$, df = 28, $P > 0.001$). The host species from which the bacteria were isolated had no significant effect on the overall inhibition capabilities of the bacteria ($\chi^2 = 0.001$, df = 1, $P = 0.981$; see Table S1 in the supplemental material). Individual models for each bacterial strain indicated that 10 of the 15 bacteria exhibited inconsistent inhibition across the *B. dendrobatidis* isolates (Table 1; Fig. 1). Only three bacteria consistently inhibited all three *B. dendrobatidis* isolates in the quantitative inhibition assessment, and only one of these also inhibited both *B. dendrobatidis* GPL isolates used for the initial screening (*Chryseobacterium* sp. strain 2; see Table S1 in the supplemental material). Two bacteria exhibited no or negligible inhibition of any of the three *B. dendrobatidis* GPL isolates in the quantitative assessment (Fig. 1), although, interestingly, of these, the *Agrobacterium* sp. inhibited both *B. dendrobatidis* GPL isolates in the initial screening, whereas *Enterobacter* sp. strain 2 inhibited neither isolate (see Table S1 in the supplemental material). Even though *Serratia* sp. strains 1, 2, and 3 all typed as identical bacterial species at the 16S rRNA locus and all were isolated from the same host species (*A. moreletii*), only *Serratia* sp. 3 showed a comprehensive ability to inhibit all three isolates of *B. dendrobatidis* (Fig. 1). The growth of two of the *B. dendrobatidis* isolates (*B. dendrobatidis* GPL CORN 3.2 and JEL 423) was consistently inhibited by the candidate bacteria, while the growth of the third isolate (*B. dendrobatidis* GPL VA05) was rarely impaired (Fig. 1).

TABLE 1 Statistical significance values for generalized linear models with Poisson error structure and log link to analyze the effect of each bacterial isolate on inhibition scores against the three *B. dendrobatidis* isolates

Bacterial isolate	χ^2 value	*P* value[a]
Acinetobacter sp. strain 1	9.843	0.007*
Acinetobacter sp. strain 2	1.567	0.457
Agrobacterium sp.	0.000	1.000
Arthrobacter sp.	14.756	<0.001*
Chryseobacterium sp. strain 1	14.120	<0.001*
Chryseobacterium sp. strain 2	23.789	<0.001*
Chryseobacterium sp. strain 3	3.170	0.205
Enterobacter sp. strain 1	9.442	0.009*
Enterobacter sp. strain 2	3.915	0.141
Lysobacter sp.	10.109	0.006*
Serratia sp. strain 1	11.046	0.004*
Serratia sp. strain 2	9.825	0.007*
Serratia sp. strain 3	1.273	0.529
Serratia sp. strain 4	17.723	<0.001*
Stenotrophomonas sp.	25.994	<0.001*

[a] *, a statistically significant result ($P < 0.05$), meaning statistically significantly different inhibition scores against the three *B. dendrobatidis* isolates for a given bacterial isolate. For all models, the degrees of freedom are equal to 2.

Genus-level models. There was no evidence for a significant interaction between bacterial genus and *B. dendrobatidis* isolate ($\chi^2 = 5.2$, df = 6, $P = 0.51$). However, both bacterial genus ($\chi^2 = 9.32$, df = 3, $P = 0.025$) and *B. dendrobatidis* isolate ($\chi^2 = 14.8$, df = 2, $P < 0.001$) were significant predictors of inhibition of *B. dendrobatidis* growth. *Post hoc* comparisons showed that there were no significant differences in the inhibitory capabilities of the genera *Acinetobacter*, *Chryseobacterium*, and *Serratia* ($\chi^2 = 0.54$, df = 1, $P = 0.76$) but that *Enterobacter* species had significantly lower inhibitory capabilities than the other three genera (*Acinetobacter*, *Chryseobacterium*, and *Serratia*; $\chi^2 = 8.77$, df = 1, $P = 0.003$; Fig. 2). Similarly, there was no significant difference in the degree of inhibition of CORN 3.2 and JEL 423 by the four bacterial genera ($\chi^2 = 0.46$, df = 1, $P = 0.47$), but all four bacterial genera

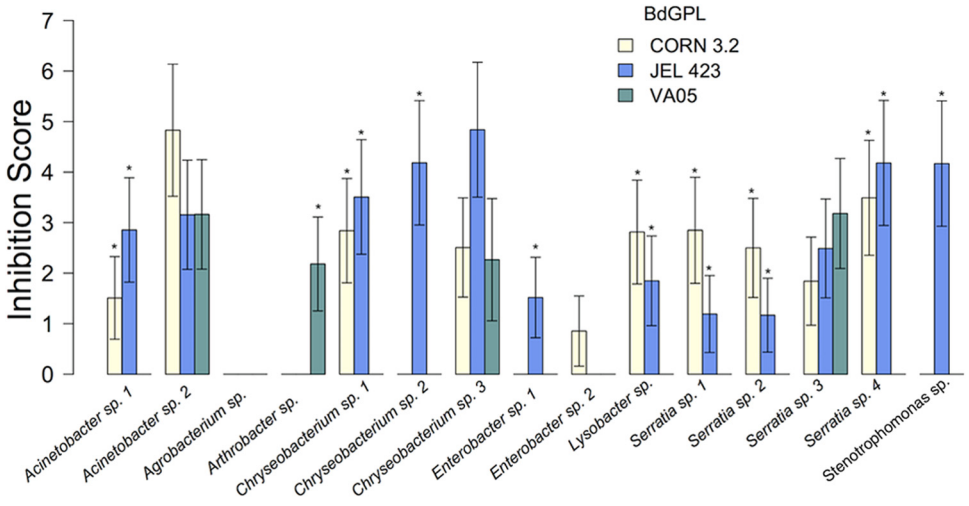

FIG 1 Average (±1 SEM) inhibition scores for 15 bacteria from quantitative *in vitro* challenges against three *B. dendrobatidis* GPL (BdGPL) isolates. *, within each bacterium, *B. dendrobatidis* isolates with inhibition scores significantly different from those for *B. dendrobatidis* isolates without an asterisk.

were significantly less likely to inhibit the growth of *B. dendrobatidis* GPL VA05 ($\chi^2 = 14.2$, df = 1, $P < 0.001$) than the growth of any of the other *B. dendrobatidis* isolates (Fig. 2).

The results from the ordinal analyses mirrored the results from the Poisson mixed models; the model with the lowest DIC (the best-supported model) contained *B. dendrobatidis* GPL isolate and bacterial genus as main effects, without an interaction. The genus *Enterobacter* was associated with significantly lower inhibition scores (mean difference = −2.03; 95% credible interval = −3.53 to −0.57). In addition, *B. dendrobatidis* GPL VA05 was also associated with significantly lower inhibition scores (mean difference = −1.42, 95% credible interval = −2.46 to −0.43). Parameter estimates for the best-supported model, as well as a model selection table containing DIC values for all five models, are provided in Tables S2 and S3 in the supplemental material.

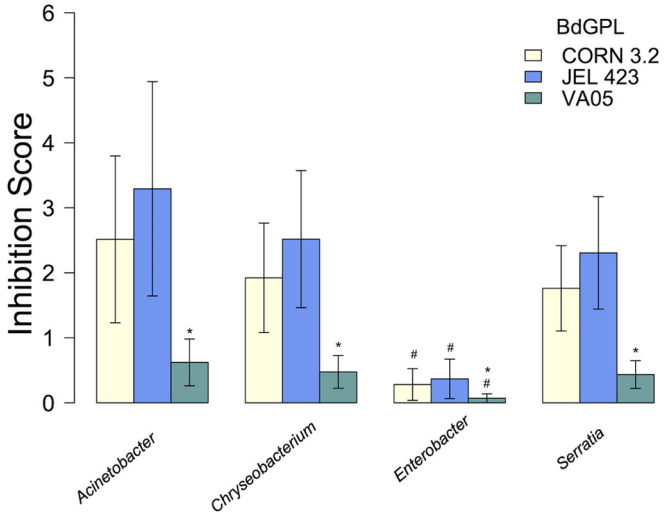

FIG 2 Average (±1 SEM) inhibition scores for multiple bacteria from four genera used to challenge three *B. dendrobatidis* GPL isolates. Inhibition scores against VA05 isolates were significantly lower than those against the other *B. dendrobatidis* GPL isolates (*), and *Enterobacter* spp. showed a significantly lower inhibition of the range of *B. dendrobatidis* GPL isolates than the other bacteria (#).

DISCUSSION

Here we show that symbiotic bacteria from the skin of amphibians exhibit differences in inhibitory capabilities across *B. dendrobatidis* GPL isolates, with only a small proportion of candidate probiotics showing broad-spectrum inhibition against the global panzootic *B. dendrobatidis* lineage. This is strong evidence that candidate bacteria tested *in vitro* for use in probiotic *B. dendrobatidis* mitigation *in situ* are unlikely to be consistently successful when confronting a variety of fungal genotypes. Because of the enormous genetic variability of *B. dendrobatidis* GPL (10, 18, 19, 24, 25), the propensity for *B. dendrobatidis* to rapidly evolve *in situ* (10, 18, 26), and the panglobal, ongoing dissemination of *B. dendrobatidis* through numerous vectors (11, 27), amphibians and their microbiomes can be expected to confront an ever changing and diverse distribution of *B. dendrobatidis* genotypes. Thus, the pathogen represents a "moving target" for potential interventions (28), and the mitigation of chytridiomycosis in the wild also needs to account for complex interactions between the host, the pathogen, and the environment, as well as multiple pathogen genotypes, in order to be successful (28–30).

We did not test our wild study animals for the presence of *B. dendrobatidis*; however, between 2006 and 2008 Kaiser and Pollinger (31) sampled amphibians at the same study site in Belize and found only a 5% *B. dendrobatidis* prevalence on *A. moreletii* frogs and a 0% prevalence on *A. callidryas* frogs. Museum specimens date the arrival of *B. dendrobatidis* in the general region (Mexico and Guatemala) to the late 1960s or early 1970s (32), suggesting that both host species are persisting in spite of the long-term presence of *B. dendrobatidis* in the area. The finding that a reasonable proportion of the bacteria isolated from these two host species in this study inhibited at least one of the *B. dendrobatidis* isolates suggests that these populations may possess a microbiome capable of at least partially mitigating *B. dendrobatidis* infection.

If manipulation of amphibian skin microbiota is to be of value for mitigating *B. dendrobatidis* infection in the wild, amphibian

microbiomes will need to be managed for a functional redundancy that provides a broad-spectrum capacity against the evolving threat represented by *B. dendrobatidis*. Studies have repeatedly illustrated the importance in a complex microbiome for resilience of the community in response to a pathogenic infection (33–35). A bacterial consortium approach that treats microbiomes as a suite of functional traits rather than a substrate for the insertion of candidate bacteria is likely to offer a more comprehensive protection of hosts from *B. dendrobatidis* and other threatening amphibian pathogens (12, 28, 36). How the different members of such consortia will be determined is currently unknown, but our results highlight the limitations of a taxonomic approach for understanding what bacterial communities may afford resistance to *B. dendrobatidis*: both species and genus showed a limited potential to identify potentially inhibitory bacteria in our study. That said, devising probiotic strategies that incorporate bacterial genus as a criterion might yield better results than bacterial species-specific approaches, and a recently developed open access database for antifungal bacterial isolates from amphibian skin will allow researchers to optimize approaches to identifying candidate probiotics (37). Ultimately, understanding functional redundancy in amphibian skin microbiomes will require a deeper understanding of how bacteria inhibit *B. dendrobatidis* growth and of their ability to infect hosts. Mining of the *B. dendrobatidis* genome for virulence factors will be fraught with difficulty, as aneuploidy and polyploidy are common across *B. dendrobatidis* isolates and changes in ploidy levels do not map to infectivity and virulence in any predictable fashion (18). However, our identification of some bacteria exhibiting broad-spectrum *B. dendrobatidis* inhibition capabilities and a significant effect of the genus on *B. dendrobatidis* growth and reproduction suggests some bacterial phylogenetic conservation of the ability to inhibit *B. dendrobatidis*. This bodes well for the presence of bacterial genetic factors that are responsible for impairment of the ability of *B. dendrobatidis* to infect and cause disease in amphibian hosts. Current criteria for selecting candidate probiotic bacteria include successful inhibition of *B. dendrobatidis*, residency in the normal microbiota of the host, and an ability to persist on the skin of inoculated individuals (12). We propose that candidate probiotics should also exhibit inhibitory activity against a range of *B. dendrobatidis* isolates, particularly the hypervirulent *B. dendrobatidis* GPL.

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