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Further Identification of the Cutaneous Bacteria of Spotted Salamanders, *Ambystoma maculatum*, in Western New York, USA

Amphibian skin serves as the first line of defense against disease-causing pathogenic fungi and bacteria (Varga et al. 2019), and cutaneous bacteria may play a role in ameliorating development of disease from skin pathogenic organisms like *Batrachochytrium dendrobatidis* (*Bd*) and *B. salamandrivorans* (*Bsal*) (Culp et al. 2007; Walke et al. 2015; Muleltz-Wolz et al. 2017). Many bacteria commonly found on amphibians have antifungal properties (Walke et al. 2014; Woodhams et al. 2019) and the skin microbiotic community, or dermosphere (de Assis et al. 2017), has been shown to positively affect the outcome of viral and fungal infections (Becker and Harris 2010; Park et al. 2014; Campbell et al. 2019; Varga et al. 2019). The vast majority of studies that have investigated the salamander cutaneous microbiome looked exclusively at plethodontid salamanders, and there is a dearth of research on the ambystomid salamanders. Spotted Salamanders (*Ambystoma maculatum*) appear to have some innate immunity to *Bsal* disease due to antimicrobial peptides (Barnhard et al. 2020; Pereira and Woodley 2021), but cutaneous bacterial species and their antifungal metabolites may still be important in protection against chytrid fungi (Gray et al. 2015). Stevens et al. (2019) and Stevens (2020) identified several species of cutaneous bacteria of Spotted Salamanders and discussed their antifungal capacities. The purpose of this study was to continue that work and further identify cutaneous bacterial species of Spotted Salamanders.

RICHARD T. STEVENS
Department of Biology, Monroe Community College,
321 State St., Rochester, New York 14608, USA
e-mail: rstevens@monroecc.edu

Methods were similar to Lauer et al. (2007) and Muletz-Wolz et al. (2017) and were intended to identify a limited number of culturable bacterial species. Adult and juvenile salamanders were hand captured at Mendon Ponds Park (MPP), Mendon, Monroe County, New York, USA (43.02472°N, 77.57277°W) using sterile gloves during the spring breeding migration and on rainy nights from March to May 2020. Larval salamanders were captured by dip netting in August 2020. Captured salamanders were washed for 30 s with distilled water to remove non-resident bacteria (Lauer et al. 2007) and swabbed with a single sterile cotton swab on all body surfaces. Each swab (one per salamander) was streaked on an R-2A agar plate, taken to the laboratory and incubated at room temperature for 72 h or until bacterial colonies became apparent. Selected unique colonies were sub-cultured until pure colonies were obtained and then grown in liquid media. Cell pellets were

TABLE 1. Cutaneous bacteria found on Spotted Salamanders, *Ambystoma maculatum*, from western New York, USA.

Family	Genus	Species	Host life stage
Alcaligenaceae	*Achromobacter*	*aegrifaciens*	Adult
Alcaligenaceae	*Advenella*	*mimigardefordensis*	Adult
Bacillaceae	*Bacillus*	*mobilis*	Larva
Bacillaceae	*Bacillus*	*mycoides*	Juvenile
Bacillaceae	*Bacillus*	*simplex*	Larva
Bacillaceae	*Bacillus*	*wiedmannii*	Metamorph
Enterobacteriaceae	*Serratia*	*liquefaciens*	Juvenile
Enterobacteriaceae	Unclassified	unclassified	Adult
Erwiniaceae	*Pantoea*	*allii*	Juvenile
Erwiniaceae	*Pantoea*	unclassified	Larva
Flavobacteriaceae	*Flavobacterium*	*olei*	Adult
Flavobacteriaceae	*Flavobacterium*	*pectinovorum*	Adult
Flavobacteriaceae	*Flavobacterium*	*sacchrophilum*	Adult
Flavobacteriaceae	*Flavobacterium*	unclassified	Adult
Microbacteriaceae	*Microbacterium*	*oxidans*	Adult
Oxalobacteraceae	*Janthinobacterium*	*agaricidamnosum*	Adult
Oxalobacteraceae	*Janthinobacterium*	*lividum*	Adult, Metamorph
Pseudomonadaceae	*Pseudomonas*	*fluorescens*	Adult
Pseudomonadaceae	*Pseudomonas*	*koreensis*	Adult
Pseudomonadaceae	*Pseudomonas*	*migulae*	Adult
Pseudomonadaceae	*Pseudomonas*	*moraviensis*	Metamorph
Pseudomonadaceae	*Pseudomonas*	*rhizosphaerae*	Adult
Pseudomonadaceae	*Pseudomonas*	*vancouverensis*	Juvenile
Sphingobacteriaceae	*Pedobacter*	*steynii*	Adult
Sphingobacteriaceae	*Sphingomonas*	*aerolata*	Adult
Sphingobacteriaceae	*Sphingomonas*	*aurantiaca*	Adult
Staphylococcaceae	*Staphylococcus*	*xylosus*	Juvenile
Weeksellaceae	*Chryseobacterium*	*ureilyticum*	Metamorph

collected by centrifugation and frozen at -15°C, sent to a commercial genomics laboratory (CD Genomics, Shirley, New York, USA) for Sanger sequencing of the entire 1.5 kb of the 16S rRNA gene, and classified to putative species level using Blast software (http:blast.ncbi.nlm.nih.gov/Blast.cgi).

A total of 27 species of bacteria from 13 genera were identified from 9 adults, 2 juveniles, one larva, and one new metamorph (Table 1). An average of 1.8 species of bacteria were identified from each adult (range 1–4 species per adult) and four species of bacteria were identified from one juvenile and six species from the other. Bacteria from 11 taxonomic families were found. The genera *Psuedomonas* (6 species), *Flavobacterium* (4 species), and *Bacillus* (4 species) were the most common genera found. Only two of the 27 species identified, *Janthinobacterium lividum* and *B. mycoides*, were found on Spotted Salamanders previously at MPP (Stevens 2020); yet the genera identified between that study, which included an additional site nearby as well as MPP, and the current sample were fairly similar. The small degree of overlap in species between sampling years indicates that there may be a high degree of bacterial species diversity in Spotted Salamanders and that there may be many more species inhabiting Spotted Salamander skin yet to be discovered. This study was not intended to identify all cutaneous bacterial species, and previous work on salamanders has identified hundreds of species present. Muletz-Wolz et al. (2017) found 480 taxa of bacteria on three species of *Plethodon* in Maryland and Virginia, USA, and Bletz et al. (2018) found 708 species of bacteria in wild European Fire Salamanders (*Salamandra salamandra*).

Several of the genera found, including *Janthinobacterium*, *Pseudomonas*, and *Flavobacterium*, have been shown to be strongly antifungal in previous studies. For example, *J. lividum*, found in two Spotted Salamanders, created the antifungal metabolites violacein and indole-3-carboxaldehyde (Park et al. 2014). This bacterium has been experimentally shown to help prevent symptoms of *Bd* in infected animals and has been used as an inoculant against *Bd* in toads that were reintroduced into habitats where they had been extirpated (Kueneman et al. 2016). *Pseudomonas* is another antifungal genus that was common on adult salamanders, and was found on one juvenile and the metamorph. Six species of *Pseudomonas* were found in this study, along with three species in 2019 (Stevens 2020), and one species in 2017 (Stevens et al. 2019). *Pseudomonas fluorescens*, found on two adult Spotted Salamanders, and has been used agriculturally as an antifungal and antibacterial treatment (Verschuere et al. 2000). *Pseudomonas fluorescens* has been found in plethodontid salamanders (Culp et al. 2007; Lauer et al. 2008) and has been found to be inhibitory to *Bd* (Myers et al. 2012). *Pseudomonas* spp. have been widely found in salamanders (Lauer et al. 2008; Fitzpatrick and Allison 2014; Muletz-Wolz et al. 2017) and *P. reactans* has been used as an anti-*Bd* treatment in Red-backed Salamanders (Harris et al. 2009). Several other bacterial taxa found on Spotted Salamanders have been found to be antifungal including *Serratia liquefaciens* (Kalbe et al. 1996), *Microbacterium* spp. (Culp et al. 2007; Muletz-Wolz et al. 2017), and *Chryseobacterium* spp. (Lauer et al. 2007; Antwis and Harrison 2017).

Although sample sizes were small in this study and statistical tests were not done, the bacterial species found on adult

salamanders were fairly distinct from the species found on juveniles and larva. The one metamorph sampled had *J. lividum* which was also found in one adult, but there was no other overlap in bacteria species among the host age classes. *Bacillus* spp. was found in juveniles, larva, and the metamorph but not in adults. Other studies have also found differences in the cutaneous bacteria between host age classes (Longo et al. 2015; Sabino-Pinto et al. 2017; Sanchez et al. 2017). These differences may be due to habitat differences among age classes, physiological changes as the salamander matures, or a combination of factors (Sabino-Pinto et al. 2017; Sanchez et al. 2017). Host age-specific microbiome patterns warrant more research in Spotted Salamanders, as does the overall cutaneous bacterial diversity of this species. The cutaneous bacterial community could play an important role in the relatively strong immunity of this species to *Bsal*.

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