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Bacterial flora on Cascades frogs in the Klamath mountains of California

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ABSTRACT

Amphibians are experiencing global declines due in part to the infectious disease chytridiomycosis. Some symbiotic bacteria residents on frog skin have been shown to inhibit the growth of *Batrachochytrium dendrobatitis* (Bd) but few studies have attempted to fully describe the resident bacterial flora of frog skin. We cultured and sequenced 130 bacterial isolates from frogs collected from the California Klamath Range, recovering predominantly Gram-negative bacteria from 20 higher order taxa and 31 genera. There were also a large number of unclassifiable isolates. Forty-three isolates were assessed for their ability to inhibit the growth of Bd *in vitro*; of these, two had strong and three had slight anti-Bd activity. We suggest that many bacterial species may play a secondary role in Bd resistance, acting synergistically with inhibitory species. Future research is required in order to characterize these interactions. Understanding the relationships between bacterial strains may be important in predicting and managing the effects of future anti-Bd treatments such as antimicrobial compounds or probiotic bacteria.

1. Introduction

Across much of the world, amphibians are undergoing population decline and extinction associated with infectious disease, habitat change, environmental contaminants, and invasive predator and competitor species. One important contributor to the decline is chytridiomycosis caused by the fungal pathogen *Batrachochytrium dendrobatidis* (Bd) [1–8]. Bd is a recently emerged, aquatic pathogen [9] that invades the keratinized epidermis of amphibians resulting in osmotic imbalance through the depletion of electrolytes [4,10]. The host experiences skin sloughing, reddening of the ventral epidermis, lethargy, loss of righting reflex, and death [11]. While most fatalities occur in post-metamorphic animals [12], tadpoles may be infected in keratinized structures (such as mouthparts) and transmit the fungus to both pre- and post-metamorphic individuals [13,14].

Amphibians of different species and at different sites exhibit pronounced variation in resistance to Bd [12] and studies have been conducted attempting to characterize this variation *via* looking at the amphibian immune system, the production of antimicrobial peptides, and the presence of resident symbiotic bacteria within the muccal coat. Symbiotic bacteria are a particularly interesting area of study as the community make-up seems to be species specific [15] and certain strains appear to inhibit the growth of Bd through producing broad-spectrum antimicrobial chemicals, competing for space or nutrients [16], or facilitating the growth of other bacteria with anti-Bd effects [17–23]. Alternatively, mucus may support opportunistic bacterial pathogens. For example, red-legged disease of frogs

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is commonly associated with infection with *Aeromonas hydrophila* (although commonly in frogs already infected with Ranavirus) [24] and Davis et al. [25] found that the pore structures present in frog skin with Bd infection tended to allow opportunistic bacteria to evade host resistance and cause disease.

It is necessary to evaluate the overall community of frog bacterial skin flora because of structural and ecological interactions in symbiotic bacterial communities. After bacteria colonize host epithelial mucus, some occupy adherent, matrix-enclosed microbial accretions called biofilms [26] which offer bacteria the advantages of a stable colony that is resistant to shearing forces and environmental and chemical biocidal agents. The surface microbiota in biofilms protects against pathogen colonization in humans [27], coral [28], and probably amphibians as well. A community approach to bacterial skin flora facilitates examination of the interactive dynamics of a diverse bacterial community, be those interactions deleterious, unimportant, or beneficial to frog health.

In this study we focus on the bacterial flora of the Cascades frog (*Rana cascadae*), a medium-sized diurnal frog that inhabits mountainous waters in the Cascades Mountains and the Klamath ranges of the north-west United States [29,30]. The Cascades frog has almost completely disappeared from the southernmost part of its range in Northern California, but populations further to the north appear robust at present [31]. The Cascades frog is susceptible to Bd [32] and chytridiomycosis has led to dramatic declines in a closely-related species in California's Sierra Nevada mountains [5,6], suggesting that Bd may have contributed to declines in the Cascades frog as well. In this study we intend to: (1) Survey a cultivable subset of bacterial flora from the skin of Cascades frogs in four sites in the California portion of the Klamath range, (2) describe the known ecological characteristics of resident bacterial species, (3) determine whether some bacterial species were over-represented in each community or associated with the presence of Bd on frog individuals, and (4) assess a subset of cultured bacteria for anti-Bd activity.

2. Materials and methods

Post-metamorphic Cascades frog individuals were sampled opportunistically at four lakes in the Trinity Alps Wilderness in the California Klamath Range in August 2008. These lakes are Found (515319 W, 4543215 N), Eagle Creek (519403 W, 4560985 N), Shimmy (516555 W, 4539439 N), and Section Line (513095 W, 4560993 N). The elevations of these four lakes ranged from 1870 to 2150 m and the perimeters ranged from 230 to 530 m. Introduced trout had been present at Section Line Lake, but the fish had been removed at least five years prior to the time of this study. The three remaining lakes have not supported fish for at least a decade. Other amphibian species recorded included Pacific chorus frogs (*Pseudacris regilla*), adult western toads (*Anaxyrus boreas*) (observed at Found and Shimmy Lakes), and long-toed salamanders (*Ambystoma macrodactylum*) which use Found and Eagle Creek Lakes as breeding sites.

Frogs were captured with a hand net and assessed for gender, size, and age. Post-metamorphic Cascades frog life stages were classified using snout-vent lengths: individuals < 30 mm in length were classified as metamorphs, males > 45 mm and females > 50 mm were classified as adults, and remaining individuals were considered subadults (Pope, Garwood and Larson, unpub. data). For assessment of Bd, we ran a sterile rayon swab (Medical Wire & Equipment, Corsham, Wiltshire, England) along the skin of animals, swabbing each of the following areas five times: the ventral surface of the abdomen, the left and right inner thighs, and webbing of each hind foot [33]. Each animal was then rinsed twice with sterile water to remove transient bacteria [22] after which bacteria were collected from each individual using a separate sterile swab and isolated following methods described in Woodhams et al. (2007). The swabs were streaked in the field onto R2A agar (Becton-Dickinson, Franklin Lakes, New Jersey, USA), a commonly used medium for isolation of aquatic bacteria. This medium and these techniques were chosen to replicate the published protocols of Woodhams et al. [34]. Cultures were transferred to the University of California, Davis, and maintained at room temperature for 3–5 days. All colonies with phenotypes which showed any differences in color, opacity, colony size, border, and profile were isolated by removing a single colony with a sterile loop, re-streaking onto R2A agar, and incubating at room temperature for an additional 3–5 days. Although 256 bacterial isolates were obtained during culture, only 130 were retained through passage and available for characterization in this study.

For molecular identification of the bacteria, the 16S rRNA gene was chosen because of the availability of highly conserved flanking primer sites and an extensive reference database (Genbank, NCBI, http://www.ncbi.nlm.nih.gov/genbank/). In order to avoid cross-contamination of samples, all master mixes were prepared in a tissue culture hood with the user wearing a clean laboratory coat, sterile latex gloves and a respirator. Sterile, plugged pipette tips were used, and all equipment and surfaces were cleaned with DRNAse Free (Argos, Elgin, IL). DNA extraction and PCR were performed in separate rooms. Individual bacterial colonies were removed from the isolation plates using a loop and emulsified into 200 μl of sterile deionized water. DNA was extracted by boiling the solution at 95 °C for 20 min and then DNA was stored at −20 °C. Bacterial DNA was amplified using a PCR method modified from a prior publication [35]. The master mix solution included 17.5 μl per reaction of Green Go Taq (Promega Corporation, Madison, WI), 7.5 μl water, 2 μl of forward primer 8flp, 2 μl of reverse primer 1492rpl, and 0.3 to 0.5 μl of bacterial DNA. Run conditions were 95 °C for 2 min, then 35 cycles of 95 °C for 30 s, 55 °C for 1 min, 72 °C for 2 min, and then hold at 72 °C for 7 min. PCR products were separated on a 1.2% agarose gel and extracted using Qiagen PCR Purification kits and the spin column gel extraction protocol (Qiagen, Valencia, CA). Gel extracts were direct-sequenced at Elim Biopharmaceuticals (Hayward, CA) or Davis Sequencing (Davis, CA). Electropherograms were assessed for quality visually and only completely unambiguous sequence data were used in further analysis. Sequences were compared to those reported in the database with the criterion

of ≥98% match for identification of isolate to bacterial species. Where bacteria could not be identified to genus, identification was reported at the level of family (or at worst case phylum).

Bd swabs were air-dried out of direct sunlight and placed in a sterile vial. Vials were frozen at −20 °C upon return from the field. To prevent moving Bd between field sites, all sampling gear was disinfected using 0.1% solution of quaternary ammonia [36]. DNA was extracted from swab samples using PrepMan Ultra (Applied Biosystems, Carlsbad, CA) following established protocols [37], with the exception that extracted DNA was not diluted prior to PCR. We used a real-time PCR to assess the number of Bd genome equivalents (GE) in each sample following standard protocols [33,38], with the exceptions that samples were analyzed singly rather than in triplicate [39] and reaction volumes were scaled down to 12.5 μl from 20 μl. However, known positive standards (courtesy A. Hyatt) and negative controls (water) were included in each run. Real-time PCR assays were conducted using an Applied Biosystems StepOnePlus real-time PCR system.

Bd challenge studies were performed as previously described [40] with modifications. Bd strain JEL215 from a mountain yellow legged frog was transferred from broth culture onto TGhL agar and grown for 4–8 days until sufficient motile zoospores were visible on the agar using an inverted microscope. Sterile water (5 ml) was added to each petri dish and allowed to sit for 5 min. The plates were gently tipped to the side and 750 μl was pipetted out and transferred to 1% tryptone plates and spread with a sterile spreader. One plate was used to make 4 plates. Plates were air-dried in a laminar flow hood for 3–4 h and then bacteria were streaked on one half of the plate and a control known to not inhibit growth was streaked on the other half of the plate. The plates were visualized at 48 h and 8 days and if Bd growth around the bacterial streak was visible, it was defined as either inhibitory (complete lack of Bd growth), slightly inhibitory (decrease in Bd growth), or not inhibitory (no visual decrease in Bd growth).

Data were maintained in Excel (Microsoft, Redmond, WA) and analyzed with the statistical package "R" (R-Development Core Team, http://www.r-project.org). The cutoff for statistical significance was $P = 0.05$. Prevalence of Bd infection with 95% confidence intervals was calculated in "R" by proportions test with correction for continuity. Although data are reported at multiple taxonomic levels, *e.g.* to species where possible, statistical data analysis was performed for families. Chi-square contingency tests were performed in order to evaluate whether the distribution of families of bacteria among lakes, frog life stages, or frogs that did or did not have Bd infection.

3. Results

A total of 133 Cascades frogs were assessed for Bd and skin-associated bacteria at four Klamath lakes including 9 adults and 11 metamorphs at Eagle Creek Lake, 16 adults and 12 subadults at Found, 14 adults, 2 metamorphs, and 12 subadults at Section Line, and 29 adults, 23 metamorphs, and 5 subadults at Shimmy. Bd was detected in 23.3% (12.3–38.9 95% C.I.) of post-metamorphic individuals, with prevalence of 0% (0–24.1 95% C.I.) at Shimmy Lake, 14.3% (0.75–58.0) at Eagle Creek Lake, 27.3% (7.3–60.6) at Section Line Lake, and 54.5% (24.5–81.9) at Found Lake. Sampling performed in July 2008 documented Bd at Shimmy Lake (Piovia-Scott, unpub. data).

We recovered bacterial isolates from 46 of the frogs sampled and sequenced 130 isolates of bacteria with a mean of 2.84 isolates per frog (±0.13 standard error). Among sequenced isolates, many could not be identified to species because of lack of homology with bacteria represented in the database. One-hundred five isolates were confirmed Gram-negative and only 25 Gram-positive. Two strains were identifiable to phylum only (one each of Actinobacteria and Bacteriodetes) and 10 isolates identifiable only to order (eight in Micrococcineae and one in Bacteriales). Isolates with several unresolvable genera were found in the families "Burkholderiales Genera incertae sedis", Corynebacteriaceae, Enterobacteriaceae, Flavobacteriaceae, Flexibacteraceae, Micrococcineae, Nocardiaceae, Oxalobacteraceae, and Sphingomonadaceae (Table 1). 18 families and 31 genera were identified with the most commonly represented higher taxa including: Neisseriaceae (16.2% of isolates), Comamonadaceae (10.8% of isolates), and Flavobacteriaceae (9.2%). Common genera were: *Aquitalea* (10.8%), *Pseudomonas* and *Aeromonas* (6.9% each), and *Paucibacter* (6.2%), with 24.6% of isolates not specified to genus. Fourteen isolates could be confirmed to species (species listed in Table 1). Among those confirmed to species, five were aerobes and the rest facultative anaerobes (Table 2).

We examined the data for association of lake, frog stage, or Bd status with particular taxa of bacteria. Among the four lakes, the taxon richness ranged from 9 to 15. Only "Burkholderiales Genera incertae sedis", Micrococcineae, Neisseriaceae, and Oxalobacteriaceae were present at all lakes. No particular taxa were associated with particular lakes or frog life stage. The richness of taxa was not statistically significantly different across lakes ($P = 0.54$). Across life stages, number of taxa ranged from 13 in subadults, 14 in metamorphs, and 16 in adults and these differences also were not statistically significant ($P = 0.22$). Bd-negative frogs had 19 taxa while infected frogs had only nine ($P = 0.24$). There were no taxa that occurred only on Bd-positive frogs.

Two isolates of bacteria had strong anti-Bd activity *in vitro* while three had slight anti-Bd activity. The two strongly inhibitory bacteria were *Aeromonas hydrophila* (only one strain) and a Flavobacteriaceae that could not be differentiated to genus. The bacteria that caused slight Bd inhibition were two strains of *Acidovorax* sp. and a *Chryseobacterium* sp.

4. Discussion

We examined cultivable bacterial isolates from Cascades frogs in four northern California lakes and found a strong preponderance of Gram-negative bacteria from 20 higher order taxa and 31 genera. Our methods have provided additional insight into the bacterial community on the skin of Cascades frogs. This information can be used to compare across species and locations in

Table 1
Isolates recovered from amphibian samples. Total numbers of isolates for families include the numbers of the genera listed below them. Isolates that were not able to be classified to genus were reported by family.

Family[a]	Genus	Number of isolates	Percent of total isolates	Species identified
Actinobacteria (phylum)		1	0.8	
Aeromonadaceae		9	6.9	
	Aeromonas	9	6.9	*Aeromonas enteropelogenes*
Bacteriodetes (phylum)		1	0.8	
Burkholderiaceae		1	0.8	
	Burkholderia	1	0.8	
Burkholderiales Genera incertae sedis		11	8.5	
	Leptothrix	1	0.8	
	Mitsuaria	1	0.8	
	Paucibacter	8	6.2	
Caulobacteraceae		2	1.5	
	Brevundimona	2	1.5	*Brevundimonas subvibrioides*
Comamonadaceae		14	10.8	
	Acidovorax	5	3.8	
	Comamonas	1	0.8	
	Curvibacter	7	5.4	
	Delftia	1	0.8	
Corynebacteriaceae		1	0.8	
Cytophagaceae		1	0.8	
	Dyadobacter	1	0.8	*Dyadobacter alkalitolerans*
Enterobacteriaceae		4	3.1	
	Serratia	2	1.5	*Serratia fonticola*
Flavobacteriaceae		12	9.2	
	Chryseobacterium	6	4.6	
	Flavobacterium	1	0.8	*Flavobacterium hercynium*
Flexibacteraceae		1	0.8	
Micrococcineae		7	5.4	
	Arthrobacter	3	2.3	*Arthrobacter oxydans*
	Cryocola	1	0.8	*Cryocola antiquus*
	Kocuria	2	1.5	
Microbacteriaceae		1	0.8	
	Pseudoclavibacter	1	0.8	*Pseudoclavibacter helvolus*
Neisseriaceae		21	16.2	
	Aquitalea	14	10.8	*Aquitalea magnusonii*
	Chitinibacter	1	0.8	
	Chromobacter	2	1.5	
	Deefgea	2	1.5	
	Iodobacter	1	0.8	
	Vogesella	1	0.8	*Vogesella perlucida*
Nocardiaceae		7	5.4	
	Rhodococcus	5	3.8	
Oxalobacteraceae		7	5.4	
	Herbaspirillum	1	0.8	*Herbaspirillum rubrisubalbicans*
	Massilia	1	0.8	
Pseudomonadaceae		9	6.9	
	Pseudomonas	9	6.9	
Sphingomonadaceae		9	6.9	
	Sphingomonas	7	5.4	
Unclassifiable		10	7.7	
Xanthomonadaceae		1	0.8	
	Xylella	1	0.8	*Xylella fastidiosa*
	Total all isolates:	130		

[a] Unless otherwise noted.

order to isolate strains or species that may be important to amphibian resistance to fungal pathogens. Among the bacteria we cultured, the most commonly represented families were Neisseriaceae, "Burkholderiales Genera incertae sedis", Comamonadaceae, Flavobacteriaceae, and Aeromonadaceae. For most of these taxa, very little is known about even the basic biology of the organisms although several known pathogens were detected. Establishing a reference database and culture collection of symbiotic bacteria is important in order to better understand and characterize the community structure of the microbiome associated with frog skin.

Despite the high level of diversity, there was a lack of specific association of taxa or taxon richness between frogs of different sites, life-stages, or Bd infection status although it is possible that part of this may be a Type II statistical error, potentially improved with further sampling. The culture methods were chosen to support a large number of aquatic bacteria and avoid bias toward those with specific nutritional, temperature et al. requirements. Despite this, some bacteria present on frog skin may not have grown under these conditions or may have been overlooked despite our efforts to identify all colonies that grew successfully on the plates. Possible future methods to

Table 2
Number of bacterial isolates identified in each family in four lakes in the Klamath range of northern California, three life stages of Cascades frog, and frogs with and without Bd infection.3.

Family	Number per lake				Number per life stage			Number per frog by Bd status	
	Eagle		Section		Adult	Metamorph	Subadult	Bd−	BD−
	Creek	Found	Line	Shimmy				negative	positive
Actinobacteria	0	1	0	0	0	0	1	1	0
Aeromonadaceae	1	0	1	7	5	2	2	9	0
Bacteriodetes	0	1	0	0	1	0	0	0	1
Burkholderiaceae	1	0	0	0	0	1	0	0	0
Burkholderiales	0	1	0	1	1	0	1	1	1
Burkholderiales Genera incertae sedis	1	2	2	6	2	5	4	9	2
Caulobacteraceae	0	1	1		1	0	1	2	0
Comamonadaceae	0	1	5	8	7	3	4	8	4
Corynebacteriaceae	0	0	1	0	0	0	1	1	0
Cytophagaceae	0	0	0	1	0	1	0	1	0
Enterobacteriaceae	0	0	0	4	1	2	1	4	0
Flavobacteriaceae	4	0	2	6	7	4	1	11	1
Flexibacteraceae	0	0	0	1	0	1	0	1	0
Micrococcineae	3	1	1	3	5	3	0	7	0
Neisseriaceae	3	10	4	4	10	5	6	15	6
Nocardiaceae	1	5	1	0	5	0	2	3	4
Oxalobacteraceae	4	1	1	1	3	3	1	6	0
Pseudomonadaceae	1	0	4	4	8	1	0	9	0
Sphingomonadaceae	0	3	2	4	5	3	1	5	4
Unclassifiable	0	1	0	4	4	1	0	4	1
Xanthomonadaceae	0	0	0	1	1	0	0	1	0

increase sensitivity might include incubation of swabs in R2A broth prior to streaking on plates, use of other media, longer incubation periods, as well as use of an agar overlay. A complementary molecular approach might yield a more accurate assessment of bacterial diversity, however most of the taxa identified are not well-represented in a database such as GenBank, so it is impossible to gain much knowledge about the likely ecological role such bacteria might play. Indeed, by having cultured isolates, we are able to test *in vivo* activities of these bacteria including their interactions with frogs, other bacteria, and Bd as well.

While 16S rRNA sequencing is a common tool for determining bacterial species, it provides only some of the information required to confirm species identity or to evaluate variability in genes within species. The 16S sequence often could not differentiate between closely related taxa or returned no closely related species matches from GenBank database. It is likely that some bacteria we found are previously undescribed species. More work should be performed to further describe these isolates by DNA sequencing of additional genes and further bacteriological studies such as electron microscopy, assessment of growth and colony morphological properties, and biochemical characterization. Valuable future directions would include assessing sympatric amphibian species and water *per se* to determine host-associations of the bacteria observed.

5. Bacterial isolates

For the remainder of the discussion we will attempt to describe some of the families we recovered and suggest further avenues for targeted research on amphibian skin microbes.

Several prior studies of antimicrobial bacteria and their contribution to disease resistance have focused on the powerful fungal inhibitor *Janthinobacterium lividum* [20]. Although *J. lividum* was not recovered in this study, we did find *Herbaspirillum rubrisulbalbicans*, which is related to *J. lividum* and is a mild plant pathogen [41]. Despite this relationship, when we attempted to assess this strain for anti-Bd activity it was not inhibitory. Further studies should be conducted on the members of the family Oxalobacteraceae to determine if strong anti-fungal activity is truly limited to *J. lividum*.

Of the 31 genera recovered, 6.9% of all isolates were among the genus *Pseudomonas*. The family Pseudomonadaceae contains organisms with strong anti-fungal properties including some that are used as biological control agents in agriculture [42] and is potentially important in amphibian resistance to fungal infection. Members of *Pseudomonas* have been recovered repeatedly from amphibian skin, including *P. tolaasii* and *P. aeruginosa* from harlequin toads (*Atelopus elegans*) [43], *P. reactans* from red-backed salamanders (*Plethodon cinereus*) [20] and *P. flourescens*, *P. orientalis* and *P. borealis* in mountain yellow-legged frogs (*Rana muscosa*). While *Pseudomonas* species are potentially important in Bd resistance, this effect is likely to be strain-specific as the Pseudomonads isolated in this study did not show any anti-Bd activity. More strains should be isolated, identified and challenged in order to better understand the community interactions of this family

Another well-represented taxon in our study (6.9% of all isolates) was the genus *Aeromonas*. The family Aeromonadaceae is almost universally present in water sources [44] and includes pathogens of fish [45], amphibians [46]

and humans [47]. The *Aeromonas hydrophila* strain recovered in this study demonstrated strong anti-Bd activity *in vitro* which may be explained *via* two possible paths: (1) *A. hydrophila* produces strong antimicrobial exotoxins, enterotoxins [48], or extracellular products such as nucleases, amylases, and lipases which may have a role in microbial inhibition [48]. (2) *A. hydrophila* may competitively exclude Bd from the epidermal layer [40]. While *A. hydrophila* has been reported to co-infect frogs with Bd, it may be an opportunistic invader [46,49] as Bd infection has been correlated with reduced defensive peptide expression [50] and healthy frogs generally inhibit the growth of this bacteria [51].

Other interesting pathogens recovered in our study are multiple members of the family Flavobacteriaceae; genus *Flavobacterium*. Several *Flavobacterium* species are pathogens of fish, including *F. psychrophilum, F. columnare* and *F. branchophilum* which cause salmonid Bacterial Cold Water Disease, cotton-wool disease, and Bacterial Gill Disease (BGD) respectively. Several of the members of this genus have been identified to have anti-Bd properties in laboratory studies [40]. Another important member of the Flavobacteriaceae is the genus *Chryseobacterium.* 11 isolates of this genus were recovered in this study, one of which had high anti-Bd activity and one that had mild anti-Bd activity. Other studies have also shown anti-Bd properties of *Chryseobacterium* [20,22,34,52] making this genus potentially very important to amphibian resistance to pathogens although the distribution of this genus in systems afflicted with Bd is unknown.

Most of the other taxa reported in this study are either known commensals, aquatic flora, or have no known ecological role. While pathogenic species have demonstrably important impacts on amphibian mortality and morbidity it is important to also report commensal species as they may contribute to the community by providing a food source or attachment surface in the biofilm for species with antifungal properties.

Some families of potentially important non-pathogenic microbes are Comamonadaceae, Micrococcineae, Burkholderiales Genera incertae sedis and Enterobacteriaceae. The family Comamonadaceae is very well represented in this study (11% of all isolates) and members of this family have been noted in the literature as frequent colonists of frog skin. The genus *Comamonas* (1 isolate) contains species frequently found in water sources and has been recovered from mountain yellow-legged frogs with oral chytridiomycosis [53]. The genus *Curvibacter* (7 isolates) is an abundant water and soil-borne phylotype that has been reported associating with amphibians in other microbial community systems [15]. The Micrococcineae family includes members of the genus *Arthrobacter*; a common cutaneous bacterium on amphibians [22,52] that has been shown to have anti-Bd properties in laboratory studies [20,22,34,52]. The Burkholderiales Genera incertae sedis family includes genera such as *Paucibacter* (9 isolates) and an unclassified isolate that was identified as being close to *Ideonella.* Members of the genus *Paucibacter* have been reported to cleave and hydrolyze amino acids from organic compounds such as cellulose [54], potentially creating a food source for other species. Members of the genus *Ideonella* have been recovered in abundant numbers in other studies from Western chorus frogs (*Pseudocris triseriata*) [15] and reported in GenBank from a *Hydra* [55]. Finally, the family Enterobacteriaceae includes members of the genus *Serratia* (4 isolates); common water-borne saprobes that have been isolated from vertebrate abscesses [56].

The nature of resistance to Bd is not fully understood. Some species of amphibians are more resistant than others and while frogs can develop adaptive immunity [57] it has been demonstrated that a robust innate immunity – beginning at the integument – is required for resistance to Bd infection [58,59]. The commensal mucosal bacteria of frogs occur in complex and variable communities, possibly within the structure of bacterial biofilms. The study of each species individually may be partially misleading since inter-species interactions among bacteria are common and the effect of the bacterial community as a whole may differ from the sum of the effects of its component parts. A focus on the entire bacterial community is necessary in order to predict and manage the consequences of any manipulation of the system such as might occur if frogs were treated with antimicrobial compounds or probiotic bacteria. The data presented in this study illustrate the considerable diversity in this community and can be used in the future as a starting point for genetic comparison across sites, to identify at-risk communities of frogs, and to understand the basic community dynamics of frog mucosal bacteria.

Conflict of interest statement

The authors of this publication declare that the research conducted herein was done in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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