A Comparison of Bacteria Cultured from Unionid Mussel Hemolymph between Stable Populations in the Upper Mississippi River Basin and Populations Affected by a Mortality Event in the Clinch River

Authors: Eric Leis, Sara Erickson, Diane Waller, Jordan Richard, and Tony Goldberg

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REGULAR ARTICLE

A COMPARISON OF BACTERIA CULTURED FROM UNIONID MUSSEL HEMOLYMPH BETWEEN STABLE POPULATIONS IN THE UPPER MISSISSIPPI RIVER BASIN AND POPULATIONS AFFECTED BY A MORTALITY EVENT IN THE CLINCH RIVER

Eric Leis[1]*, Sara Erickson[1], Diane Waller[2], Jordan Richard[3], and Tony Goldberg[4]

[1] *La Crosse Fish Health Center, Midwest Fisheries Center, U.S. Fish and Wildlife Service, 555 Lester Ave, Onalaska, WI 54650 USA*

[2] *Upper Midwest Environmental Sciences Center, U.S. Geological Survey, 2630 Fanta Reed Rd, La Crosse, WI 54603 USA*

[3] *Southwestern Virginia Field Office, U.S Fish and Wildlife Service, Southwestern Virginia Field Office, 330 Cummings Street, Abingdon, VA 24210 USA*

[4] *University of Wisconsin–Madison, Department of Pathobiological Sciences and Global Health Institute, 1656 Linden Drive, Madison, WI 53706 USA*

ABSTRACT

The diagnosis of bacterial disease in freshwater unionid mussels has been hindered by a lack of baseline information regarding the microbial communities associated with these animals. In this study, we cultured and identified bacteria from the hemolymph of stable mussel populations from Wisconsin portions of the upper Mississippi River basin and compared the results to those from mussel populations experiencing a mortality event in the Clinch River in Virginia and Tennessee. Several bacterial genera were consistently identified across mussel species and locations, appearing to be part of the natural bacterial flora. One noteworthy bacterial species identified from the Clinch River was *Yokenella regensburgei*, which occurred in relatively high prevalence during the mortality event but was absent from samples acquired afterward. Its role in the mortality event, if any, is unknown but deserves further investigation. We suggest that future studies of freshwater mussel health incorporate hemolymph as a sample type due to its relative separation from the aquatic environment, its role in the circulatory system, and the fact that it can be collected nonlethally.

KEY WORDS: freshwater mussel, Unionidae, microflora, bacteriology, hemolymph, disease

INTRODUCTION

Freshwater mussels are exposed to the microorganisms that they filter and accumulate from the aquatic environment. Bacteria are a food source, but also can be found in body tissues outside of the gut, including the hemolymph, in apparently healthy animals (Starliper et al. 1998, 2008; Antunes et al. 2010). In general, the characteristic bacterial flora of freshwater mussels is largely unknown, despite the emergence of microbiome research examining correlations between bacterial and archaeal communities and health and resilience across a variety of animal species (e.g., gut biota of humans and fish, livestock, etc.; see Ingerslev et al. 2014; Ghanbari et al. 2015; Reese et al. 2018; Trinh et al. 2018). In mussels, bacterial diversity in fluids and tissues has been associated with healthy, responsive animals (Starliper et al. 2008), whereas high bacterial loads have been associated with sick or moribund animals (see Sparks et al. 1990, Starliper et al. 2011).

*Corresponding Author: eric_leis@fws.gov

Historically, mass mortality events of freshwater mussels have occurred that suggest infectious causes (Neves 1987), but in most cases, the causative agent has not been identified (see review in Grizzle and Brunner 2009). While samples may be collected and analyzed from such events, the lack of data regarding the normal microbiota of healthy mussel populations has made it difficult to identify potential pathogens as well as other potential commensal or mutualistic relationships (Starliper et al. 1998; Starliper 2008). The reports by Starliper, focused on populations from the southeastern USA (Starliper et al. 1998; Starliper and Morrison 2000; Starliper 2001, 2005, 2008, 2011; Starliper et al. 2008, 2011), constitute much of the knowledge base regarding the bacterial communities associated with freshwater mussels. To develop much-needed specific diagnostic assays, we must better understand mussel-microbe interactions and identify pathogens, tasks feasible only with bacteriology data from diverse unionid species across broader geographic regions.

Standardized diagnostic methods for freshwater fish typically utilize the kidney for the collection of bacteriological samples (USFWS and AFS–FHS 2012) due to its function as a filtration organ. However, similar methods are lacking for freshwater mussels, primarily because of limited attention to the diseases of these taxa (Grizzle and Brunner 2009). Typically, previous bacteriology studies of unionids have utilized lethal sampling to collect fluids and mixed tissue or whole body homogenate samples (Starliper et al. 1998, 2008, 2011; Starliper and Morrison 2000; Starliper 2001, 2005), while others compared the microbiota between specific tissues (Sparks et al. 1990; Chittick et al. 2001; Nichols et al. 2001; Antunes et al. 2010). Although bacteria were cultured from most tissue types, interpretations are confounded by lack of organ specificity (mixed tissue or whole body homogenates) as well as the risk to sample integrity due to the closeness of the internal organs to the aquatic environment and the disinfection procedures used to reduce contamination. Moreover, whole body and soft tissue samples generally require sacrifice of the mussel, which should be avoided, especially for imperiled fauna.

A sample type that has received less attention in the assessment of freshwater mussel health is hemolymph (Sparks et al. 1990; Starliper 2008; Antunes et al. 2010). This fluid, which plays an important role in immunity as well as many other critical functions, makes up approximately 50% of the weight of mussel tissue (Thorp and Covich 2010). The interaction of hemolymph with the organs and tissues of the mussel, its relative compartmentalization from the aquatic environment, and the accessibility through the adductor sinus for nonlethal sampling provide many advantages (Gustafson et al. 2005; Burkhard et al. 2009). Furthermore, this sample may be particularly useful in examining potential septicemia (Sparks et al. 1990).

In this study, we cultured and identified bacteria from the hemolymph of unionid mussels from apparently stable populations in the Wisconsin portion of the Upper Mississippi River (UMR) basin as well as from samples obtained from a mussel mortality event in the Clinch River in Tennessee and Virginia. Our primary objective was to determine the community composition of the culturable bacteria present within these populations and the prevalence of specific taxa.

METHODS

We collected a variety of freshwater mussel species on June 16, 2017, August 23, 2017, August 29, 2017, October 6, 2017, and October 26, 2017, from the Wisconsin stretches of the La Crosse River (43°54′52.51″N, 91°4′34.93″W), Chippewa River (44°45′38.80″N, 91°40′44.80″W), Lake Onalaska (a backwater lake of Pool 7 of the UMR; 43°53′45.85″N, 91°16′10.41″W), Black River (43°52′18.65″N, 91°14′42.39″W), and Goose Island (Pool 8 of the UMR; 43°44′46.20″N, 91°13′34.17″W), respectively (Fig. 1). We obtained samples from Pheasantshell (*Actinonaias pectorosa*) mussels from the Tennessee reaches (Wallen Bend, 36°35′2.65″N, 83°0′49.96″W; Kyle's Ford, 36°33′57.05″N, 83°2′29.57″W) of the Clinch River on November 2, 2017, during an active mortality event (Richard 2018) and postmortality event on February 1, 2018, again from Kyle's Ford as well as Sycamore Island (Virginia, 36°37′16.36″N, 82°49′6.20″W) (Fig. 1). Mussels were hand-collected and held in source water until a sufficient sample size (~20–30) was acquired for each site. During the postmortality sampling event, we processed approximately half of the Pheasantshells in the field and, due to inclement weather, transported the others in source water to the laboratory, where they were held (up to 72 h) before sampling. Following collection, we used either a reverse pliers or a child's nasal speculum and stopper to open the shell slightly. The anterior adductor mussel was cleaned using an individually wrapped sterile rayon swab soaked in 70% isopropyl alcohol. We withdrew approximately 100 μL of hemolymph from the anterior adductor muscle using a 1 mL insulin needle and syringe. The hemolymph sample was then sinuously streaked onto a tryptic soy agar (TSA) plate using a sterile inoculation loop. TSA plates were incubated for 1 wk in a 21°C incubator. Bacterial colonies with unique macroscopic morphologies were sampled from each plate using a sterile bacteriology loop and placed in sterile 2.0 mL microconical screw-cap collection tubes. Following the manufacturer's instructions, we then extracted DNA using 100 μL of PrepMan™ Ultra Sample Preparation Reagent (ThermoFisher Scientific). Polymerase chain reaction (PCR) primers targeting the 16S rRNA gene (Table 1) were used to amplify and sequence this gene from each isolate. The master mix consisted of 46 μL Platinum PCR Supermix as well as 100 pmol of each selected forward and reverse primer (Table 1). Two μL of extracted DNA was added to the master mix for each reaction. PCR products were exo-SAP purified, and Sanger sequencing was performed by the Whitney Genetics Laboratory (U.S. Fish and Wildlife Service; Onalaska, WI). We edited sequences using Geneious (version 11.1.5) and conducted BLASTn queries using the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Accession numbers reported in the Supplemental Data represent the top listed, named species that shared the most similarity to our

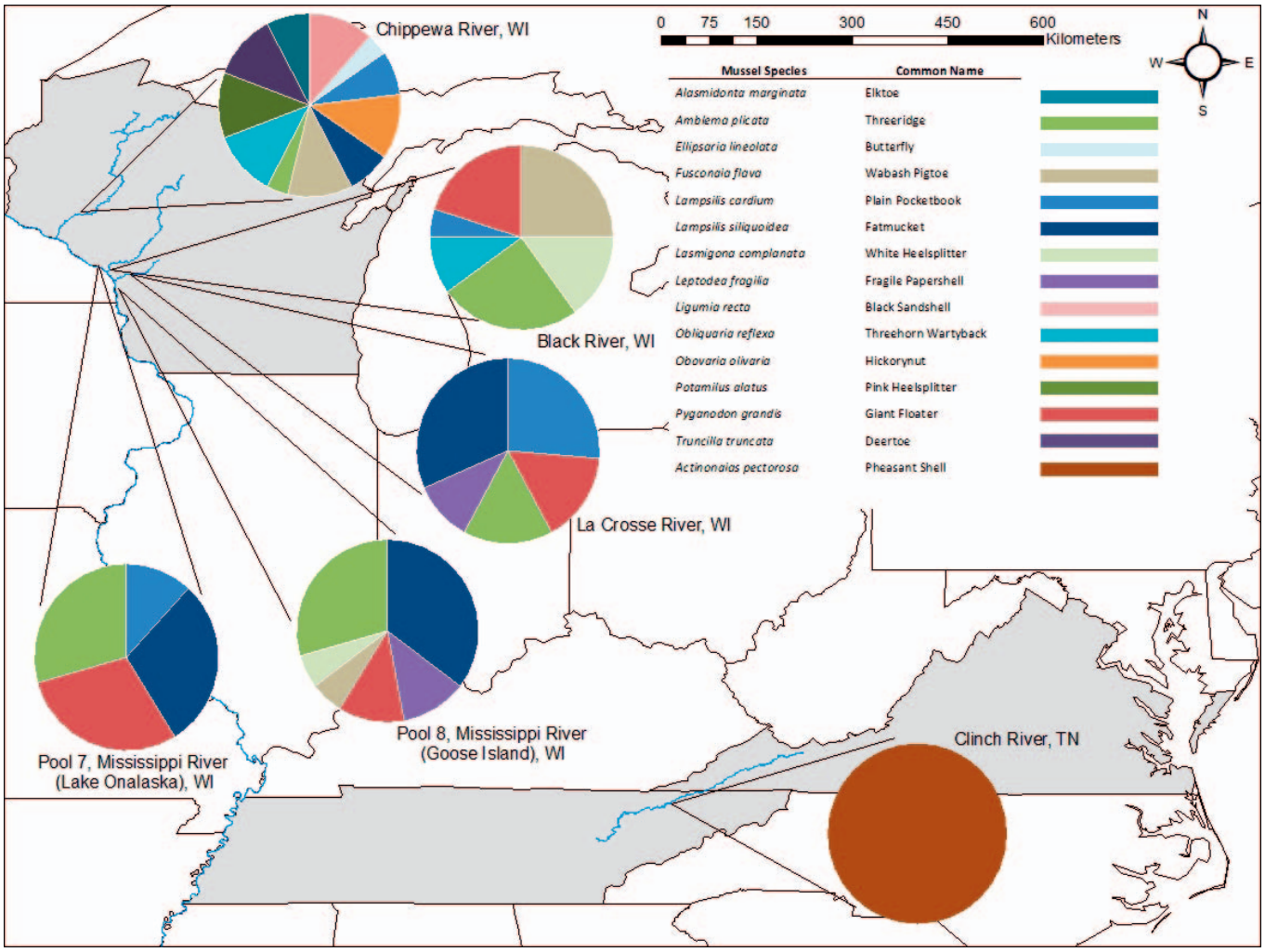

Figure 1. Relative proportion of mussel species sampled at each location.

query. Ambiguities are also reported (i.e., multiple species, or in a few cases genera, that shared the same degree of similarity).

RESULTS

We obtained unionid mussels ($n = 99$) representing 14 species from five sites in the Upper Mississippi River Basin (Fig. 1). We cultured bacteria representing 47 genera (Table 2) from the hemolymph of 73 mussels (74%), identifying 190 colonies through molecular methods. Two colonies were not identified. The most prevalent bacterial genera from the UMR overall were *Bacillus* spp. (19%) and *Aeromonas* spp. (21%). Most genera had a prevalence $<10\%$, and approximately half were single-incidence isolates. Mussels sampled from the UMR basin were healthy in appearance with the lone exception being one gaping Plain Pocketbook (*Lampsilis cardium*) from the La Crosse River; we identified only *Pseudomonas* spp. from this animal. We detected bacteria displaying high levels of similarity to two fish pathogens, *Yersinia ruckeri* and *Aeromonas salmonicida*. We identified *Y. ruckeri* from one Black Sandshell (*Ligumia recta*) and one Three Horn Wartyback (*Obliquaria reflexa*) from the Chippewa River (Supplemental Data). We identified *A. salmonicida* from one Plain Pocketbook, one Wabash Pigtoe (*Fusconaia flava*), two Deertoe (*Truncilla truncata*), one Fat Mucket (*Lampsilis siliquoidea*), and one Hickorynut (*Obovaria olivaria*) in the Chippewa River; one Giant Floater and one Plain Pocketbook from the Black River; two Fat Muckets and one Wabash Pigtoe from the Goose Island

Table 1. Primers used in PCR amplification and sequencing of 16S rRNA genes of bacterial isolates cultured from unionid mussels.

Primer	Sequence (5′–3′)	Reference
8F	AGAGTTTGATCCTGGCTCAG	Turner et al. 1999
27F	AGAGTTTGATCMTGGCTCAG	Lane 1991
518F	TACCAGGGTATCTAATCC	Faisal et al. 2017
800R	CCAGCAGCCGCGGTAATACG	Faisal et al. 2017
1160F	AATCATCACGGCCCTTACGC	Faisal et al. 2017
1387R	GGGCGGWGTGTACAAGGC	Marchesi et al. 1998
1492R (I)	GGTTACCTTGTTACGACTT	Turner et al. 1999
1541R	AAGGAGGTGATCCAGCCGCA	Loffler et al. 2000

Table 2. Bacteria cultured and identified from hemolymph collected from mussels in the upper Mississippi River basin.

Location	Mussel species	Number sampled	Bacteria	Prevalence (% of mussels)
La Crosse River	**Fatmucket**	6	*Agrococcus*	17
	Lampsilis siliquoidea		*Bacillus*	33
			Erwinia	17
			Exiguobacterium	33
			Kocuria	17
			Microbacterium	17
			Arthrobacter	17
	Fragile Papershell	2	*Bacillus*	50
	Leptodea fragilis		*Pseudomonas*	50
	Giant Floater	3	*Bacillus*	67
	Pyganodon grandis		*Chryseobacterium*	50
			Aeromonas	50
			Pseudomonas	50
	Plain Pocketbook	5	*Acinetobacter*	20
	Lampsilis cardium		*Brevundimonas*	20
			Chryseomicrobium	20
			Comamonas	20
			Exiguobacterium	80
			Microbacterium	20
			Pseudarthrobacter	20
	Threeridge	3	*Bacillus*	67
	Amblema plicata		*Brevundimonas*	33
			Erwinia	33
			Exiguobacterium	33
			Stenotrophomonas	33
			Luteimonas	33
Chippewa River	**Black Sandshell**	3	*Aeromonas*	33
	Ligumia recta		*Deefgea*	33
			Yersinia	33
	Deertoe	3	*Aeromonas*	67
	Truncilla truncata		*Bacillus*	33
			Brevundimonas	33
			Chromobacterium	33
			Enterobacteriaceae (*Serratia, Yersinia, Rahnella*)	33
			Microbacterium	33
			Pseudomonas	67
			Sporosarcina	33
	Elktoe	2	*Aeromonas*	50
	Alasmidonta marginata		*Chromobacterium*	50
			Leuconostoc	50
			Pantoea	50
			Pseudoxanthomonas	50
			Stenotrophomonas	100
	Fatmucket	2	*Acidovorax*	50
	Lampsilis siliquoidea		*Aeromonas*	50
			Chromobacterium	50
	Hickorynut	3	*Aeromonas*	33
	Obovaria olivaria		*Bacillus*	33
	Pink Heelsplitter	3	*Agrobacterium* (*Rhizobium*)	33

Table 2, continued.

Location	Mussel species	Number sampled	Bacteria	Prevalence (% of mussels)
	Potamilus alatus		*Bacillus*	33
			Moraxella	33
			Pseudomonas	33
	Plain Pocketbook	2	*Aeromonas*	67
	Lampsilis cardium		*Agrobacterium* (*Rhizobium*)	33
			Bacillus	33
			Chromobacterium	67
			Bacillales (*Viridibacillus, Bacillus, Lysinibacillus, Kurthia, Paenibacillus*)	33
	Threehorn Wartyback	3	*Acinetobacter*	33
	Obliquaria reflexa		*Aeromonas*	100
			Brevundimonas	100
			Lysinibacillus	33
			Vogesella	33
			Yersinia	33
	Threeridge	1	*Sphingomonas*	100
	Amblema plicata			
	Wabash Pigtoe	3	*Aeromonas*	33
	Fusconaia flava			
Lake Onalaska	**Fatmucket**	5	*Aeromonas*	20
	Lampsilis siliquoidea		*Thermomonas*	20
	Giant Floater	5	*Cellulomonas*	20
	Pyganodon grandis		*Microbacterium*	20
	Plain Pocketbook	2	*Cellulomonas*	50
	Lampsilis cardium		*Cellulosimicrobium*	50
			Microbacterium	50
			Pseudomonas	50
	Threeridge	5	Alpha proteobacterium	20
	Amblema plicata		*Bacillus*	20
			Bosea	20
			Curtobacterium	20
			Fictibacillus	20
			Flavobacterium	20
			Pseudomonas	20
			Pseudoxanthomonas	20
			Sphingopyxis	20
Black River	**Giant Floater**	4	*Aeromonas*	25
	Pyganodon grandis		*Brevundimonas*	25
			Enterobacteriacea (*Erwinia, Pantoea*)	25
			Staphylococcus	25
	Plain Pocketbook	1	*Aeromonas*	100
	Lampsilis cardium		*Stenotrophomonas*	100
	Three Ridge	5	*Bacillus*	20
	Amblema plicata		*Pseudomonas*	40
			Staphylococcus	20
			Stenotrophomonas	20
	Threehorn Wartyback	2	*Deefgea*	50
	Obliquaria reflexa		*Staphylococcus*	50
	Wabash Pigtoe	5	*Acidovorax*	20

Table 2, continued.

Location	Mussel species	Number sampled	Bacteria	Prevalence (% of mussels)
	Fusconaia flava		*Bacillus*	20
			Flectobacillus	20
			Morganella	20
			Rhodococcus	20
			Serratia	20
			Staphylococcus	20
	White Heelsplitter	3	*Acidovorax*	33
	Lasmigona complanata		*Aeromonas*	66
Mississippi River	**Fatmucket**	6	*Acidovorax*	17
	Lampsilis siliquoidea		*Acinetobacter*	33
			Aeromonas	50
			Arthrobacter	17
			Bacillus	50
			Chryseobacterium	17
			Microbacterium	33
			Pseudomonas	17
			Shewanella	17
			Staphylococcus	17
	Fragile Papershell	2	*Chitinibacter*	50
	Leptodea fragilis			
	Giant Floater	2	*Bacillus*	50
	Pyganodon grandis		*Bosea*	50
			Chryseobacterium	50
			Rheinheimera	50
	Threeridge	5	*Stenotrophomonas*	50
	Amblema plicata		*Brevundimonas*	20
			Microbacterium	20
			Stenotrophomonas	60
			Variovorax	20
	White Heelsplitter	1	*Acidovorax*	100
	Lasmigona complanata		*Bacillus*	100
			Flavobacterium	100
			Staphylococcus	100
			Stenotrophomonas	100
	Wabash Pigtoe	1	*Pseudarthrobacter*	100
	Fusconaia flava		*Aeromonas*	100

backwater of Pool 8 in the UMR; one Giant Floater from the La Crosse River; and three Pheasantshells from the Clinch River (Supplemental Data). Note that some ambiguity (see Supplemental Data) was observed in the identifications of *A. salmonicida*, likely due to the diversity of genetically similar taxa within Aeromonad species.

During an active mortality event, we sampled 19 Pheasantshells from the Clinch River in Tennessee and cultured bacteria from 89% of the hemolymph samples. Again, *Bacillus* (16%), *Aeromonas* (42%), and *Pseudomonas* (21%) were among the most prevalent genera (Table 3). We also identified *Yokenella regensburgei* from 42% of the Pheasantshells; this bacterium was not observed in samples obtained from the UMR.

In the postmortality sampling event of 14 Pheasantshells, we cultured bacteria from 100% of the samples with the most prevalent isolates identified as *Bacillus* spp. (53%) and *Pseudomonas* spp. (53%) (Table 3). It was noteworthy that *Y. regensburgei* was not identified from this later sampling event.

DISCUSSION

Hemolymph from 74% of the mussels from the UMR, 89% of Pheasantshells sampled during the mortality event, and 100% of Pheasantshells sampled after the mortality event yielded at least one bacterial colony. In both geographic areas, *Bacillus*, *Pseudomonas*, and *Aeromonas* were among the most

Table 3. Bacteria cultured and identified from hemolymph collected from mussels in the Clinch River.

Date	Mussel species	Number sampled	Bacteria	Prevalence (% of mussels)
November 2017	**Pheasantshell**	19	*Aeromonas*	42
	Actinonaias pectorosa		Micrococcaceae (*Arthrobacter*/*Pseudarthrobacter*)	5
			Bacillus	16
			Enterobacteriaceae (*Yokenella*/*Klebsiella*)	5
			Flavobacterium	5
			Lysinibacillus	5
			Massilia	5
			Moraxella	5
			Pseudomonas	21
			Streptococcus	11
			Yokenella	42
February 2018	**Pheasantshell**	14	*Arthrobacter*	7
	Actinonaias pectorosa		*Bacillus*	53
			Cellulomonas	7
			Exiguobacterium	7
			Klebsiella	7
			Kocuria	7
			Massilia	7
			Microbacterium	13
			Paeniglutamicibacter	7
			Planococcus	7
			Pseudomonas	53
			Sanguibacter	7
			Sphingomonas	7
			Streptomyces	7

prevalent bacterial genera identified from mussel hemolymph. Many of the species identified from the UMR and Clinch Rivers also had been reported previously from unionid mussels in the Mississippi, Illinois, Clinch, and Tennessee rivers (Sparks et al. 1990; Starliper et al. 2008, 2011).

Aeromonas spp., a group known for varying levels of pathogenicity (Sreedharan et al. 2011), were identified with the highest prevalence (42%) during the peak of the mortality event on the Clinch River. This genus was not identified during the postmortality sampling and was reported from only 21% of the mussels sampled from apparently healthy populations in the UMR. In previous studies, *Aeromonas* spp. have been among the most prevalent bacteria cultured from both healthy and diseased mussels (Sparks et al. 1990; Atunes et al. 2010; Starliper et al. 2011). We suggest that future work investigate associations between *Aeromonas* spp. and unionid health and disease, especially studies examining bacterial growth and mussel immune function under stressful conditions.

Yersinia ruckeri and *A. salmonicida* are important fish pathogens with regulatory implications. Fish infected with these bacteria are not only at risk for disease, but they may not be approved for stocking, thereby putting them at risk for depopulation. Since captive mussel propagation efforts typically occur in fish hatcheries, we suggest testing mussels for these pathogens before incorporating them into hatchery operations. Additionally, strong consideration should be given to depurating the mussels, in isolation, before introducing them to hatchery facilities, because this has been shown to effectively eliminate *A. salmonicida* (Starliper 2005).

The diversity of the microbial community in the hemolymph may be an important indicator of population health. Measures of bacterial diversity reportedly decrease in stressed Zebra mussels (*Dreissena polymorpha)* (Gu and Mitchell 2002) and diseased Pacific oysters (*Crassostrea gigas)* (Lokmer et al. 2016). In fact, the diversity of microbial communities in the hemolymph of Pacific oyster was a predictor of response to environmental stress (Lokmer et al. 2016). Our results provide baseline data on microbial diversity

in native mussel populations in the UMR for comparison, especially if future stressful events occur.

An important next step is to compare microbial community composition in nonnative bivalves that co-occur with native mussels, such as dreissenids and *Corbicula*. Other studies have investigated bacterial communities associated with Zebra mussels, but none, to our knowledge, have concurrently examined native mussels (e.g., Frischer et al. 2000; Gu and Mitchell 2002; Winters et al. 2010, 2011). Zebra mussels have caused significant shifts in bacterial community structure (Frischer et al. 2000; Lohner et al. 2007), which could have consequences for the stability of native mussel microbiota. Similarly, *Corbicula* are efficient filter feeders that reduce bacterial abundance in streambeds (Hakenkamp et al. 2001) and could also potentially alter the microbial community composition.

Bacteria have been routinely isolated from the hemolymph of aquatic invertebrates in varying stages of health (see the table in Zhang et al. 2018). It is therefore plausible that mussels are under constant invasion from bacteria in the aquatic environment. However, the consistent presence of taxonomically related bacteria across mussel species and geographic locations suggests a characteristic unionid hemolymph microbiome. Members of the genus *Bacillus* have several characteristics that appear probiotic in nature. For example, several *Bacillus* spp. (including some sharing high levels of similarity to species identified in this study; see Supplemental Data) convert urea into calcium carbonate (Wei et al. 2015; Anbu et al. 2016), a major component of the freshwater mussel shell. Furthermore, members of this group also are known for their antimicrobial properties (Yilmaz et al. 2006). The well-studied *Bacillus subtilis* is a calcium carbonate producer that has been used as a probiotic in chicken feed to thicken eggshells and inhibit pathogens (Fathi et al. 2018; Hosseindoust et al. 2018). This species also has been shown to produce fructooligosaccharides (Silva et al. 2016), which reportedly increase calcium absorption in mammals (Morohashi et al. 1998). *Bacillus subtilis* also has been recommended as a probiotic in shrimp culture due to its inhibition of *Vibrio*, a common shellfish pathogen (Vaseeharan and Ramasamy 2003). Similarly, other genera were identified that have species and/or strains with similar potential probiotic characteristics: *Exiguobacterium* (production of the shell component chondroitin, Bhotmange and Singhal 2015), *Brevundimonas* (calcium carbonate production, Wei et al. 2015), *Chromobacterium* (violacein production, Durán and Menck 2001), *Sporosarcina* (calcium carbonate production, Wei et al. 2015; Kim et al. 2016), *Pseudomonas* (calcium carbonate production, Li et al. 2015), *Stenotrophomonas* (production of osmoprotective and antifungal properties, Wolf et al. 2002), *Lysinibacillus* (calcium carbonate production, Lee et al. 2017; chondroitin production, Bhotmange and Singhal 2015; antimicrobial properties, Ahmad et al. 2014), *Acinetobacter* (calcium carbonate production, Zamarreno et al. 2009), and *Microbacterium* (calcium carbonate production, Xu et al. 2017).

Many of the bacteria isolated from unionid mussels were similar genetically to genera with species and/or strains targeted for bioremediation efforts (see Supplemental Data). There are many examples describing the use of environmental bacteria, including some genetically similar to the hemolymph isolates, with the potential for environmental detoxification (Schippers et al. 2005; Hegazi et al. 2007; Genovese et al. 2008; Seeger et al. 2010; Chatterjee et al. 2011; Irawati et al. 2012; Wanjohi et al. 2015; Huët and Puchooa 2017; Poornima and Velan 2018). Historical issues with contamination have been documented in both the Mississippi (Schramm 2004) and Clinch river (Price et al. 2014) systems. Although water quality in the UMR has improved significantly since the 1970s (Schramm 2004), the presence of these bacterial species in freshwater mussels in the UMR may be a response to persistent pollutants, especially in the sediments where mussels reside. The microbiome of an animal plays a critical role in chemical detoxification within the host (see the review in Adamovsky et al. 2018), and we do not know the extent to which the bacteria residing within the mussels may be providing this service. Future research examining whether the species and strains of bacteria associated with freshwater mussel hemolymph are indeed active in the detoxification of aquatic pollutants will be critical in examining this aspect of symbiosis as well as to assess whether mussel microbiomes may be an indicator of environmental pollutants.

In the Clinch River, *Y. regensburgei* was identified from 42% of the Pheasantshells sampled during an active mortality event but, interestingly, was not detected from the same population just a few months later. Isolates from the *Yokenella* genus have been shown to degrade hydrocarbons from soils contaminated with oily sludge (Bhattacharya et al. 2003); its presence could indicate elevated levels of contaminating hydrocarbons during the period of peak mortality, levels that may have subsided thereafter. Interestingly, *Y. regensburgei* also was identified during the peak of a mortality event involving Ebonyshell (*Fusconaia ebena*) from the Tennessee River, Alabama (Starliper et al. 2011). In human medicine, *Y. regensburgei* is considered an opportunistic pathogen (Lo et al. 2011; Jain et al. 2013); it also has been identified from a variety of environmental samples including well water and the gastrointestinal tracts of insects (Kosako et al. 1984). Such observations warrant further investigations of the relationship of *Y. regensburgei* with freshwater mussels, perhaps including *in vivo* exposures of mussels to hydrocarbons and experimental assessment of the mitigating effects (if any) of *Y. regensburgei* on toxicosis.

The occurrence of a bacterial species in both apparently healthy and sick mussels does not necessarily indicate either a commensal or pathogenic relationship. Changes in the environment, condition of the host, and balance of the microbial community can facilitate pathogenesis. Additionally, the virulence of a bacterial species can vary significantly

among strains (see, e.g., Olivier 1990). Indigenous bacteria isolated from Zebra mussel whole body homogenates were pathogenic when administered in high doses and under elevated water temperatures (Gu and Mitchell 2002). Studies of the pathogenicity of suspect bacteria under different conditions are needed to elucidate the mechanisms and conditions that encourage bacterial pathogenesis in freshwater mussels.

In our study, the TSA media and culture conditions undoubtedly limited the diversity of bacterial species that were identified. Incubation temperature, time, and media are all important factors to consider when attempting to recover specific bacteria of interest (Starliper and Morrison 2000) or to maximize growth of greater microbial diversity. For example, incubation of digestive gland samples from *Elliptio complanata* at both 20°C and 35°C yielded a greater number and type of isolates than at a single temperature (Chittick et al. 2001). Additional research is needed to determine optimal media and culture conditions for growth of bacteria from freshwater mussels. Furthermore, research using metagenomic analysis will help identify unculturable species as well as examine functional profiles of all hemolymph bacteria, especially in regard to pathways pertaining to calcium carbonate production and pollutant detoxification.

CONCLUSIONS

Our study established reference data on the diversity of culturable bacteria from the hemolymph of unionid mussels across multiple species and geographic regions in the USA. Hemolymph proved highly suitable for assessing the microbiota of freshwater mussels by nonlethal methods. Isolates genetically similar to two potential fish pathogens, *A. salmonicida* and *Y. ruckeri*, were detected in mussels from two sites in the upper Mississippi River basin. *Yokenella regensburgei* was identified from Pheasantshell mussels during a mortality event, and further work is necessary to determine the importance of this bacterium.

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