Antifungal Bacteria on Woodland Salamander Skin Exhibit High Taxonomic Diversity and Geographic Variability

Carly R. Muletz-Wolz,[a,e] Graziella V. DiRenzo,[a,b] Stephanie A. Yarwood,[c] Evan H. Campbell Grant,[d] Robert C. Fleischer,[e] Karen R. Lips[a]

Department of Biology, University of Maryland, College Park, Maryland, USA[a]; Department of Ecology, Evolution, and Marine Biology, University of California, Santa Barbara, Santa Barbara, California, USA[b]; Department of Environmental Science & Technology, College Park, Maryland, USA[c]; United States Geological Survey Patuxent Wildlife Research Center, S.O. Conte Anadromous Fish Research Laboratory, Turners Falls, Massachusetts, USA[d]; Center for Conservation Genomics, Smithsonian Conservation Biology Institute, National Zoological Park, Washington, DC, USA[e]

ABSTRACT Diverse bacteria inhabit amphibian skin; some of those bacteria inhibit growth of the fungal pathogen *Batrachochytrium dendrobatidis*. Yet there has been no systematic survey of anti-*B. dendrobatidis* bacteria across localities, species, and elevations. This is important given geographic and taxonomic variations in amphibian susceptibility to *B. dendrobatidis*. Our collection sites were at locations within the Appalachian Mountains where previous sampling had indicated low *B. dendrobatidis* prevalence. We determined the numbers and identities of anti-*B. dendrobatidis* bacteria on 61 *Plethodon* salamanders (37 *P. cinereus*, 15 *P. glutinosus*, 9 *P. cylindraceus*) via culturing methods and 16S rRNA gene sequencing. We sampled co-occurring species at three localities and sampled *P. cinereus* along an elevational gradient (700 to 1,000 meters above sea level [masl]) at one locality. We identified 50 anti-*B. dendrobatidis* bacterial operational taxonomic units (OTUs) and found that the degree of *B. dendrobatidis* inhibition was not correlated with relatedness. Five anti-*B. dendrobatidis* bacterial strains occurred on multiple amphibian species at multiple localities, but none were shared among all species and localities. The prevalence of anti-*B. dendrobatidis* bacteria was higher at Shenandoah National Park (NP), VA, with 96% (25/26) of salamanders hosting at least one anti-*B. dendrobatidis* bacterial species compared to 50% (7/14) at Catoctin Mountain Park (MP), MD, and 38% (8/21) at Mt. Rogers National Recreation Area (NRA), VA. At the individual level, salamanders at Shenandoah NP had more anti-*B. dendrobatidis* bacteria per individual ($\mu = 3.3$) than those at Catoctin MP ($\mu = 0.8$) and at Mt. Rogers NRA ($\mu = 0.4$). All salamanders tested negative for *B. dendrobatidis*. Anti-*B. dendrobatidis* bacterial species are diverse in central Appalachian *Plethodon* salamanders, and their distribution varied geographically. The antifungal bacterial species that we identified may play a protective role for these salamanders.

IMPORTANCE Amphibians harbor skin bacteria that can kill an amphibian fungal pathogen, *Batrachochytrium dendrobatidis*. Some amphibians die from *B. dendrobatidis* infection, whereas others do not. The bacteria that can kill *B. dendrobatidis*, called anti-*B. dendrobatidis* bacteria, are thought to influence the *B. dendrobatidis* infection outcome for the amphibian. Yet how anti-*B. dendrobatidis* bacterial species vary among amphibian species and populations is unknown. We determined the distribution of anti-*B. dendrobatidis* bacterial species among three salamander species ($n = 61$) sampled at three localities. We identified 50 unique anti-*B. dendrobatidis* bacterial species and found that all of the tested salamanders were negative for *B. dendrobatidis*. Five anti-*B. dendrobatidis* bacterial species were commonly detected, suggesting a stable, functional association with these salamanders. The number of

Address correspondence to Carly R. Muletz-Wolz, craemuletz@gmail.com.

anti-*B. dendrobatidis* bacteria per individual varied among localities but not among co-occurring salamander species, demonstrating that environment is more influential than host factors in structuring the anti-*B. dendrobatidis* bacterial community. These anti-*B. dendrobatidis* bacteria may serve a protective function for their salamander hosts.

KEYWORDS *Batrachochytrium dendrobatidis*, pathogen inhibition, salamanders, skin bacteria, symbionts

The skin microbiome of vertebrates serves as a barrier against pathogens (1–3) and can mediate disease risk (4, 5). Lower disease risk in vertebrates has been associated with different characteristics of the microbiome, such as high bacterial species richness (6–8), specific microbial community assemblages (2, 7, 9, 10), and the presence of microbes that produce metabolites that inhibit growth of pathogens (1, 11, 12). For amphibians, inter- and intraspecies-specific variations in the skin microbiome (13–15) may contribute to variation in responses to infection by the deadly fungal pathogen *Batrachochytrium dendrobatidis* (16, 17). Yet we know little about how microbial diversity differs among amphibian host species and environments and how this relates to *B. dendrobatidis*-associated disease risk. Characterizing these patterns is a step toward understanding evolutionary and ecological processes structuring functionally important microbial skin assemblages, which may aid in development of conservation strategies.

B. dendrobatidis has caused mass die-offs and extirpations of susceptible amphibians on a global scale (18–21), and yet no effective treatments exist for amphibians in the wild. An active line of research has been to identify bacteria that inhibit *B. dendrobatidis* growth, here referred to as anti-*B. dendrobatidis* bacteria, and to use their geographic distribution to predict fungal disease outcome in the field (11, 22, 23) or to use them in trials of efficiency of bioaugmentation to mitigate *B. dendrobatidis*-associated disease symptoms (24–27). To date, roughly 255 anti-*B. dendrobatidis* bacterial operational taxonomic units (OTUs) that have been identified from the skin of 37 amphibian species have been deposited in a reference database (28). Nine of these bacterial species have been used in bioaugmentation trials (2, 24, 25, 29, 30).

Bioaugmentation trials using anti-*B. dendrobatidis* bacteria have had mixed success. For instance, three studies found no effect of an anti-*B. dendrobatidis* bacterial species, *Janthinobacterium lividum*, in reducing *B. dendrobatidis*-associated disease symptoms (29, 31, 32), even though *J. lividum* had previously been shown to be effective against *B. dendrobatidis* (25–27). This suggests that a bacterial species may not mitigate the impact of *B. dendrobatidis* on host health for all host species or in all environments.

Host and environmental factors may influence anti-*B. dendrobatidis* bacterial composition or whether specific bacterial species are inhibitory. For instance, Bresciano et al. (33) found several anti-*B. dendrobatidis* bacterial species at high-elevation sites but did not detect these anti-*B. dendrobatidis* bacteria at low elevations, suggesting that environmental factors impact the geographic distribution of anti-*B. dendrobatidis* bacteria. If an amphibian population does not naturally harbor a bacterial species, that bacterial species may not colonize or persist on the host if used in bioaugmentation trials (34). Further, temperature can impact whether specific bacterial taxa produce inhibitory metabolites, and this may also depend on the species of host from which the bacteria were isolated (35, 36).

We quantified differences in the number and identity of anti-*B. dendrobatidis* bacteria on three *Plethodon* species at three localities and along an elevational gradient in the central Appalachian Mountains. We chose *Plethodon* salamanders because previous sampling had indicated a low prevalence of *B. dendrobatidis* even though *B. dendrobatidis* often occurs on other amphibians in the region (37). This contrasts with widespread population declines caused by *B. dendrobatidis* in closely related plethodontid salamanders in Central America (38). The skin microbiome of *Plethodon* salamanders has been shown to limit *B. dendrobatidis* infection (24, 27, 37,

TABLE 1 Summary of salamanders sampled[a]

Locality	No. of sites	Elevation(s) (m)	Species	No. of salamanders sampled	No. of salamanders with anti-*B. dendrobatidis* bacteria	Avg no. of isolates per salamander (range)	Avg no. of anti-*B. dendrobatidis* isolates per salamander (range)
Catoctin	1	404	*P. cinereus*	7	4	8.9 (5–14)	1 (0–2)
	1	404	*P. glutinosus*	7	3	5.7 (2–9)	0.6 (0–2)
Subtotal				14	7	7.3	0.8
Shenandoah	1	702	*P. cinereus*	4	3	5.5 (1–10)	2.3 (0–3)
	2	797 ± 6	*P. cinereus*	6	6	9 (5–14)	3.7 (1–7)
	2	881 ± 18	*P. cinereus*	4	4	6.3 (4–11)	3.8 (1–7)
	1	974	*P. cinereus*	5	5	9.4 (6–16)	4.4 (3–6)
	4	697–974	*P. cylindraceus*	7	7	5.6 (2–8)	2.4 (1–4)
Subtotal				26	25	7.2	3.3
Mt. Rogers	2	997, 1,053	*P. cinereus*	11	5	2.7 (1–6)	0.6 (0–2)
	1	997	*P. glutinosus*	8	2	2 (0–3)	0.25 (0–1)
	1	1,053	*P. cylindraceus*	2	1	2 (0–4)	0.5 (0–1)
Subtotal				21	8	2.4	0.4
Total				61	40	5.6	1.7

[a]Data represent numbers of salamanders with at least one anti-*B. dendrobatidis* bacterial species and total numbers of bacterial isolates and anti-*B. dendrobatidis* bacterial isolates detected per salamander. The salamanders at Shenandoah NP had a higher (96%) prevalence of anti-*B. dendrobatidis* bacteria and a higher number (mean = 3.3) of anti-*B. dendrobatidis* bacterial species per individual than the salamanders at Catoctin Mountain Park, MD (50%; mean = 0.8) or Mt. Rogers National Recreation Area, VA (38%; mean = 0.4), regardless of species. At Shenandoah NP, *P. cinereus* had similar proportions of anti-*B. dendrobatidis* bacterial isolates along an elevational gradient.

39), and we expected anti-*B. dendrobatidis* bacteria to be prevalent. We also expected that different salamander species and populations would harbor different anti-*B. dendrobatidis* bacterial species given known species-level (14, 40) and population-level (13, 15, 33) differences in amphibian skin bacterial communities. However, we expected a few anti-*B. dendrobatidis* bacterial species, such as *J. lividum*, to be widely distributed across species and localities given previous reports of these taxa being present on *Plethodon* salamanders (41, 42). Our overall objectives were to (i) describe the geographic and taxonomic distribution of anti-*B. dendrobatidis* bacteria, (ii) determine how host and site factors influence the number of anti-*B. dendrobatidis* bacteria detected, and (iii) determine if the *B. dendrobatidis* inhibition score was related to bacterial phylogenetic relatedness. The inhibitory properties of anti-*B. dendrobatidis* bacteria may inform research on other emerging fungal pathogens such as *B. salamandrivorans*, which also poses a significant threat to global amphibian biodiversity (43, 44).

RESULTS

We did not detect *B. dendrobatidis* on any of the salamanders.

We collected and tested 341 bacterial isolates from 61 *Plethodon* salamanders at three localities (Catoctin MP, $n = 103$; Shenandoah NP, $n = 187$; Mt. Rogers NRA, $n = 51$), with an average of 6 (range, 0 to 17) bacterial morphotypes per individual (Table 1). We identified 119 of those bacterial isolates, representing a total of 50 anti-*B. dendrobatidis* bacterial OTUs across all salamanders (Table 1), as anti-*B. dendrobatidis* (Fig. 1). The 50 anti-*B. dendrobatidis* bacterial isolates belonged to four bacterial phyla (*Actinobacteria* [$n = 5$], *Proteobacteria* [$n = 33$], *Bacteroidetes* [$n = 11$], and *Firmicutes* [$n = 1$]), with *B. dendrobatidis* inhibition levels ranging from 1% to 100% (Fig. 1). We found that the more closely related anti-*B. dendrobatidis* bacterial species did not share similar *B. dendrobatidis* inhibition scores (Fig. 1) ($P = 0.865$ [Mantel]; Mantel statistic $r = -0.023$).

Most (40/61, 66%) salamanders harbored at least one anti-*B. dendrobatidis* bacterial species, with a higher proportion at Shenandoah NP (25/26, 96%) than at Catoctin MP (7/14, 50%) and Mt. Rogers NRA (8/21, 38%) (χ^2 contingency table test: $\chi^2 = 12.9$, df = 2, $P < 0.0001$) (Table 1).

Most anti-*B. dendrobatidis* bacteria were rare; 35 anti-*B. dendrobatidis* bacterial species were found on only one salamander individual. Ten anti-*B. dendrobatidis*
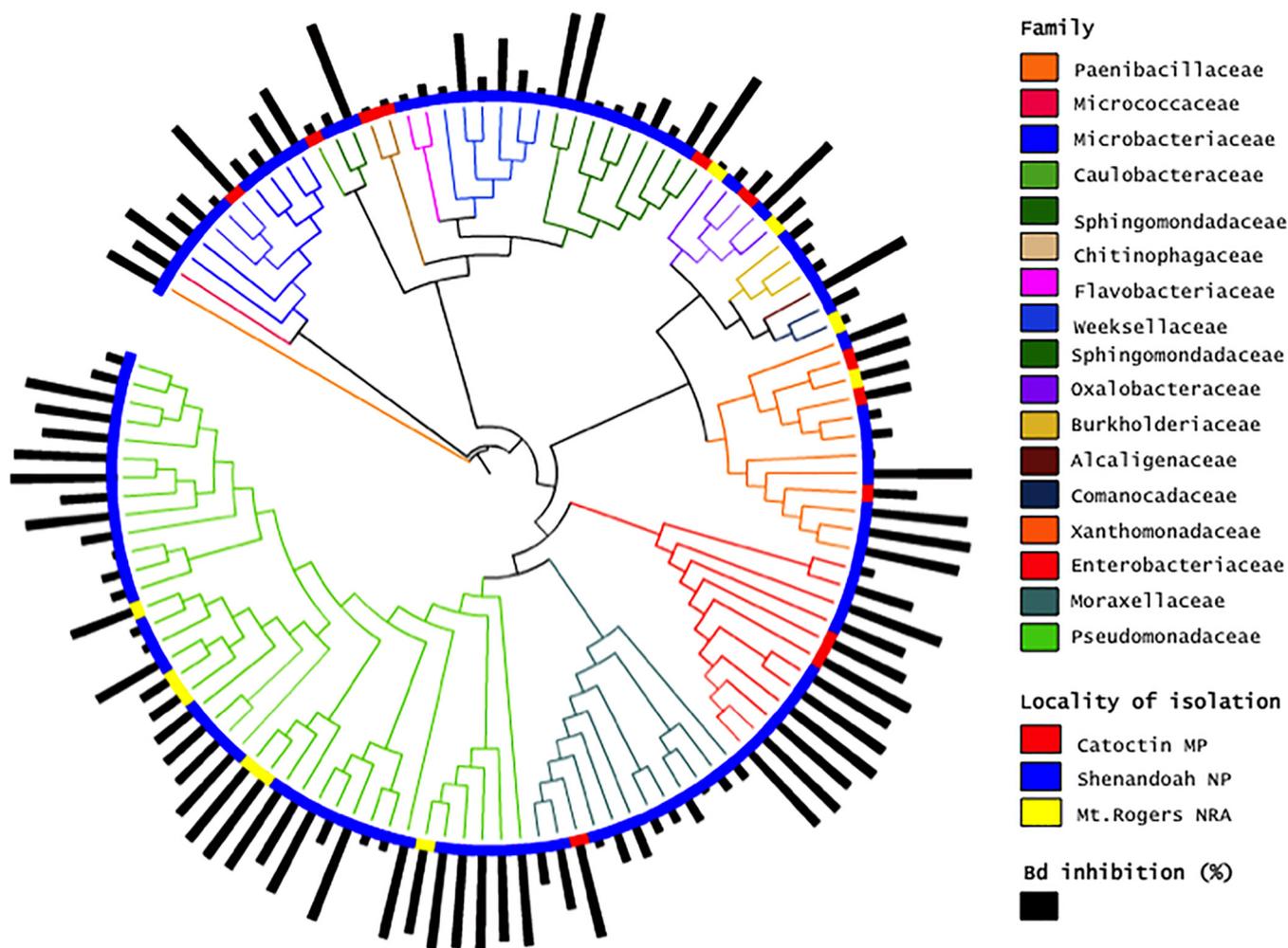
FIG 1 Phylogenetic distribution of *B. dendrobatidis* inhibition by anti-*B. dendrobatidis* bacteria across 17 families of bacteria. Branches represent individual isolates that were cultured from *Plethodon* salamanders sampled at three localities. Each bar represents the relative percent *B. dendrobatidis* inhibition (1% to 100%) by each isolate. We found that *B. dendrobatidis* inhibition values were not related to the phylogenetic relationship of the anti-*B. dendrobatidis* bacteria (*P* > 0.05 [Mantel test]), suggesting functional trait redundancy. Bd, *B. dendrobatidis*.

bacterial species were detected either on one salamander species or at one locality. A few anti-*B. dendrobatidis* bacterial species were widespread; specifically, five anti-*B. dendrobatidis* bacterial species (*Acinetobacter rhizosphaerae*, *Luteibacter rhizovicinus*, two *Pseudomonas* spp., and one *Stenotrophomonas* sp.) were found in multiple localities and on multiple salamander species (Table 2). These five bacterial species have also been commonly detected on other amphibian species globally (Woodhams et al. 2015 [28]) (Table 2). An OTU corresponding to *Luteibacter rhizovicinus* was the only anti-*B. dendrobatidis* bacterial OTU found at all three sampled localities, and a *Pseudomonas* sp. OTU was the only anti-*B. dendrobatidis* bacterial OTU found on all three salamander species. No anti-*B. dendrobatidis* bacteria were found at all localities and on all salamander species.

We identified 13 novel anti-*B. dendrobatidis* bacterial species that did not match any bacteria previously identified on amphibian skin (28). Twelve of those bacterial species occurred on only a single individual, while one was detected on two heterospecific individuals at one site.

Locality was the only significant predictor of the proportion of anti-*B. dendrobatidis* bacterial species per individual (generalized linear mixed-effects model [GLMM]: LR Chisq = 12.0107, df = 2, *P* = 0.0025). The average proportion of anti-*B. dendrobatidis* bacterial species per individual was higher at Shenandoah NP, with 46% (3.3/7.2) of

TABLE 2 Summary of the five common anti-*B. dendrobatidis* bacterial species that we detected on at least two salamander species and in at least two localities[a]

Anti-*B. dendrobatidis* bacterial OTU	Mean % inhibition (range)	Localities	No. of sites	Salamander species	No. of salamanders	No. of other amphibian species in Woodhams et al. database (2015)
Acinetobacter rhizosphaerae (denovo45)	28 (1–99)	Catoctin, Shenandoah	6	*P. cylindraceus, P. cinereus*	9	4
Luteibacter rhizovicinus (denovo18)	35 (11–58)	Catoctin, Shenandoah, Mt. Rogers	4	*P. cylindraceus, P. cinereus*	4	2
Pseudomonas sp. (denovo13)	58 (4–100)	Shenandoah, Mt. Rogers	7	*P. cylindraceus, P. cinereus, P. glutinosus*	14	25
Pseudomonas sp. (denovo20)	63 (1–100)	Shenandoah, Mt. Rogers	7	*P. cylindraceus, P. cinereus*	11	26
Stenotrophomonas sp. (denovo22)	89 (45–100)	Catoctin, Shenandoah	4	*P. cylindraceus, P. cinereus*	4	1

[a]We indicate the number of amphibian species on which the same anti-*B. dendrobatidis* bacterial OTUs have been detected in previous studies using the database of Woodhams et al. (28) (total, 37 species).

bacterial species isolated being anti-*B. dendrobatidis* bacteria, while 11% (0.8/7.3) at Catoctin MP and 17% (0.4/2.4) at Mt. Rogers NRA were anti-*B. dendrobatidis* bacteria (Table 1). Among the host (species, sex, body condition, cover object) and site (leaf litter depth, soil pH, substrate temperature) characteristics that we examined, none were significant predictors of the proportion of anti-*B. dendrobatidis* bacteria per individual (GLMM: $P > 0.05$). On *P. cinereus* in Shenandoah NP, the proportions of anti-*B. dendrobatidis* bacteria per individual were similar among all elevations (GLM: t-value $= 0.566$, $P = 0.58$) (Table 1).

DISCUSSION

Geographic and taxonomic distribution of anti-*B. dendrobatidis* bacteria. We identified a large number of anti-*B. dendrobatidis* bacterial taxa on the three *Plethodon* species, but few were widespread. Other studies have also found high diversity and little overlap in anti-*B. dendrobatidis* bacteria among individual amphibians (11, 14, 22). High taxonomic turnover across the landscape with functional trait redundancy has been reported in other microbial communities (45, 46). Turnover in the regional pool of microorganisms is likely due to different environmental conditions driving environmental filtering of microbial species but not microbial traits (45, 47, 48). This indicates that selection for certain bacteria may be driven by the function of the bacteria and not species identity. For instance, Loudon et al. (49) found that bacteria with anti-*B. dendrobatidis* properties are more likely to colonize a host than those that lack anti-*B. dendrobatidis* properties, suggesting that certain bacteria may be more prevalent on amphibian skin given their functional properties regardless of species identity.

Five anti-*B. dendrobatidis* bacterial species were widely distributed across multiple localities and multiple salamander species, suggesting a symbiotic association between these anti-*B. dendrobatidis* bacteria and *Plethodon* salamanders. Specifically, *Pseudomonas* spp. were the most widespread in both our study and a previously published study (28). Several ecological and physiological factors could explain this relationship, including (i) their ability to utilize available glucose (50) or to produce siderophores (51), both of which would reduce resource availability for competitors, as well as (ii) selection by the host for pseudomonads given their capacity to produce numerous antimicrobial compounds (52, 53). If generalist anti-*B. dendrobatidis* bacteria, such as pseudomonads, are important as an amphibian defense mechanism against *B. dendrobatidis*, then the abundance of these bacterial species may be a key factor in protection (54, 55). In some cases, high cell density is needed to produce inhibitory metabolites because the metabolites are produced in high quantities only when bacteria form a biofilm and undergo quorum sensing (56, 57). *B. dendrobatidis* likely reduces the growth of some anti-*B. dendrobatidis* bacteria (58), and augmenting these bacterial populations on susceptible amphibians during *B. dendrobatidis* epidemics may prove helpful.

We expected *J. lividum* to be common because it was previously detected, using molecular approaches, on most individuals in two of three *P. cinereus* populations in Virginia (27, 42, 59). However, using culture methods, we found anti-*B. dendrobatidis* bacterial OTUs in the *Janthinobacterium* genus on only three *P. cinereus* salamanders at two of our three sampled localities (Catoctin MP and Shenandoah NP). *Janthinobacterium* spp. may be widely distributed but not always common across the range of *P. cinereus*. Alternatively, *Janthinobacterium* or other anti-*B. dendrobatidis* bacterial species may commonly occur but may be in low abundance (59) and less likely to be cultured.

Influence of host and site factors on anti-*B. dendrobatidis* bacterial distribution. We found no support for the notion of host species factors influencing the number of anti-*B. dendrobatidis* bacteria. Other studies have shown that levels of skin bacterial richness differ among amphibian species (2, 15, 40). However, the processes that structure the antifungal bacterial community may differ from those structuring the total bacterial community, given different selection pressures (60, 61). For instance, bacteria can diversify more in the presence of a parasite (62), and even a single gene locus change can modify community structure to the same extent as the loss of an apex predator (63). Alternatively, the host biology of *Plethodon* salamanders may explain the differences (64). We studied terrestrial, direct-developing amphibian species within a single genus that are ecologically similar throughout their lifetime (65), in contrast to previous studies that compared species from different families of amphibians that have both aquatic and terrestrial life stages (15, 40, 66).

Location was a key predictor of the prevalence and number of anti-*B. dendrobatidis* bacteria, indicating environmental differentiation. However, none of the site characteristics that we examined (substrate temperature, soil pH, and leaf litter depth) explained this pattern. Across the three localities, salamanders at Shenandoah NP had a higher number of anti-*B. dendrobatidis* bacteria than salamanders at Catoctin MP or Mt. Rogers NRA. Other environmental properties such as long-term trends in temperature may have influenced salamander bacterial diversity (67), as both Catoctin MP and Mt. Rogers NRA have experienced higher rates of warming than Shenandoah NP (68).

Spatial scale may explain variations in the number of anti-*B. dendrobatidis* bacteria among localities but not variations along the elevational gradient. Environmental differences (e.g., temperature, moisture) of localities 150 km apart (e.g., Catoctin MP and Shenandoah NP) may be more likely to drive diversity patterns than the small-scale variations in environmental conditions from 700 meters above sea level (masl) to 1,000 masl within Shenandoah NP. For instance, Sunagawa et al. (45) found temperature, rather than other environmental factors (e.g., salinity, nutrients) or geography, to be the most important factor driving ocean microbiome richness, but only when large temperature differences were examined. In other systems, differences in geochemistry, such as pH (45, 69), total nitrogen (70), or dissolved organic matter (71), can influence bacterial composition more than climate or elevation. We suggest future research on habitat chemistry to identify the factors driving environmental differentiation.

We did not detect *B. dendrobatidis* in this study, but *B. dendrobatidis* has been previously detected at all three of the localities that we sampled (37, 72; K. R. Lips, unpublished data). *B. dendrobatidis* is commonly detected in amphibian communities throughout the eastern United States, with prevalence estimates of 10% to 40% (reviewed in reference 37). This suggests that these salamanders may be exposed to *B. dendrobatidis* and that anti-*B. dendrobatidis* bacteria or other factors (e.g., immuno-genetic properties) (73, 74) may be limiting *B. dendrobatidis* infection on these salamanders.

We found evidence for potential herd immunity to *B. dendrobatidis* (11, 75) at Shenandoah NP, with 96% of salamanders harboring at least one anti-*B. dendrobatidis* bacterial species. The concept of herd immunity suggests that an infectious disease will be less likely to affect a population if the proportion of protected individuals is above a threshold value, generally between 80% and 95% (75). Woodhams et al. (11) suggested that this value is approximately 80% in the amphibian-*B. dendrobatidis* system, as a population of *Rana muscosa* with 86% of individuals harboring anti-*B.*

dendrobatidis bacteria persisted with *B. dendrobatidis*, whereas a population with 62% died off following *B. dendrobatidis* invasion. We found that 50% and 38% of salamanders at Catoctin MP and Mt. Rogers NRA, respectively, had anti-*B. dendrobatidis* bacteria; more anti-*B. dendrobatidis* bacteria could have been present, but we were unable to detect them due to the limitations of culturing (76).

Association between *B. dendrobatidis* inhibition and anti-*B. dendrobatidis* bacterial phylogeny. *B. dendrobatidis* inhibition strength was not related to bacterial phylogeny, indicating that phylogenetic information is not predictive of *B. dendrobatidis* inhibition strength. Nonetheless, certain genera contain a large proportion of anti-*B. dendrobatidis* bacterial species, chiefly *Pseudomonas* and *Stenotrophomonas*, and detection of bacterial sequences in those genera likely indicates that many of those species have anti-*B. dendrobatidis* traits (94). Two possible, nonexclusive mechanisms may explain the lack of phylogenetic conservatism of *B. dendrobatidis* inhibition: (i) genes involved in inhibition are widely distributed among bacterial phylogenies, and (ii) horizontal gene transfer of antifungal genes may occur among unrelated bacteria. First, functions involving few genes are often widely distributed among bacterial phylogenies (77), and relatively few genes can be involved in the biosynthetic pathways that produce secondary metabolites. For instance, the sequences that produce the anti-*B. dendrobatidis* metabolites violacein (78) and 2,4-diacetylphloroglucinol (79) consist of five and eight genes, respectively (80, 81). Second, horizontal gene transfer of genes important in *B. dendrobatidis* inhibition may be occurring among unrelated bacteria, as homologous recombination can occur among bacterial lineages with levels of DNA divergence as high as 25% (82). For instance, the transfer of genes encoding antifungal compounds between different bacterial species was previously observed (83, 84). While we cannot determine which mechanism(s) operated, we found that anti-*B. dendrobatidis* bacterial inhibition strength was phylogenetically dispersed among the members of at least four bacterial phyla, demonstrating the wide taxonomic breadth of this trait.

Conservation implications. There are no proven strategies to prevent infection, mortality, or population declines of amphibians caused by *B. dendrobatidis*. The use of anti-*B. dendrobatidis* bacterial augmentation has been championed as a conservation strategy (34, 85), but results have been inconsistent, in part because environmental conditions may control bacterial communities on amphibian skin. We found that individual salamanders had a mostly unique anti-*B. dendrobatidis* bacterial profile, with a few shared bacterial species, suggesting that enhancing the functional traits of the existing anti-*B. dendrobatidis* bacterial community (86) and using only widely distributed anti-*B. dendrobatidis* bacteria may be beneficial bioaugmentation strategies (34). One method to enhance the functional traits of a community is to use prebiotics, which selectively promote growth or activity in the community. For instance, polysaccharide β-glucan has been commercially used as a prebiotic to improve resistance of fish to infection by pathogens (87). Additionally, using ubiquitous anti-*B. dendrobatidis* bacterial species in bioaugmentation trials, such as those identified here, may increase the likelihood that the bacteria will persist for longer time periods on target amphibians (34). In two bioaugmentation studies, introduction of anti-*B. dendrobatidis* bacteria onto amphibian skin did not affect the skin microbial community (2, 88), indicating that adding probiotics is likely not detrimental to the host in terms of their resident microbial community. Yet augmented anti-*B. dendrobatidis* bacterial populations often do not persist on amphibian hosts for longer than a few months (25, 27), and reinoculation over time may be needed (89).

Inhibitory metabolites produced by anti-*B. dendrobatidis* bacteria likely have general antimicrobial properties (52) and may provide a general defensive benefit against pathogens to the host (9, 13, 23, 42). Therefore, our results may also be useful if the deadly, closely related fungal pathogen *Batrachochytrium salamandrivorans* (43) reaches this salamander biodiversity hot spot (44).

MATERIALS AND METHODS

Field sampling. We sampled three species of terrestrial woodland salamanders, *Plethodon cinereus*, *P. glutinosus*, and *P. cylindraceus*, at three localities within a 497-km stretch in the central Appalachians in spring 2012 (Table 1). We chose the three localities, Catoctin Mountain Park (MP), MD, Shenandoah National Park (NP), VA, and Mt. Rogers National Recreation Area (NRA), VA, because they were within the range of localities where we had previously tested these species for *B. dendrobatidis* and found <1% *B. dendrobatidis* prevalence (37) and because they were within the distribution of *P. cinereus* and either *P. glutinosus* or its sister species *P. cylindraceus* (65). At Catoctin MP, we sampled *P. cinereus* ($n = 7$) and *P. glutinosus* ($n = 7$) salamanders at one site. At Shenandoah NP, we sampled 19 *P. cinereus* salamanders at six sites along an elevational gradient and sampled 7 *P. cylindraceus* salamanders at four of those sites (Table 1). At Mt. Rogers NRA, we sampled two *P. cylindraceus* and six *P. cinereus* salamanders at one site and eight *P. glutinosus* and five *P. cinereus* salamanders at a second site (Table 1). We had permits from state and federal agencies for handling and swabbing live amphibians (DNR permit no. 50269 [Maryland], VDGIF permit no. 042151 [Virginia], and NPS permit no. SHEN-2011-SCI-0014 [Shenandoah National Park]), and we received approval for the research from the University of Maryland Institutional Animal Care and Use Committee (R-11-11).

We collected two skin swabs from each salamander, one to culture bacteria and one to test for the presence of *B. dendrobatidis*. We used a new pair of nitrile powder-free gloves to handle each salamander and rinsed each salamander twice for 30 s with sterile water to remove transient microbes (42). We placed each individual salamander into a new plastic bag and swabbed it 20 times on the left side (dorsal/ventral sides and front/back limbs [five strokes each]) with a MW-113 swab (Medical Wire, United Kingdom). We stored the swab in a 1.5-ml tube on ice until we returned to the laboratory, where samples were stored in a −80°C freezer until analysis for *B. dendrobatidis* quantification. Then, we swabbed the right side of each salamander 20 times and immediately streaked the swab onto a R2-A nutrient agar plate in a zigzag fashion and wrapped the plate in Parafilm in the field. We stored plates at ambient temperature (12°C to 23°C) throughout incubation.

We recorded GPS coordinates, leaf litter depth, and soil pH (Kelway soil tester) at each site. For each individual, we (i) measured the substrate temperature at capture (Fluke infrared thermometer), (ii) recorded the cover object, (iii) identified the species and sex, and (iv) measured mass and snout-to-vent length (SVL) to quantify their body condition. From previous research on their effects on microbial diversity, we expected that a number of variables, such as the sources of the environmental microbes (e.g., leaf litter depth and cover object [59, 90]), pH (70), temperature (45), species (40), sex (91), and body condition (92), might influence the distribution of anti-*B. dendrobatidis* bacteria.

Microbiology procedures. We isolated morphologically distinct bacterial colonies into pure cultures based on color, form, elevation, margin, substance, and opacity. We preserved isolates in 20% glycerol in a −80°C freezer until challenge assays were conducted.

We conducted bacterium-*B. dendrobatidis* challenge assays with each bacterial isolate ($n = 341$) and *B. dendrobatidis* isolate JEL 404 (Maine, USA) using a modified version of a protocol by Bell et al. (93). To begin, we grew cryopreserved bacteria on 1% tryptone plates for 3 days, passaged the bacteria in 3 ml of 1% tryptone broth, and grew the bacteria on a shaker at 100 rpm for an additional 3 days. Then, we made bacterial isolate/*B. dendrobatidis* cocultures, *B. dendrobatidis* monocultures, and negative controls in 3-ml culture tubes. For the bacterial isolate/*B. dendrobatidis* cocultures, we added 100 μl of a bacterial isolate and 100 μl of *B. dendrobatidis* to 1 ml of 1% tryptone broth. We grew bacteria and *B. dendrobatidis* together to mimic natural conditions, as bacteria and *B. dendrobatidis* likely interact on amphibian skin. Nonetheless, Becker et al. (94) demonstrated that testing *B. dendrobatidis*/bacterial isolate cocultures versus bacterial monocultures against *B. dendrobatidis* had no effect on the magnitude of *B. dendrobatidis* inhibition. For *B. dendrobatidis* monocultures, we added 100 μl of *B. dendrobatidis* to 1.1 ml of 1% tryptone broth. For a negative control, we added 1.2 ml of 1% tryptone broth to the 3-ml tubes. We grew these cultures on a shaker at 100 rpm for 3 days. To obtain microbially produced metabolites, termed cell-free supernatant (CFS [93]), we centrifuged the bacterial isolate/*B. dendrobatidis* cocultures, *B. dendrobatidis* monocultures, and negative controls at 10,000 rpm for 5 min. Then, we used 18-gauge hypodermic needles attached to 3-ml syringes (*B. dendrobatidis* Vacutainer no. 309657) to remove the supernatant and filtered the supernatant through 0.22-μm-pore-size filters in 13-mm-diameter-syringe-filter holders (Millipore GSWP01300/SX0001300). We harvested *B. dendrobatidis* zoospores by flooding 1-week-old *B. dendrobatidis* plates grown at room temperature (RT) with 1% tryptone and filtering the broth through a sterilized coffee filter in a glass funnel.

To set up the assays, we added 50 μl of approximately 1×10^6 zoospores/ml of JEL 404 (counted with a hemocytometer; approximately 50,000 zoospores in each well) to a 96-well plate in all wells except those designated for use as negative controls. Into the bacterium-*B. dendrobatidis* sample wells, we added 50 μl of the CFS from each bacterium-*B. dendrobatidis* sample in each of four wells. We used four controls (two negative controls and two positive controls [PC]) in each 96-well assay using four wells for each control. The positive controls were as follows: 50 μl of *B. dendrobatidis* zoospores plus 50 μl of *B. dendrobatidis* CFS (PC) and 50 μl of *B. dendrobatidis* zoospores plus 50 μl of water (nutrient-depleted positive control [NDPC]). The negative controls were as follows: 50 μl of *B. dendrobatidis* zoospores heat-killed (HK) at 60°C for 60 min plus 50 μl of *B. dendrobatidis* CFS (HK *B. dendrobatidis*) and 50 μl of 1% tryptone broth plus 50 μl of *B. dendrobatidis* CFS. We measured the level of absorbance (optical density at 492 nm [OD_{492}]) in each well using a microplate reader on days 0, 1, 4, 7, 8, and 10 of the experiment.

B. dendrobatidis inhibition score calculations. We used the day of maximum growth (day 7 or 8) of the PC for each assay as the day to quantify *B. dendrobatidis* inhibition. To calculate *B. dendrobatidis*

inhibition scores, we divided the average OD reading from the bacterium-*B. dendrobatidis* sample ($n = 4$) by the average OD reading from the NDPC for that assay ($n = 4$), after correcting for the average OD of the heat-killed *B. dendrobatidis* (HK, $n = 4$), and subtracted that value from 1. We subtracted the OD of the HK to remove the value corresponding to the baseline absorbance of *B. dendrobatidis* zoospores. The equation we used was as follows: inhibition score $= 1 - [(\text{sample}_{OD} - HK_{OD})/(NDPC_{OD} - HK_{OD})]$. We interpreted values greater than zero as being representative of anti-*B. dendrobatidis* bacteria, indicating that the bacterium-*B. dendrobatidis* sample wells had less growth than the NDPC wells as measured by OD. Prior to the OD readings, we visually inspected each well using an inverted microscope and coded the visual observations of the sample wells as no, weak, moderate, or strong inhibition in comparison to the NDPC wells.

To identify anti-*B. dendrobatidis* bacteria, we used a conservative approach: (i) both the OD readings and visual observations had to support inhibition, and (ii) we used the NDPC rather than the PC to quantify *B. dendrobatidis* inhibition strength. We used the NDPC rather than the PC because the experimental wells in challenge assays contained 50 μl of CFS in which bacteria and *B. dendrobatidis* had previously been cultured, while the PC wells contained 50 μl of previously unused medium. The growth of *B. dendrobatidis* in experimental wells could have been affected by depletion of the nutrients used by the bacteria and *B. dendrobatidis*. Therefore, comparing the experimental wells to the NDPC wells accounts for the issue of nutrient depletion and is a more conservative approach in identifying anti-*B. dendrobatidis* bacteria.

We attempted to minimize the variation of bacterial population size and growth stage by allowing each culture to grow for 3 days. At approximately 3 days, most bacterial cultures reached the later phases of growth, when inhibitory metabolites are produced (95). This is a commonly accepted practice and is currently the best method for testing a large number of bacterial isolates (35, 60, 93, 94).

Molecular procedures. We sequenced the 16S rRNA gene of all anti-*B. dendrobatidis* bacterial isolates using Sanger sequencing. We selected a single colony of each isolate from agar plates using a sterilized toothpick, added the colony to 25 μl of sterile deionized water, and boiled the mixture for 10 min at 95°C to extract bacterial DNA. We used PCR to amplify a 1,037-bp fragment of the 16S rRNA gene using the 8F/1045R primer set. Each 25-μl PCR assay mixture consisted of 1.25 U of AmpliTaq Gold DNA polymerase (ThermoFisher) in proprietary buffer, 2.5 μM MgCl$_2$, 200 nM concentrations of deoxynucleoside triphosphates (dNTPs), a 600 nM concentration of each primer, and 2 μl DNA template. PCR conditions were 95°C for 8 min, followed by 30 cycles of 95°C for 45 s, 52°C for 30 s, and 72°C for 30 s and a final extension (72°C for 3 min). We followed the sequencing methods outlined by Muletz et al. (37) to sequence the cleaned PCR products. Individual sequences were assembled and edited in Sequencher 5.1 to obtain a consensus sequence per anti-*B. dendrobatidis* bacterial isolate.

We tested all sampled salamanders for *B. dendrobatidis* using quantitative PCR (qPCR). We extracted DNA from swabs using a MoBio PowerSoil DNA extraction kit following the manufacturer's protocol. We used primers developed by Boyle et al. (96) and used iTaq supermix with Rox (Bio-Rad) following their qPCR protocol. We ran all DNA samples in duplicate and used standards of 100, 10, 1, and 0.1 zoospore genomic equivalents (ZGEs) developed from a Puerto Rican *B. dendrobatidis* isolate, JEL 427. If one of the duplicates returned a positive signal, it was run a third time. Samples were considered positive if they amplified twice before 0.1 ZGEs.

Sequence analysis. We identified anti-*B. dendrobatidis* bacteria to the lowest taxonomic level with MacQIIME 1.9.1 (97) using the *pick_de_novo_otus.py* command with default parameters. This command clustered sequences into operational taxonomic units (OTUs) using UCLUST based on 97% pairwise identity and assigned taxonomy using UCLUST (Edgar 2010) with the Greengenes reference database (98) (release 13_8). We aligned the bacterial isolate sequences using the Ribosomal Database Project (http://rdp.cme.msu.edu) alignment tools following Dunitz et al. (99), including an archaeal outgroup in the alignment. We built a phylogenetic tree using Fasttree (100) implemented in MacQIIME and used the package "ape" (101) in the R environment (102) to root the tree and trim the outgroup.

Comparison of identified anti-*B. dendrobatidis* bacteria to published database entries. We compared our anti-*B. dendrobatidis* bacterial 16S rRNA sequences to those in a published database (28) to identify common and novel anti-*B. dendrobatidis* bacteria. As described above, we identified bacterial OTUs using the *pick_de_novo_otus.py* command with default parameters in MacQIIME 1.9.1. We then split the OTU table using the *split_otu_table.py* command to ensure that it would contain only anti-*B. dendrobatidis* bacteria and filtered out zeros (not anti-*B. dendrobatidis* bacteria) using the *filter_otus_from_otu_table.py* command. We summed the number of anti-*B. dendrobatidis* bacterial OTUs in the Woodhams database, which contains a total of 255 OTUs. Next, we used a custom blast analysis in Geneious 8.1 (103) to query our anti-*B. dendrobatidis* bacterial isolate sequences against those 255 anti-*B. dendrobatidis* bacterial OTUs. We used a megablast program, having Geneious return results as query-centered alignment data only and returning only the top hit. We considered anti-*B. dendrobatidis* bacterial sequences in our data set to be novel if all sequences in that OTU had <97% pairwise identity to the OTUs in the Woodhams data set. For common anti-*B. dendrobatidis* bacteria in our data set, we counted the number of amphibians in the Woodhams data set that had OTUs that matched at >97% pairwise identity. Note that while these OTUs matched antifungal database entries, such a result does not necessarily indicate that these bacteria exhibit antifungal activity but does indicate that they are strong candidates for exhibiting antifungal activity.

Statistical analysis. All statistical analyses were performed in R 3.1.3 (102).

To determine if the numbers of salamanders with at least one anti-*B. dendrobatidis* bacterial OTU differed among the three localities, we used a χ^2 contingency table test. To determine if the number of anti-*B. dendrobatidis* bacteria per salamander was related to host or environmental characteristics, we

used a generalized linear mixed-effects model (GLMM) with a binomial distribution in the package "lme4" (104). We included species and locality as the main explanatory variables, along with additional covariates of host (sex, body condition, cover object) and site (leaf litter depth, soil pH, substrate temperature). As the response variable, we used the proportion of anti-*B. dendrobatidis* bacteria per salamander (i.e., the total number of anti-*B. dendrobatidis* bacteria divided by the total number of bacteria isolated) to account for variations in the number of isolates cultured per individual. We also ran this analysis using the raw counts of anti-*B. dendrobatidis* bacterial OTUs as the response variable with a Poisson distribution, and our results were the same. We included site as a random effect in the model to account for pseudoreplicating sites at localities. To quantify salamander body condition, we used the residuals of the linear regression of body mass on SVL for *P. cinereus* and for the *P. glutinous* complex (*P. cylindraceus* and *P. glutinosus*) separately, as *P. cinereus* is smaller in size than the *P. glutinosus* complex. To determine which variables in the model significantly predicted the proportion of anti-*B. dendrobatidis* bacteria per salamander, we used the *Anova* function in the "car" package (105) with the type III margin sum of squares reported. Then, we used Tukey's honestly significant difference (HSD) test for *post hoc* comparisons to determine significant differences between categorical levels of fixed effects using the *lsmeans* function in the package "lsmeans" (106). We assessed model goodness of fit by visually inspecting residuals.

We used a generalized linear model to determine if the number of anti-*B. dendrobatidis* bacteria per salamander was related to elevation for *P. cinereus* sampled along an elevational gradient in Shenandoah NP. As described above, we used the proportion of anti-*B. dendrobatidis* bacteria per salamander as the response variable. We assessed goodness of fit for the model by visually inspecting residuals and used a quasibinomial distribution to account for overdispersion.

We tested if *B. dendrobatidis* inhibition scores were phylogenetically conserved across anti-*B. dendrobatidis* bacteria using a Mantel test. We generated a distance matrix of *B. dendrobatidis* inhibition scores among anti-*B. dendrobatidis* bacterial isolates using Euclidean distances (94) and a corresponding distance matrix of percent sequence identity from the anti-*B. dendrobatidis* bacterial isolate sequence alignment. We used the "vegan" package (107) to conduct the Mantel test with 10,000 permutations. We visualized inhibition scores of anti-*B. dendrobatidis* bacteria on the bacterial phylogeny using iTol (108) (http://itol.embl.de/).

Accession number(s). We deposited the 16S rRNA sequences for the 119 anti-*B. dendrobatidis* bacterial isolates in GenBank (accession numbers KU738912 to KU739030). All other data from this study, including inhibition data and sequence alignments, have been deposited on figshare (https://figshare .com/s/f96dafa91c0b15291818).

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