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THE BACTERIAL COMMUNITY ASSOCIATED WITH THE LEECH *MYZOBDELLA LUGUBRIS* LEIDY 1851 (HIRUDINEA: PISCICOLIDAE) FROM LAKE ERIE, MICHIGAN, USA

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Summary:

Leeches are widespread in the Great Lakes Basin, yet their potential to harbor disease-causing agents has not been investigated. The purpose of this study was to identify the bacterial community of the commonly occurring leech, *Myzobdella lugubris*, within the Lake Erie Watershed. Leech samples were collected from the pectoral fins of channel catfish, *Ictalurus punctatus*, and freshwater drum, *Aplodinotus grunniens*, from Lake Erie in commercial trap nets and pooled into two samples based on host attachment. Bacteria from within the viscera of *M. lugubris* were identified by sequencing their 16S rRNA (rDNA) gene of amplified community bacterial DNA extracted from pooled leech homogenate samples and were checked for similarity in two public databases: the Ribosomal Database Project and BLAST. Bacteria belonging to the phylum Bacteroidetes, β-proteobacteria, Verrucomicrobia, and unclassified Bacteria were present in the leech samples. A large number of bacteria found within leeches attached to channel catfish consisted of sequences that could not be classified beyond the Domain Bacteria. However, many of these sequences were homologous (< 45 %) to the phylum Bacteroidetes. One of the five genera detected in the leech homogenates was *Flavobacterium psychrophilum*, a serious fish pathogen that causes Bacterial Cold Water Disease. While the occurrence of genera varies, bacteria associated with the two fish species were similar.

KEY WORDS: *Myzobdella lugubris*, leech, bacterial community, Lake Erie.

Résumé : COMMUNAUTÉ BACTÉRIENNE ASSOCIÉE À LA SANGSUE MYZOBDELLA LUGUBRIS LEIDY 1851 (HIRUDINEA : PISCICOLIDAE) DU LAC ÉRIÉ, MICHIGAN, USA

Les sangsues sont répandues dans le bassin des Grands Lacs, cependant, leur capacité d'héberger des agents pathogènes n'avait pas été investiguée. Le but de cette étude était d'identifier la communauté bactérienne de la sangsue commune, Myzobdella lugubris, *du Lac Érié. Les échantillons de sangsue ont été collectés sur des poissons-chats,* Ictalurus punctatus, *et des poissons perciformes,* Aplodinotus grunniens. *Les bactéries présentes dans les viscères de* M. lugubris *ont été identifiées par le séquensage de leur 16S rRNA (rDNA) et les échantillons ont été comparés à deux bases de données publiques : Ribosomal Database Project et BLAST. Des bactéries appartenant aux phyla Bacteroidetes, β-proteobacteria, Verrucomicrobia et d'autres bactéries non classifiées étaient présentes dans les échantillons de sangsues. De nombreuses bactéries trouvées dans des sangsues parasitant le poisson-chat n'ont pas pu être classifiées au-delà du genre. Cependant, beaucoup étaient homologues (< 45 %) au phylum Bacteroidetes. Un des cinq genres détectés dans l'ensemble des sangsues était* Flavobacterium psychrophilum, *une bactérie pathogène responsable de la "Maladie des eaux froides" chez les poissons. Les bactéries associées à la sangsue étaient les mêmes pour les deux espèces de poisson mais d'occurrences variables.*

MOTS CLÉS : Myzobdella lugubris, *sangsue, communauté bactérienne, Lac Erié.*

INTRODUCTION

Leeches (Hirudinea: Piscicolidae) are ubiquitous throughout North America (Klemm 1991; Muzzall *et al.* 1987; Klemm *et al.* 2003). Most recently we identified leeches prevalent on Lake St. Clair and Lake Erie fish (Schulz *et al.*, submitted, 2010). Epidemiological studies demonstrated that leeches are more widespread in Lake St. Clair than originally thought, with a prevalence of 7.79 %, a mean intensity of 6.45 leeches/fish, and an abundance of 0.50 leeches/fish (Schulz *et al.*, submitted, 2010). *Myzobdella lugubris*, an intermittent, haematophagous feeder, dominated the leech populations in both lakes and heavily parasitized the channel catfish (*Ictalurus punctatus*) and freshwater drum (*Aplodinotus grunniens*).

It is known that leeches can harbor potentially harmful bacteria (Graf, 1999; Kikuchi & Fukatsu, 2005; Laufer *et al.*, 2008). While there have been a few studies describing bacteria within leeches used for medicinal purposes (Graf, 1999; Silver *et al.*, 2007; Laufer *et al.*, 2008), to the best of our knowledge, this is one of the first studies describing bacterial communities of leeches which feed exclusively on fish. The European medicinal leech, *Hirudo* spp., is of particular interest due its use in aiding venous congestion complications that occur following surgical procedures in humans and its unique relationship with *Aeromonas* spp. (Sawyer, 1986; Silver *et al.*, 2007). *Hirudo* spp. contains multiple *Aeromonas* spp., such as *A. veronii* Biovar sobria, *A. hydrophilia*, and *A. jandaei* in its digestive tract (Graf, 1999; Silver

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et al., 2007; Laufer *et al.*, 2008). *Aeromonas jandaei* is also a symbiont in the digestive tract of the North American medicinal leech, *Macrobdella decora* (Siddall *et al.*, 2007). Additionally, a recent study has shown that the bladder of *H. verbana* harbors several species of bacteria, including a *Niabella* spp., which is similar to *Flavobacterium johnsoniae* (Kikuchi *et al.*, 2009). Infections have also been known to occur in human post-operative patients, for which *Hirudo* spp. have been used for bloodletting (Silver *et al.*, 2007; Laufer *et al.*, 2008).

Pathogenic bacteria have also been found within the digestive organs of leeches. Kikuchi & Fukatsu (2005) found a *Rickettsia* spp. infection in the frog leeches *Torix tagoi* and *T. tukubana*, and the fish leech *Hemiclepsis marginata*. *Rickettsia* is a known pathogen that seriously affects many fish species (Cusack *et al.*, 2002). Also, leeches that fed on infected fish contained pathogenic strains of *Aeromonas* spp. and *Pseudomonas* spp. in their digestive tracks (Snieszko & Bullock, 1968). Additionally, Dombrowski (1952) demonstrated that *Piscicola geometra* transmits *Pseudomonas punctata* to carp, which is known to cause moralities in farmed fish (Altinok *et al.*, 2006). Leeches are also known to disperse *A. hydrophila* from host to host (Negele 1975). Additionally, *Aeromonas* spp. can cause lethal infections in farmed and wild fish (Harikrishnan & Balasundaram, 2005), as well as septicemia and diarrhea in humans (Janda & Abbott, 1998). To this end, we used 16S rRNA (rDNA) gene sequencing to identify bacterial communities associated with the internal organs of leeches.

MATERIAL AND METHODS

Study area and fish sampling

Fish were collected from the western portion of Lake Erie, where *Myzobdella lugubris* are found to occur (41° 46’ 00.74” N, 83° 24’ 58.09” W, Fig. 1). Lake Erie is the smallest and shallowest of all the Great Lakes (GL), and is located near the southeastern portion of Michigan. Lake Erie supports a commercial fishery for a wide variety of fish, including the channel catfish (*Ictalurus punctatus*), freshwater drum (*Aplodinotus grunniens*), bigmouth buffalo (*Ictiobus cyprinellus*), and white perch (*Morone americana*), to name a few (Baldwin *et al.*, 2002).

Leech collection

Fish were collected during October of 2008 from western Lake Erie by commercial trap nets. In addition to the abundance of fish hosts and leeches, this specific location was chosen due to the accessibility by commercial fishermen. The trap nets had 1.8 m deep pots of 5.1 cm stretch mesh, 7.6 cm stretch mesh hearts and wings, and 91.4 m long leads of 10.2 cm stretch mesh. Fish were removed from the nets after 48 hrs and examined for the presence of leeches. The attachment sites were recorded for each fish and leeches were separated into whirlpaks containing lake water according to the fish number and attachment site. Leeches remained alive until returned to the laboratory, where they were identified as *Myzobdella lugubris* based on the morphological key of Peckarsky *et al.* (1990). Leeches, ranging in size from 7.5 mm to 20 mm, were disinfected by dipping them in absolute ethanol for 30 sec prior to dissection (Graff, 1999). Leech viscera were aseptically removed, with the assistance of a dissecting microscope, and placed in a microcentrifuge tube containing 80 % ethanol (Thermo Fisher Scientific, Pittsburgh, PA). Due to the small size of the leeches, internal organs were homogenized and then pooled. Sequence analysis was performed on eight leeches which were collected from the pectoral fins of channel catfish and four leeches which were collected also from the pectoral fins of freshwater drum.

Sequence analysis

Genomic bacterial community DNA was harvested from the leech homogenates (30-50 mg) using the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA) following the manufacturer’s protocol. The PCR amplification of the bacterial 16S gene were performed using the universal eubacterial primer set 27f-1387r (27f: 5’-AGAGTTTGATC(AC)TGGCTCAG-3’ and 1387r (5’-GGG CGG WGT GTA CAA GGC-3’) (Marchesi *et al.*, 1998). The PCR mixtures (25 µl/reaction) contained 20 pmol 27F and 1387R primers, 22 mM Tris-HCL (pH 8.4), 55 mM KCL, 1.65 mM $MgCl_2$, 220 µM dNTP’s, 0.55 units recombinant *Taq* DNA Polymerase, and 50 ng template DNA (all reagents from Invitrogen Life Technologies, Carlsbad, CA, USA unless otherwise stated). The PCR amplification was carried out for 30 cycles of 94 °C for 4 min., 56 °C for 30 sec. and 72 °C for 1.5 min. and final 7 min. incubation at 72 °C (modified after Sambrook and Russell 2001).

The expected size of PCR products was 1.36 kb. The PCR products were used to construct 16S gene clone libraries using a TOPO TA Cloning Kit® (with pCR®2.1-TOPO® vector and One Shot® TOP10 Chemically Competent *E. coli*, Invitrogen) following the manufacturer’s protocol. All clones were cultured on Luria-Bertani agar plates (Fisher Scientific Inc., Pittsburgh, PA), containing 50 µg/ml Kanamycin, as directed by the protocol supplied in the TOPO TA Cloning Kit®. Clones were screened for positive transformation with PCR using the primer set M13 forward (5’-GTT TTC CCA GTC ACG AC-3’) and M13 reverse (5’-CAG GAA ACA GCT ATG ACC-3’).

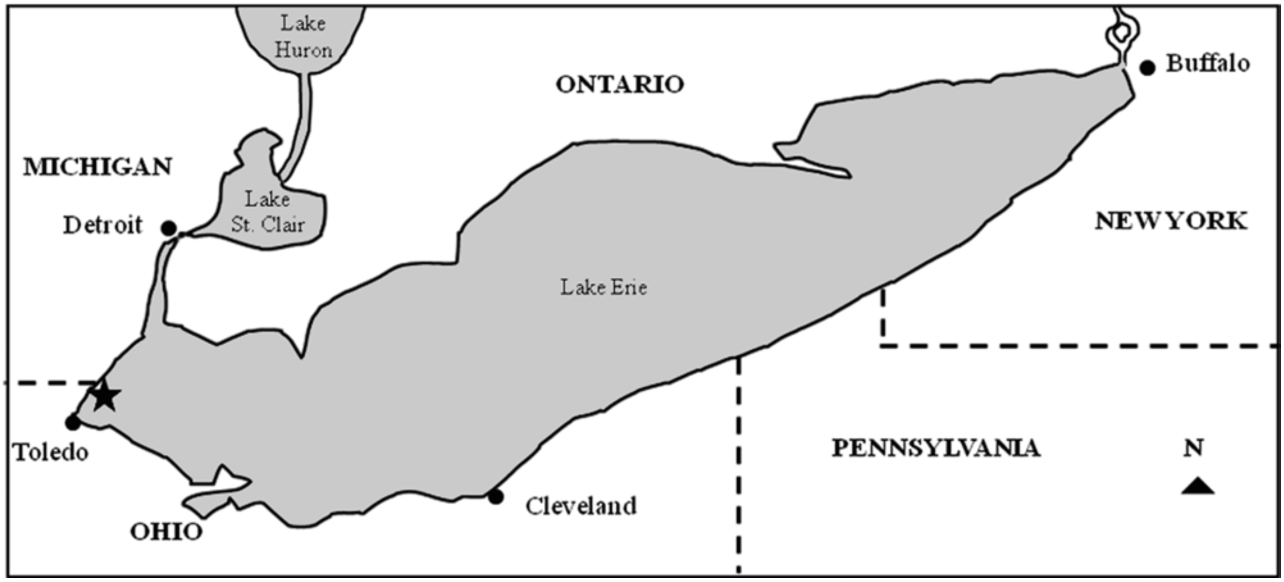

Fig 1. – The Lake Erie Watershed is connected in the east to Lake Ontario by the Welland canal and in the west to Lake Huron via the Detroit River, Lake St. Clair, and the St. Clair River. The black star indicates the commercial fishing trap nets (41° 46’ 00.74” N, 83° 24’ 58.09” W) in Lake Erie from which leeches were collected during this study.

Phylum	Genus species	# of sequences	Average # of bp
Bacteroidetes	*Flavobacterium johnsoniae*	1	603
	Flavobacterium psychrophilum	3	705
	Flavobacterium sp.	7	609
β-proteobacteria	*Chromobacterium violaceum*	2	585
	Thiobacillus denitrificans	3	446
	Vogesella sp.	56	452
Unclassified	Bacteroidetes* (no BLAST match)	10	469
	Bacteroidetes* (*Marinomonas* sp.⁺)	29	489
	γ-proteobacteria* (*Shewanella woodyi*⁺)	1	529
Verrucomicrobia	Verrucomicrobium genera incertae sedis	1	582

* Closest taxonomic match according to RDP search
\+ Closest taxonomic match according to BLAST search

Table I. – The sequence alignment of 113 screened clones from channel catfish and freshwater drum leech homogenate samples. Sequences were classified using the Ribosomal Database Project II (RDP) and the Basic Local Alignment Search Tool (BLAST) of the NCBI. The average sequence length (reported as number of base pairs) is also presented for each taxonomic group.

A total of 190 screened clones were sequenced using a 3730 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA). Sequences were aligned and classified using the Ribosomal Database Project II (RDP II) Release 9.47 produced by Wang *et al.* (2007). The RDP-II provides aligned and annotated rRNA sequences and uses a naive Bayesian classifier to assign sequences to the RDP Taxonomy. It also provides taxonomic assignments from domain to genus, with confidence estimates for each assignment. Sequencing was unidirectional and the average sequence length was 498 bp. Fifty-five clones from the channel catfish leech homogenate and 58 clones from the freshwater drum leech homogenate were successfully aligned (Table I). The RDP “Seq Match” was used to identify bacteria, while the RDP “Classifier” (95 % confidence interval) was used to compare relative abundance of sequences. Multiple sequences were rechecked with the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information of the National Institute of Health. All sequences from this study were submitted to GenBank and received the Accession numbers GU942570-GU942682 (Table II).

RESULTS

Sequence analysis revealed the presence of four bacterial groups within leech viscera (β-proteobacteria, Bacteroidetes, Verrucomicrobia, and RDP-unclassified Bacteria), although the occurrence at which each group was found in the two samples differed. With the exception of Verrucomicrobia, which only occurred in the freshwater drum leech

Sample #	# of BP	RDP Classification	RDP % match	Accession #	BLAST Classification	BLAST % match
1	444	Bacteroidetes	21	GU942630	No match	N/A
2	499	Bacteroidetes	32	GU942629	*Marinomonas* sp. MWYL1 (NC_009654.1l)	88
3	462	Bacteroidetes	32	GU942627	*Marinomonas* sp. MWYL1 (NC_009654.1l)	87
4	496	Bacteroidetes	44	GU942633	*Marinomonas* sp. MWYL1 (NC_009654.1l)	87
5	317	Bacteroidetes	14	GU942631	*Thioalkalivibrio* sp. HL-EbGR7 ctg53 (NZ_ABYM01000001.1l)	81
6	524	Bacteroidetes	29	GU942652	No match	N/A
7	442	Bacteroidetes	26	GU942651	No match	N/A
8	503	Bacteroidetes	45	GU942656	*Marinomonas* sp. MWYL1 (NC_009654.1l)	88
9	514	Bacteroidetes	34	GU942663	No match	N/A
10	493	Bacteroidetes	38	GU942645	*Marinomonas* sp. MWYL1 (NC_009654.1l)	88
11	375	Bacteroidetes	20	GU942647	*Marinomonas* sp. MWYL1 (NC_009654.1l)	85
12	460	Bacteroidetes	15	GU942638	*Marinomonas* sp. MWYL1 (NC_009654.1l)	87
13	418	Bacteroidetes	28	GU942636	*Marinomonas* sp. MWYL1 (NC_009654.1l)	87
14	448	Bacteroidetes	27	GU942640	*Marinomonas* sp. MWYL1 (NC_009654.1l)	87
15	470	Bacteroidetes	39	GU942635	*Marinomonas* sp. MWYL1 (NC_009654.1l)	87
16	492	Bacteroidetes	45	GU942642	No match	N/A
17	464	Bacteroidetes	33	GU942644	No match	N/A
18	417	Bacteroidetes	26	GU942650	*Marinomonas* sp. MWYL1 (NC_009654.1l)	86
19	443	Bacteroidetes	25	GU942649	*Marinomonas* sp. MWYL1 (NC_009654.1l)	87
20	490	Bacteroidetes	33	GU942665	*Marinomonas* sp. MWYL1 (NC_009654.1l)	87
21	480	Bacteroidetes	33	GU942664	*Marinomonas* sp. MWYL1 (NC_009654.1l)	87
22	456	Bacteroidetes	20	GU942662	*Marinomonas* sp. MWYL1 (NC_009654.1l)	87
23	373	Bacteroidetes	29	GU942660	No match	N/A
24	483	Bacteroidetes	37	GU942659	No match	N/A
25	519	Bacteroidetes	38	GU942658	*Marinomonas* sp. MWYL1 (NC_009654.1l)	87
26	475	Bacteroidetes	40	GU942654	*Marinomonas* sp. MWYL1 (NC_009654.1l)	87
27	490	Bacteroidetes	36	GU942655	No match	N/A
28	370	Bacteroidetes	15	GU942672	*Marinomonas* sp. MWYL1 (NC_009654.1l)	85
29	432	Bacteroidetes	20	GU942675	*Marinomonas* sp. MWYL1 (NC_009654.1l)	87
30	468	Bacteroidetes	44	GU942674	*Marinomonas* sp. MWYL1 (NC_009654.1l)	87
31	443	Bacteroidetes	21	GU942669	*Marinomonas* sp. MWYL1 (NC_009654.1l)	87
32	468	Bacteroidetes	32	GU942668	No match	N/A
33	471	Bacteroidetes	38	GU942671	*Marinomonas* sp. MWYL1 (NC_009654.1l)	87
34	478	Bacteroidetes	40	GU942667	*Marinomonas* sp. MWYL1 (NC_009654.1l)	87
35	449	Flavobacteriaceae	100	GU942681	*Flavobacterium psychrophilum* JIP02/86 (NC_009613.1l)	94
36	423	*Flavobacterium* sp.	86	GU942628	*Flavobacteria bacterium* BAL38 (AAXX01000001.1l)	94
37	495	*Flavobacterium* sp.	94	GU942653	*Flavobacteria bacterium* BAL38 (AAXX01000001.1l)	92
38	452	*Flavobacterium* sp.	88	GU942641	*Flavobacteria bacterium* BAL38 (AAXX01000001.1l)	94
39	528	*Flavobacterium* sp.	98	GU942639	*Flavobacteria bacterium* BAL38 (AAXX01000001.1l)	93
40	517	*Flavobacterium* sp.	100	GU942679	*Flavobacterium psychrophilum* JIP02/86 (NC_009613.1l)	98
41	474	*Flavobacterium* sp.	100	GU942677	*Flavobacterium psychrophilum* JIP02/86 (NC_009613.1l)	97
42	415	*Flavobacterium* sp.	86	GU942673	*Flavobacteria bacterium* BAL38 (AAXX01000001.1l)	94
43	501	*Flavobacterium* sp.	92	GU942670	*Flavobacteria bacterium* BAL38 (AAXX01000001.1l)	94
44	457	Neisseriaceae	100	GU942648	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	92
45	439	Neisseriaceae	100	GU942646	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	92
46	441	Neisseriaceae	100	GU942626	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	92
47	528	Proteobacteria	92	GU942680	*Shewanella woodyi* ATCC 51908 (NC_010506.1l)	88
48	520	*Vogesella* sp.	99	GU942634	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	92
49	495	*Vogesella* sp.	80	GU942632	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	92
50	457	*Vogesella* sp.	95	GU942657	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	93

Table II. – Sequence classification for each sample in the study - classification was first determined according to the RDP, followed by classification according to BLAST. Sample numbers 1-55 are from channel catfish tissue homogenate, whereas samples 56-113 are from freshwater drum tissue homogenate.

Table II continued

Sample #	# of BP	RDP Classification	RDP % match	Accession #	BLAST Classification	BLAST % match
51	412	*Vogesella* sp.	100	GU942643	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	92
52	350	*Vogesella* sp.	91	GU942637	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	91
53	505	*Vogesella* sp.	92	GU942666	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	90
54	359	*Vogesella* sp.	99	GU942661	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	92
55	435	*Vogesella* sp.	91	GU942625	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	93
56	435	Bacteroidetes	30	GU942575	*Marinomonas* sp. MWYL1 (NC_009654.1l)	87
57	544	Bacteroidetes	40	GU942587	*Marinomonas* sp. MWYL1 (NC_009654.1l)	86
58	550	Bacteroidetes	25	GU942588	*Marinomonas* sp. MWYL1 (NC_009654.1l)	88
59	552	Bacteroidetes	26	GU942581	*Marinomonas* sp. MWYL1 (NC_009654.1l)	86
60	521	Bacteroidetes	27	GU942602	*Marinomonas* sp. MWYL1 (NC_009654.1l)	87
61	533	*Flavobacterium* sp.	100	GU942619	*Flavobacteria bacterium* BAL38 (NZ_AAXX01000001.1l)	95
62	603	*Flavobacterium* sp.	95	GU942682	*Flavobacterium johnsoniae* UW101 (NC_009441.1l)	92
63	630	Unclassified Neisseriaceae	99	GU942676	*Chromobacterium violaceum* ATCC 12472 (NC_005085.1l)	96
64	540	Unclassified Neisseriaceae	96	GU942678	*Chromobacterium violaceum* ATCC 12472 (NC_005085.1l)	94
65	582	Verrucomicrobiaceae genera incertae sedis	86	GU942580	*Verrucomicrobium spinosum* DSM 4136 (NZ_ABIZ01000001.1l)	85
66	431	*Vogesella* sp.	98	GU942576	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	92
67	434	*Vogesella* sp.	97	GU942579	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	93
68	489	*Vogesella* sp.	96	GU942578	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	93
69	437	*Vogesella* sp.	94	GU942574	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	93
70	436	*Vogesella* sp.	98	GU942615	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	93
71	450	*Vogesella* sp.	100	GU942618	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	93
72	523	*Vogesella* sp.	98	GU942617	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	92
73	470	*Vogesella* sp.	95	GU942616	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	92
74	560	*Vogesella* sp.	98	GU942623	*Chromobacterium violaceum* ATCC 12472 (NC_005085.1l)	89
75	566	*Vogesella* sp.	96	GU942622	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	89
76	415	*Vogesella* sp.	98	GU942621	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	93
77	476	*Vogesella* sp.	97	GU942620	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	92
78	490	*Vogesella* sp.	95	GU942607	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	92
79	481	*Vogesella* sp.	95	GU942624	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	93
80	508	*Vogesella* sp.	93	GU942606	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	93
81	552	*Vogesella* sp.	98	GU942609	*Chromobacterium violaceum* ATCC 12472 (NC_005085.1l)	89
82	516	*Vogesella* sp.	94	GU942608	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	93
83	571	*Vogesella* sp.	98	GU942611	*Chromobacterium violaceum* ATCC 12472 (NC_005085.1l)	90
84	515	*Vogesella* sp.	98	GU942610	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	92
85	528	*Vogesella* sp.	99	GU942612	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	92

Table II continued

Sample #	# of BP	RDP Classification	RDP % match	Accession #	BLAST Classification	BLAST % match
86	514	*Vogesella* sp.	94	GU942614	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	93
87	566	*Vogesella* sp.	98	GU942613	*Chromobacterium violaceum* ATCC 12472 (NC_005085.1l)	90
88	465	*Vogesella* sp.	93	GU942597	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	93
89	511	*Vogesella* sp.	98	GU942598	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	92
90	553	*Vogesella* sp.	97	GU942583	*Chromobacterium violaceum* ATCC 12472 (NC_005085.1l)	90
91	519	*Vogesella* sp.	95	GU942582	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	93
92	579	*Vogesella* sp.	95	GU942585	*Chromobacterium violaceum* ATCC 12472 (NC_005085.1l)	90
93	488	*Vogesella* sp.	94	GU942591	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	93
94	605	*Vogesella* sp.	93	GU942592	*Chromobacterium violaceum* ATCC 12472 (NC_005085.1l)	90
95	589	*Vogesella* sp.	98	GU942593	*Chromobacterium violaceum* ATCC 12472 (NC_005085.1l)	90
96	498	*Vogesella* sp.	93	GU942589	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	92
97	620	*Vogesella* sp.	90	GU942590	*Chromobacterium violaceum* ATCC 12472 (NC_005085.1l)	90
98	541	*Vogesella* sp.	97	GU942584	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	93
99	578	*Vogesella* sp.	97	GU942586	*Chromobacterium violaceum* ATCC 12472 (NC_005085.1l)	90
100	530	*Vogesella* sp.	95	GU942596	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	93
101	630	*Vogesella* sp.	100	GU942595	*Chromobacterium violaceum* ATCC 12472 (NC_005085.1l)	91
102	556	*Vogesella* sp.	100	GU942594	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	93
103	560	*Vogesella* sp.	98	GU942605	*Chromobacterium violaceum* ATCC 12472 (NC_005085.1l)	89
104	608	*Vogesella* sp.	98	GU942604	*Chromobacterium violaceum* ATCC 12472 (NC_005085.1l)	90
105	590	*Vogesella* sp.	98	GU942603	*Chromobacterium violaceum* ATCC 12472 (NC_005085.1l)	90
106	512	*Vogesella* sp.	98	GU942601	*Thiobacillus denitrificans* ATCC 25259 (NC_007404.1l)	93
107	587	*Vogesella* sp.	97	GU942600	*Chromobacterium violaceum* ATCC 12472 (NC_005085.1l)	90
108	604	*Vogesella* sp.	96	GU942599	*Chromobacterium violaceum* ATCC 12472 (NC_005085.1l)	90
109	586	*Vogesella* sp.	97	GU942571	*Chromobacterium violaceum* ATCC 12472 (NC_005085.1l)	90
110	588	*Vogesella* sp.	96	GU942570	*Chromobacterium violaceum* ATCC 12472 (NC_005085.1l)	90
111	591	*Vogesella* sp.	92	GU942572	*Chromobacterium violaceum* ATCC 12472 (NC_005085.1l)	90
112	606	*Vogesella* sp.	93	GU942573	*Chromobacterium violaceum* ATCC 12472 (NC_005085.1l)	91
113	575	*Vogesella* sp.	92	GU942577	*Chromobacterium violaceum* ATCC 12472 (NC_005085.1l)	90

homogenate, all phyla were detected in both leech homogenates. In both leech homogenates there were several RDP-unclassified Bacteria; however, the RDP library did produce homologous match scores (< 45 %) to the Sphingobacteriales order (Bacteroidetes).

A number of sequences within the Bacteroidetes phylum showed high homology to *Flavobacterium* spp. (*F. johnsoniae* and *F. psychrophilium*) according to RDP and BLAST (Table II). In the freshwater drum leech homogenate, six of the nine Bacteroidetes sequences were similar to *Flavobacterium* sp. (GenBank: AAXX01000001.1l), with the highest match at 94 % (BLAST) and three of the nine sequences were similar to *F. psychrophilium* (GenBank: NC_009613.1l), with the highest match at 98 % (BLAST, Table II). In the channel catfish leech homogenate, the only two sequences homologous to the phylum Bacteroidetes were similar to *Flavobacterium* sp. (GenBank: AAXX01000001.1l) with the match at 95 % and *F. johnsoniae* (GenBank: NC_009441.1l) with the match at 92 % (Table II).

The RDP and BLAST searches revealed matches for the genera *Chromobacterium*, *Thiobacillus*, and *Vogesella* within the class β-proteobacteria. In the freshwater drum leech homogenate, three of the 11 β-proteobacteria sequences closely resembled *T. denitrificans* (GenBank: NC_007404.1l), with the highest match at 92 % (BLAST); however RDP matched the same three sequences as unclassified members of the Neisseriaceae family of bacteria (Table II). The remaining eight sequences of β-proteobacteria were similar to *Vogesella* sp., with the highest match at 100 % (RDP, Table II). In the channel catfish leech homogenate, 48 of the 50 β-proteobacteria sequences were also closely related to *Vogesella* sp., with the highest match at 100 % (RDP). The last two sequences from the channel catfish leech homogenate in the β-proteobacteria were determined to be unclassified members of the Neisseriaceae family of bacteria according to RDP, but according to BLAST, they were similar to *Chromobacterium violaceum*, with the highest match at 96 % (Table II).

One sequence from the freshwater drum leech homogenate was determined to belong to the phylum Verrucomicrobia. This sequence showed high homology to *Verrucomicrobium genera incertae sedis*, with a match of 86 % (RDP, Table II).

DISCUSSION

This is the first report addressing the microbial community of the piscicolid leech, *Myzobdella lugubris*. In other studies, bacterial communities have been considered in medicinal leeches, either as intestinal or bladder symbionts or as an infection transmitted by leeches. Although *Aeromonas* spp. were previously detected within the European medicinal leeches *Hirudinea orientalis*, *H. verbana*, and *H. medicinalis* (Graf, 1999; Kikuchi & Graf, 2007; Laufer *et al.*, 2008) and the North American medicinal leech *Macrobdella decora* (Siddall *et al.*, 2009), they were not detected in this study. This is surprising considering the abundance of *Aeromonas* spp. in the aquatic environment. Other infectious bacteria, such as *Rickettsia* sp., have been found within *Torix* spp. and *Hemiclepsis* spp. (Kikuchi & Fukatsu, 2005), but were also not detected in *M. lugubris* samples in this study.

The results indicated that there are multiple bacterial groups within the viscera of *M. lugubris*. With the exception of Verrucomicrobia, the bacterial communities within the freshwater drum homogenate and the channel catfish homogenate consisted of mostly similar phylum. Considering that freshwater drum and channel catfish have similar diets and exist in similar habitats, this is not surprising. The group that had the highest occurrence in the channel catfish leech homogenate was unclassified bacteria; however, they were similar to the order Sphingobacteriales (Bacteroidetes). The order Sphingobacteriales includes several species, such as *Sphingobacterium composti*, *S. antarcticus*, and *S. anhuiense*, which are bacteria commonly found in forest soil, composts, and aquatic environments (Shivaji *et al.*, 1992; Yoo *et al.*, 2007; Wei *et al.*, 2008). Interestingly, a bacterium from Sphingobacteriales was recently isolated from diseased fish (Loch & Faisal, personal communication). It is not unexpected since *M. lugubris* feeds exclusively on blood and tissue fluids; however, it is unknown as to how bacteria from Sphingobacteriales found its way to the interior of *M. lugubris*.

A number of sequences from the Bacteroidetes phylum were similar to *Flavobacterium* spp., including *F. psychrophilum*. *Flavobacterium psychrophilum* is the causative agent of Bacterial Cold-Water Disease and Rainbow Trout Fry Syndrome, which are known to cause high mortalities in salmonids and can increase their susceptibility to other diseases (Nematollahi *et al.*, 2003). This finding raises the question as to the potential role of *M. lugubris* in *Flavobacterium* spp. transmission among susceptible fish.

Additionally, there were several unclassified members of Neisseriaceae (β-proteobacteria) within the freshwater drum leech homogenate. Most of the sequences were similar to a *Vogesella* sp., of which there are only two known species, *V. indigofera* and *V. perlucida* (Gu & Cheung, 2001; Chou *et al.*, 2008). *Vogesella indigofera* and *V. perlucida* have both been found in groundwater (Gu & Cheung, 2001; Chou *et al.*, 2008). However, *V. indigofera* has been found within the pyloric cecae of wild fish which had leeches, specifically *Piscicola geometra*, attached to them (Goldschmidt-Clermont *et* *al.*, 2008). The bacterial community from within *P. geometra* was not investigated; therefore, it is unknown if the leeches contained *V. indigofera* or not (Goldschmidt-Clermont *et al.*, 2008); however it would be of value to determine if leeches do aid in the transmission of *Vogesella* spp. While the sequences in this study were similar to a *Vogesella* sp., further research is needed to determine the potential symbiotic, or pathogenic, relationship there might be between *M. lugubris* and *Vogesella* spp.

In the freshwater drum leech homogenate, the bacteria group Verrucomicrobia had only one sequence that was similar to a *Verrucomicrobium* species. *Verrucomicrobium* spp. are found in soil and aquatic environments and are significant for understanding the evolution of bacteria (Paissé *et al.*, 2008; Lee *et al.*, 2009). Verrucomicrobia bacteria have also been detected in toxic cyanobacterial blooms, although their role in the toxic bloom is unknown (Pope & Patel, 2008). This is the first report of a *Verrucomicrobium*-like species to be found associated with the leech bacterial community, however the phylum Verrucomicrobia is still relatively new and it is unknown as to what kind of relationship the bacteria and leeches might have.

In general, the leech bacterial community in this study seems to be much less diverse when compared to other aquatic animals collected from the Lake Erie Watershed (Winters, 2008). This may be due to the haematophagous nature of leech nutrition on only fish blood and tissue fluids. Moreover, there was little variation in bacterial community composition between *M. lugubris* collected from the channel catfish and those collected from the freshwater drum. As expected, most of the bacteria detected from within the leeches were environmental bacteria typically found in soil and the aquatic environment; however, a further investigation is warranted to determine if there is a pathogenic relationship between *M. lugubris* and *F. psychrophilum*. It is possible that the fish pathogenic bacteria found within the leech homogenates could have origins from prey fish. Our findings emphasize the potential of *M. lugubris* in transmitting these pathogens among susceptible fish hosts. The fact that some of the environmental bacteria found within the leech were also found in the aquatic environment raises the question as to whether or not these bacteria are resident to leech internal organs, or are they contaminants that were on fish mucous and/or the outermembrane which the leech engulfed as it attached for a blood meal. In the same context, one may wonder as to how aquatic bacteria such as *Aeromonas* spp. gained access to medicinal leeches, kept in the laboratory environment for generations, but were not found in fish-parasitic leeches. These types of studies are needed to better understand the role played by leeches in shaping the bacterial community structure of an ecosystem and in disease transmission.

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