Amphibian symbiotic bacteria do not show universal ability to inhibit growth of the global pandemic lineage of *Batrachochytrium dendrobatidis*

Running title: Variation in bacterial inhibition across *Bd*GPL isolates

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Abstract

Microbiomes associated with multicellular organisms influence disease susceptibility of hosts. 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* (*Bd*GPL), responsible for mass extinctions and population declines of amphibians. *Bd*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 exhibit broad-spectrum inhibition across *Bd*GPL isolates. Moreover, some bacterial genera show significantly greater inhibition than others, but overall, genus and species are not particularly reliable predictors of inhibitory capabilities. These findings indicate 'bacterial consortia' are likely to offer a more stable and effective approach to probiotics, particularly if related bacteria are selected from genera with greater anti-microbial 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 *Bd*GPL isolates.

Introduction

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 recognised. 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 a healthy gut microbiome may also contribute to the prevention or moderation of liver, heart and mental disease (reviewed in [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. Using host-associated bacteria to act as 'probiotics' for disease mitigation is already common practice in agriculture and human health (e.g. reviewed in [3,4]). 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 listed as threatened by the International Union for the Conservation of Nature [5,6]. This is in part due to pathogenic chytridomycete fungi and the resulting chytridiomycosis disease [7,8], which is arguably the most devastating infectious disease confronting wildlife today. Two fungal species have been identified, *Batrachochytrium dendrobatidis* and *B. salamandivorans*, both of which 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 [12]). Several bacteria that reside on amphibian skin have been shown to inhibit the growth and survival of *B. dendrobatidis in vitro*. The presence or 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 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 global panzootic lineage (*Bd*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 growth of single *Bd*GPL isolates and can be effective at limiting disease when applied as augments to amphibian microbiomes (e.g. [13,14,16]), it is not clear if this ability is universal across *Bd*GPL. This would be essential because amphibian communities may be host to multiple *Bd*GPL genotypes, all of which may cause mortality in susceptible hosts [19].

Here we use 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 pandemic lineage. All bacteria used in

this study are amphibian-associated, and therefore have the potential to act as probiotics in the event they inhibit *B. dendrobatidis* growth and reproduction. Our objectives were two-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, as characterized using 16S 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

Prior to starting, this study was approved by The University of Manchester Research Ethics Committee. Bacteria were collected and exported from the wild populations of *Agalychnis moreletii* and *A. callidryas* by permission of Belize Forest Department (Research and Export Permit Number CD/60/3/12) and imported to the UK by permission of DEFRA (Authorisation Number TARP/2012/224).

Bacterial sampling from *Agalychnis* frogs

Eight *A. moreletii* and eight *A. callidryas* (four males and four females of each species) were collected from Elegans Pond at 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 (~200m distance). Sterile gloves were worn throughout handling and changed between frogs to minimise cross-contamination. Frogs were rinsed twice on their dorsal and ventral surfaces using sterile water to remove any transient bacteria from their skin, then

swabbed all over their skin using sterile Eurotubo® collection swabs (Deltalab, Spain), which were placed into 1.5 ml sterile screw-top tubes containing 1ml of 1M $NaCl_2$ solution. Care was taken to ensure frogs were not harmed during this process, and frogs were released back at the pond the same evening as they were collected. Tubes containing swabs were shaken vigorously for 30 seconds and the contents poured on to R2A agar plates, which were covered in parafilm, inverted, and bacteria left to grow at ambient temperature (~25°C) for eight days. Morphologically distinct bacterial colonies were collected using individual sterile swabs and placed into screw-top tubes containing 1ml R2A broth media. Tubes were then shipped to the UK where the contents were poured on to fresh R2A agar plates and incubated at 25°C until bacteria grew (~3 days). These were then re-streaked to ensure a pure culture was obtained. In total 56 strains of bacteria were isolated and sequenced according to [21] (GenBank accession numbers KC853168 – KC853224, KF444793).

In vitro B. dendrobatidis challenges

We initially tested the anti-*Bd* capabilities of all 56 bacterial isolates using *in vitro* agar plate challenges against two *B. dendrobatidis* isolates (*Bd*GPL SFBC 014 and *Bd*GPL AUL 1.2) according to [21]. Briefly, *B. dendrobatidis* cultures were grown in 1% TGhL liquid media at 18°C until zoospore density and activity reached ~10,000 zoospores/ml (at around three days post-passage). Three ml of active *B. dendrobatidis* 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 on to 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 non-inhibitory bacterial isolate as a control.

Based on the results of the initial screening, we selected four bacterial isolates that inhibited growth of *Bd*GPL SFBC 014, four that inhibited *Bd*GPL AUL 1.2, three 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). Three previously un-assessed *B. dendrobatidis* isolates (*Bd*GPL CORN 3.2, isolated from *Mesotriton alpestris* in the UK, *Bd*GPL JEL 423 isolated from *Agalychnis lemur* in Panama, and *Bd*GPL VA05 isolated from *Alytes obstetricans* in Spain) were cultured and *in vitro* inhibition assays conducted using the methods described above, with each bacterial isolate replicated on three different plates and never paired with the same bacterial isolate twice. These *B. dendrobatidis* isolates were chosen because they exhibited good zoospore growth on 1% tryptone, 1% agar plates, and one (JEL 423) originated from within the natural range of *A. callidryas* from which some of the bacteria were isolated. *Batrachochytrium dendrobatidis* plate challenges were conducted as described above, again using 3ml of *B. dendrobatidis* cultures containing ~10,000 zoospores/ml. Care was taken to ensure similar 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 in ImageJ (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 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 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 does not allow for direct competition that may occur between *B. dendrobatidis* and bacteria (e.g. for space and resources) 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 analysed using a Generalised Linear Model with a Poisson error structure and log-link. To control for 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 models would not converge, and so GLMs were used. In addition, individual Generalised 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 had been tested for four genera (*Acinetobacter, Chryseobacterium, Enterobacter* and *Serratia*), and so differences in the overall propensity of a given genus to inhibit *Bd*GPL isolates were analysed using a Generalised 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 were fitted as random effects to control for phylogenetic structure in the data. Statistical significance was determined by stepwise removal of terms from the maximal model (Bd * Genus) and performing likelihood ratio tests between nested models. Where appropriate post-

hoc tests were performed on 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 non-significant result suggests that the combined factor levels all have 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 variance being equal to the fitted mean. To test the robustness of the distributional assumptions of the models, analyses were re-run using ordinal models using the package 'MCMCglmm' [23]. Five competing models were fitted, all with the same random effects structure as above (~Genus/Bacteria). The most complex model contained *Bd*GPL, bacterial genus, and their interaction as fixed effects. All four nested models were also fitted: *Bd*GPL and bacterial isolate as main effects without their interaction, *Bd*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. Uninformative priors were used for the random effects (G) structure specifying V = 1 and nu = 0.002. As the residual variance is not identifiable for ordinal models, it was fixed at 1.

Results

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

Genus-level models

There was no evidence for a significant interaction between bacterial genus and *B. dendrobatidis* isolate (χ^2 = 5.2, d.f. = 6, p = 0.51). However, both bacterial genus (χ^2 = 9.32, d.f. = 3, p = 0.025) and *B. dendrobatidis* isolate (χ^2 =14.8, d.f. = 2, p < 0.001) were significant predicators of inhibition of *B. dendrobatidis* growth. Posthoc comparisons showed there were no significant differences in the inhibitory capabilities of the genera *Acinetobacter, Chryseobacterium and Serratia* (χ^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;* χ^2 = 8.77, df = 1, p = 0.003, Figure 2). Similarly, there was no significant difference in the degree of inhibition of CORN 3.2 and JEL 423 by the four bacterial genera (χ^2 = 0.46, df = 1, p =0.47), but all four bacterial genera were significantly less likely to inhibit growth of *Bd*GPL VA05 (χ^2 = 14.2, df = 1, p < 0.001) than any of the other *B. dendrobatidis* isolates (Figure 2).

Results from the ordinal analyses mirrored results from the Poisson mixed models; the model with the lowest DIC (the best supported model) contained *Bd*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, -0.57]). In addition, *Bd*GPL VA05 was also associated with significantly lower scores (mean difference = -1.42, 95% credible interval [-2.46, -0.43]). Parameter estimates for the best-supported model, as well as the model selection table containing DIC values for all five models are provided as online supplementary information (Tables S2 and S3).

Discussion

Here we show that symbiotic bacteria from the skin of amphibians exhibit differences in inhibitory capabilities across *Bd*GPL isolates, with only a small proportion of candidate

probiotics showing broad-spectrum inhibition against the global pandemic *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 *Bd*GPL [10,18,19,24,25], the propensity for *B. dendrobatidis* to rapidly evolve *in situ* [10,18,26], and the pan-global, on-going 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 mitigating 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 Kaiser & Pollinger [31] sampled amphibians between 2006 and 2008 at the same study site in Belize and found only 5% *B. dendrobatidis* prevalence on *A. moreletii* and 0% prevalence on *A. callidryas*. Museum specimens date the arrival of *B. dendrobatidis* in the general region (Mexico and Guatemala) in the late 1960s or early 1970s [32], suggesting that both host species are persisting in spite of long-term presence of *B. dendrobatidis* in the area. 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 these populations may possess a microbiome capable of at least partially mitigating *B. dendrobatidis* infection.

For manipulation of amphibian skin microbiota is to be of value for mitigating *B. dendrobatidis* in the wild, amphibian microbiomes will need to be managed for functional redundancy that provide 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 to 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 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 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 optimise 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 ability to infect hosts. Mining 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 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 *Bd*GPL.

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Table 1

Statistical significance values for Generalised Linear Models with Poisson error structure and log-link to analyse the effect of each bacterial isolate on *B. dendrobatidis* inhibition scores for the three isolates. An * indicates a statistically significant result ($p < 0.05$), meaning statistically different inhibition scores between the three *B. dendrobatidis* isolates for a given bacterial isolate. For all models, the degrees of freedom are equal to 2.

Bacterial isolate	**X^2 value**	**p value**
Acinetobacter sp. 1	9.843	0.007 *
Acinetobacter sp. 2	1.567	0.457
Agrobacterium sp.	0.000	1.000
Arthrobacter sp.	14.756	<0.001 *
Chryseobacterium sp. 1	14.120	<0.001 *
Chryseobacterium sp. 2	23.789	<0.001*
Chryseobacterium sp. 3	3.170	0.205
Enterobacter sp. 1	9.442	0.009 *
Enterobacter sp. 2	3.915	0.141
Lysobacter sp.	10.109	0.006 *

Serratia sp. 1	11.046	0.004 *
Serratia sp. 2	9.825	0.007 *
Serratia sp. 3	1.273	0.529
Serratia sp. 4	17.723	<0.001 *
Stentrophomonas sp.	25.994	<0.001*

Figure 1

Average (± 1 SEM) inhibition scores for 15 bacteria from quantitative *in vitro* challenges against three *Bd*GPL isolates. Within each bacteria, *B. dendrobatidis* isolates with an * have significantly different inhibition scores to those without an *.

Figure 2

Average (± 1 SEM) inhibition scores for multiple bacteria from four genera challenged against three *Bd*GPL isolates. VA05 isolates had significantly lower inhibition scores than the other *Bd*GPL isolates (as indicated by an *), and *Enterobacter spp.* showed significantly lower inhibition of *Bd*GPL across the range of isolates (indicated by an #).