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ARTICLE

Diversity of Bacteria Cultured from the Blood of Lesser Electric Rays Caught in the Northern Gulf of Mexico

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Abstract

The prevalence and taxonomic diversity of bacteria cultured from the blood of apparently healthy Lesser Electric Rays *Narcine bancroftii* captured from open beach habitat in the north-central Gulf of Mexico are reported herein. The blood of 9 out of 10 Lesser Electric Rays was positive for bacteria, and bacterial isolates ($n = 83$) were identified by 16S rRNA gene sequencing. The majority of the isolates belonged to the phylum Proteobacteria (91.5%). *Vibrio* spp. comprised 53% of all isolates and were recovered from all Lesser Electric Rays with culture-positive blood. Among them, *V. harveyi* ($n = 14$) and *V. campbellii* ($n = 11$) were most common, followed by a group of unidentified *Vibrio* sp. ($n = 10$) related to *V. nigripulchritudo*. Isolates representing other species of Proteobacteria included *Pseudoalteromonas* ($n = 13$), *Shewanella* ($n = 5$), *Amphritea* ($n = 3$), *Nautella* ($n = 3$), and *Arenibacter* ($n = 1$). Higher bacterial diversity was observed in blood cultured on marine agar relative to blood agar, but gram-positive bacteria were isolated from the latter only. The 16S rRNA gene sequences of bacterial isolates were compared phylogenetically to those from related type strains. Most isolates were identified to the level of species, but some clustered independently from reference strains, likely representing new species of *Vibrio, Amphritea, Shewanella*, and *Tenacibaculum*. The present study is the first record of any bacterium from this ray species and reveals a taxonomically and phylogenetically diverse microbiota associated with its blood. Moreover, these data document that the presence of bacteria in elasmobranch blood is not coincident with clinical signs of disease, thereby rejecting the paradigm of septicemia indicating a disease condition in aquatic vertebrates.

Little published information exists on the biodiversity, prevalence, and physiological effects of bacteria that infect the blood and other tissues of cartilaginous fishes (class Chondrichthyes: sharks, skates, rays, and chimaeras) (Borucinska and Frascas 2002; Mylniczenko et al. 2007). However, several studies have reported the isolation of bacteria from the blood of apparently healthy elasmobranchs (Grimes et al. 1993; Borucinska and Frascas 2002; Mylniczenko et al. 2007) Based on this foundational taxonomic work with blood-borne bacteria in sharks, seemingly, the classical assumption that bacterial presence in blood indicates disease is no longer robust. Yet, other bacteria, including *Vibrio* spp., recovered

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from sharks are indeed considered opportunistic pathogens (Grimes et al. 1984a). Hence, whether the taxonomic spectrum of these elasmobranch-associated bacteria comprise opportunistic or obligate pathogens, benign commensals, or bona fide tissue-dwelling symbionts that serve a critical role in elasmobranch physiology is indeterminate. Nevertheless, documenting microbial taxonomic diversity in other elasmobranch lineages is a good first step towards deciphering the nature of these associations between elasmobranchs and bacteria. Detailed studies based on materials sampled from nonshark elasmobranchs, i.e., skates, rays, or chimaeras, are largely missing from the literature (Mylniczenko et al. 2007).

The Lesser Electric Ray *Narcine bancroftii* (order Torpediniformes, family Narcinidae; also known as *Narcine brasiliensis*) ranges in shallow waters of tropical and subtropical continental shelves to 37 m deep, including the Gulf of Mexico, the Caribbean Sea, and the islands of the West Indies (Robins and Ray 1986). Three other species of Narcinidae have geographic ranges that overlap with the Lesser Electric Ray, but the Lesser Electric Ray is the only narcinid that reportedly ranges in the north-central Gulf of Mexico (the focus area for the present study) (McEachran and de Carvalho 2002). This ray is a slow swimming fish that can be seasonally aggregated on sandbars and surf zones along open beaches and barrier islands. It can be regionally abundant in the summer months, during which time pregnant females birth viviparous offspring, but then moves to offshore deep waters in the winter (Rudloe 1989). During fall (August–October) in the northern Gulf of Mexico, Lesser Electric Rays can be observed commonly by snorkeling in waters of 0.2−3.0 m; the spiracles of the nearly completely buried rays appear as characteristic holes in the sand (S.A.B., personal observations). Perhaps because this ray species is seldom caught by commercial fishermen, has no recreational or commercial value, and is typically hidden, nearly completely buried in the sand, it is rarely included in faunal surveys of beach habitat in the Gulf of Mexico. As a result, there is little substantive information on the abundance and population structure of this species throughout its range or in the northern Gulf of Mexico. Concomitantly, we know little of its general biology, including its parasites, pathogens, and symbionts. The objective of this study was to determine if bacteria could be isolated from the blood of apparently healthy Lesser Electric Rays and, if so, to characterize the bacterial diversity present in their blood.

METHODS

Sample collection.—The studied Lesser Electric Rays were hand-netted off Fort Morgan, Alabama (30°13′45″N, 87°54′7″W), maintained alive in enclosed plastic transport containers filled with water from the collection site and fitted with water pumps and aerators powered by a car battery, and transported alive to Auburn University (within 5 h after collection). A total of 10 Lesser Electric Rays (24–47 cm in total length; 7 females and 3 males) were examined in the study. Immediately before necropsy, each ray was euthanized with an overdose (300 mg/L) of tricaine methanesulfonate (MS-222). All animal protocols were approved by the Auburn University Institutional Animal Care and Use Committee (IACUC number 2012–2098). Immediately after euthanasia, the area of skin circumscribed by the gill slits, mouth, and pectoral girdle was dried with a clean paper towel, disinfected with 70% ethanol, and cut away to expose the pericardial chamber. The exposed surfaces of the heart, including ventricle and conus arteriosus, were disinfected with 70% ethanol before a blood sample was taken by inserting a sterile syringe into the lumen of the heart. Each blood sample from each ray was immediately spread onto blood agar (BA) (MOLTOX, Boone, North Carolina) and marine agar (MA) (Difco, Sparks, Maryland) using aseptic methods. Agar plates were incubated at 28°C for 48 h under aerobic conditions. A representative of each colony type on the primary isolation plate was restreaked on MA to obtain pure cultures for identification. A total of 86 single isolates were preserved as glycerol stocks (marine broth supplemented with 20% glycerol) at −80°C until subsequent analysis. Individual blood samples were labeled NB-01 through NB-09. The isolates were designated as FMR (Fort Morgan Ray) followed by the colony number.

Bacterial identification.—Bacterial isolates were identified by partially sequencing the 16S rRNA gene. The DNA template was prepared using a rapid-boiling method as follows. Five colonies from a pure isolate were selected from a 24-h culture on MA and resuspended in a centrifuge tube with 100-μL sterile distilled H_2O. Proteinase K was added to the cell suspension to a final concentration of 30 unit/μL. After a 20-min digestion at 55°C, the lysate was heated to 100°C for 15 min and spun down at 15,000 *g* for 5 min. The supernatant was transferred to a new tube and used as template DNA. The nearly complete 16S rRNA gene of each isolate was amplified using the following primers: 63V (forward) 5′-CAGGCCTAACACATGCAAGTC-3′ and 1387R (reverse) 5′-GGGCGGWGTGTACAAGGC-3′ (Marchesi et al. 1998). The PCR conditions and reagents have been described elsewhere (Arias et al. 2006). Sequencing was conducted on the PCR-amplified products, using 63V as sequencing primer, in an ABI 3730xl sequencer at Lucigen (Madison, Wisconsin).

Sequence analysis.—Sequence trace files were edited with BioEdit version 7.1.9 (Hall 1999) to remove noise and untrusted ends. Sequences ($n = 3$) having < 500 bp or > 3 ambiguous positions were excluded from the analysis. The resulting 83 sequences were assigned to taxonomic units by (1) the Ribosomal Database Project (RDP) Naïve Bayesian Classifier (Cole et al. 2007), (2) the GreenGenes web classification tool (DeSantis et al. 2006), and (3) the Basic Local Alignment Search Tool (BLAST; Altschul et al. 1997). Query sequences that had a 98–100% and 95–98% identity match to those present in the databases were considered identified at the species and genus level, respectively (Tindall et al. 2006).

Phylogenetic analysis.—Partial 16S rRNA gene sequences were aligned using Clustal X2 (Larkin et al. 2007). Multiple

sequence alignment (MSA) was conducted by trimming the sequences to cover the entire alignment and subsequent realignment. The trimmed MSA spanned the hypervariable V2, V3, and V4 regions corresponding to the *Escherichia coli* 16S rRNA gene base pair positions (Van de Peer et al. 1996). Sequences of the type strains identified as nearest to the Lesser Electric Ray isolates by RDP and BLAST were incorporated into the phylogenetic trees as reference. Phylogenetic analysis was conducted in MEGA 5.0 software (Tamura et al. 2011). Trees were constructed using the neighbor-joining method (Saitou and Nei 1987) with the Jukes–Cantor correction (Jukes and Cantor 1969). The partial 16S rRNA gene sequences of bacterial isolates recovered from Lesser Electric Rays were submitted to the GENBANK nucleotide sequence database (accession numbers KC439161 to KC439244).

RESULTS

Isolate Identification

All blood samples but one (NB-09) were culture positive, although the number of colony types (from approximately 3–16) varied among specimens (Figure 1). A total of 86 pure isolates were recovered from the blood samples. Isolates were recovered on both MA (47 colonies) and BA (39 colonies) culture media. Three isolates yielded poor 16S rRNA gene sequence quality and were removed from the study. The remaining 83 sequences were ascribed to specific taxa using three databases. Overall, results from RDP, GreenGene, and GENBANK were in agreement and isolates were identified unambiguously to genus (sequence similarity of 95% or higher). Isolates were classified into 14 genera, 11 families, 6 orders, 4 classes, and 3 phyla. The majority of the isolates (91.5%) were ascribed to the phylum Proteobacteria, followed by the phylum Bacteroidetes (6.0%) and the phylum Actinobacteria (2.4%). In a few cases, there was a disagreement between the results obtained from different databases. For example, GreenGenes could not place four Flavobacteriaceae isolates below family; whereas, RDP ascribed

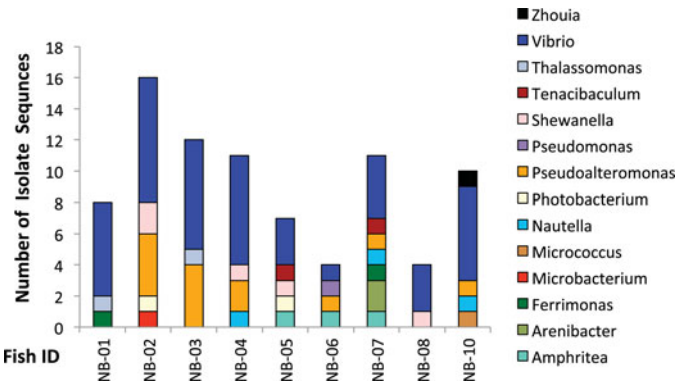

FIGURE 1. Bacteria recovered from the blood of Lesser Electric Rays (indicated as NB followed by their ID number). [Color figure available online.]

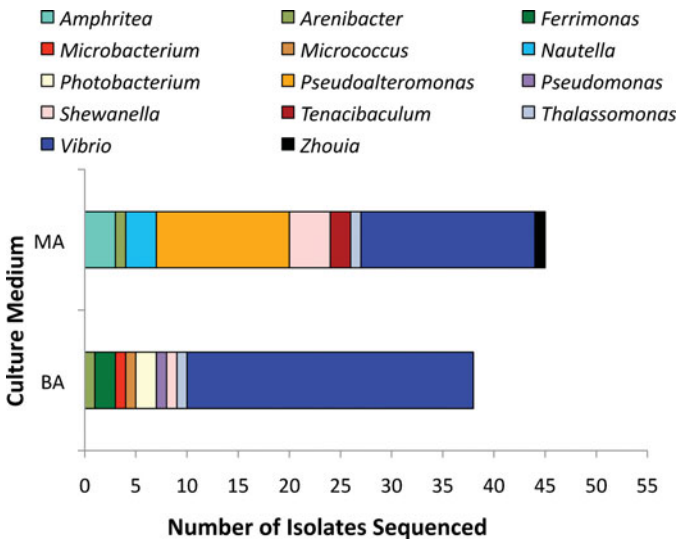

FIGURE 2. Distribution of isolates from the blood of Lesser Electric Rays cultured in marine agar (MA) and blood agar (BA). [Color figure available online.]

them to *Arenibacter* sp. Five isolates were ascribed to *Vibrio* by GreenGenes, but RDP ascribed them to Vibrionaceae. We resolved the divergence by assigning the sequence to the lowest taxonomic level.

Among the Proteobacteria, 79 isolates were from the class Gammaproteobacteria while only 3 isolates were identified as class Alphaproteobacteria. Within the Gammaproteobacteria, *Vibrio* was the predominant genus with 45 isolates. Other isolates representing genera of Gammaproteobacteria comprised *Pseudoalteromonas* ($n = 13$), *Shewanella* ($n = 5$), *Ferrimonas* ($n = 2$), *Amphritea* ($n = 3$), *Photobacterium* ($n = 2$), *Thalassomonas* ($n = 2$), *Aestuariibacter* ($n = 1$) and *Pseudomonas* ($n = 1$). All Alphaproteobacteria were assigned to *Nautella* ($n = 3$). Figure 1 shows the distribution of the predominant genera in each individual fish. *Vibrio* was the only genus recovered from all fish with culture-positive blood. In fact, it was the most common genus in all fish except in NB-07 from which only four isolates were recovered and all of them belonged to different genera. *Pseudoalteromonas* and *Shewanella* were recovered from five and four Lesser Electric Rays, respectively. The MA not only yielded more isolates but also provided a higher diversity of genera than did the BA (Figure 2), but a few genera (gram-positive bacteria) were recovered on BA only.

Phylogenetic Analysis

The majority of *Vibrio* isolates (35 out of 45) comprised three clades (Figure 3). Clade I included *V. harveyi* ($n = 14$) plus the types species, Clade II included 11 isolates of *V. campbellii* or *V. sagamiensis* (the partial 16S rRNA gene sequence used did not allow for differentiation between these two species), and Clade III included 11 isolates not ascribed to any reference or

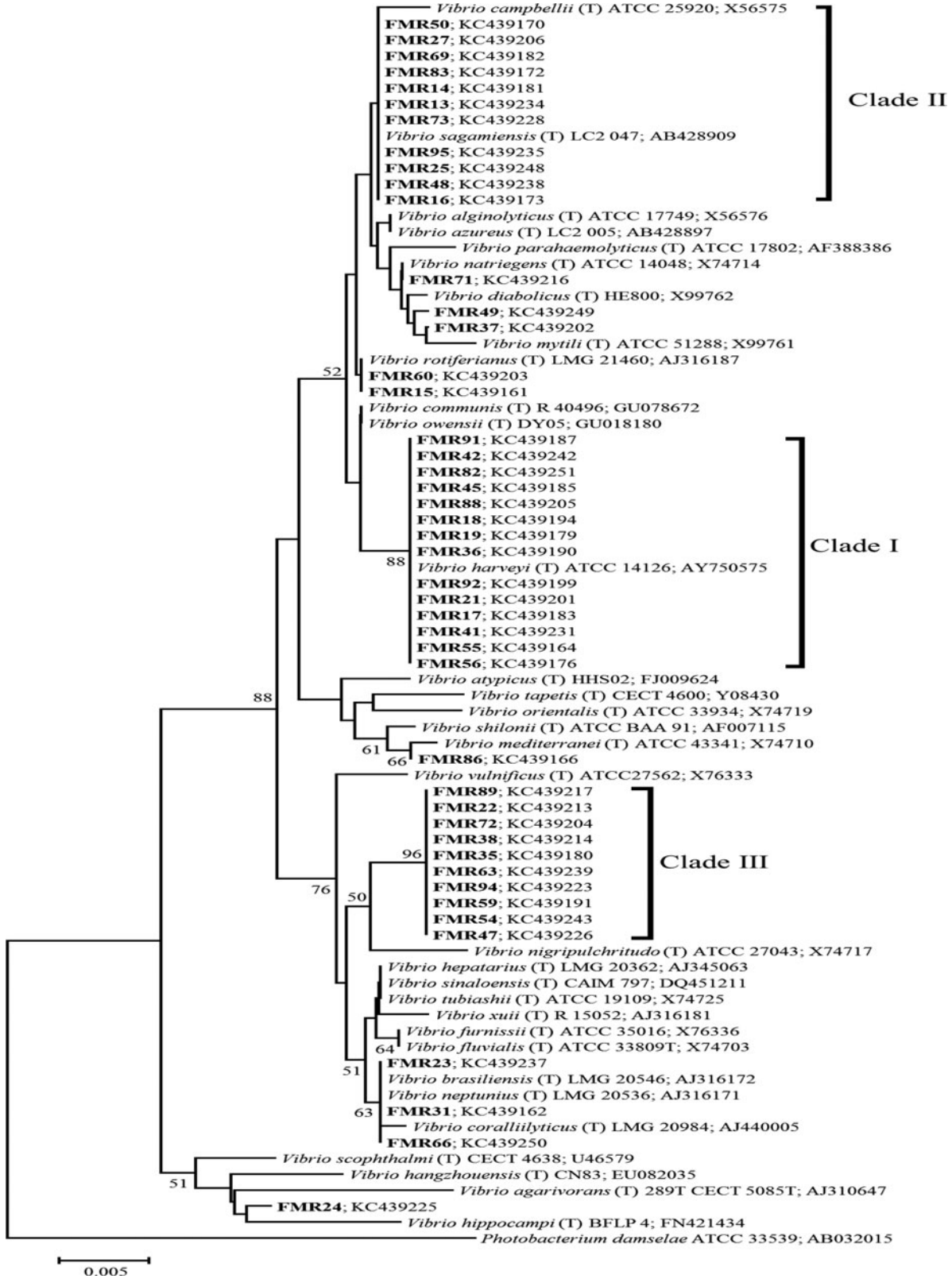

FIGURE 3. Phylogeny (partial 16S rRNA gene sequences) of bacterial isolates from the blood of Lesser Electric Rays and ascribed to species in the genus *Vibrio*. The isolate number is followed by the GENBANK accession number. Sequences from type strains, or the closest match, were used for comparison. The tree topology was obtained by the neighbor-joining method (Jukes–Cantor correction). The three main clades are indicated. The numbers at the nodes indicate bootstrap values (1,000 replicates). Scale bar = 0.5% sequence divergence.

type strain sequence but with *V. nigripulchritudo* as the closest relative.

Analysis of the non-*Vibrio* Gammaproteobacteria isolates resulted in nine principal groups (Figure 4). A *Pseudoalteromonas* clade had 13 Lesser Electric Ray isolates ascribed to *P. phenolica* ($n = 7$), *P. prydzensis* ($n = 1$), and *P. spongiae* ($n = 4$). Two of the ray isolates could not be ascribed to named species. In two instances, a few ray isolates shared identical sequences but did not cluster with any reference strain. Five isolates within the *Shewanella* clade had a nonculturable bacterium as its closest neighbor and could not be assigned to any named species of *Shewanella*. Similarly, within the *Amphritea* clade, three isolates clustered separately from all known species of *Amphritea*.

The only two Actinobacteria recovered clustered along with *Micrococcus luteus* or *M. yunnanensis* and *Microbacterium hominis* (Figure 5). Two isolates of Bacteroidetes clustered with *Arenibacter nanhaiticus*, and another was the sister taxon to *Zhouia amylolytica*. The remaining two isolates were most similar to *Tenacibaculum* sp. but could not be ascribed to a named species.

DISCUSSION

It is well known that the blood of elasmobranchs, as in other vertebrates, harbors a diverse assemblage of parasites, including flagellates, amoebas, apicomplexans, microsporidians, and ciliates (see Goertz 2004 and references therein), as well as metazoan parasites (Bullard and Dippenaar 2003; Benz and Bullard 2004; Patella and Bullard 2013). Bacteria, while markedly less studied in elasmobranchs than the aforementioned symbionts, have been detected in various elasmobranch tissues, including in the blood, liver, muscle, and epithelium (Knight et al. 1987; Terrell 2004; Mylniczenko et al. 2007). Some of these bacteria have been identified as pathogens, e.g., *Vibrio harveyi* (as *V. carchariae*) (Grimes et al. 1984a, 1984b; Grimes et al. 1993; Pedersen et al. 1998), *Aeromonas salmonicida* (Briones et al. 1998), and *Flavobacterium* sp. (Terrell 2004), but many others are described as opportunistic pathogens or have not been associated with disease previously (Mylniczenko et al. 2007).

However, one should not assume an elasmobranch is diseased if its blood is infected with parasites or bacteria nor should one assume a link between the presence or absence of parasites and that of bacteria in the blood of elasmobranchs. Doubtless, ample plausible scenarios exist for how the blood of elasmobranchs can be exposed to bacteria, and there is insufficient data to conclude whether these bacteria are commensals or symbionts of the elasmobranchs or simply reflect transient bacteremias that course asymptomatically. In any case, we still lack a firm understanding of the taxonomic and phylogenetic diversity of bacteria that live in the blood of sharks and rays.

Most isolates recovered from Lesser Electric Rays belonged to the phylum Proteobacteria (91.5%), which is a result that is in agreement with previous reports (Horsley 1977; Grimes et al. 1985). As expected, several species of *Vibrio* were isolated, including *V. harveyi* (17% of all isolates) and *V. campbellii* (13%). These *Vibrio* spp. have been previously reported as part of the normal flora in sharks (Grimes et al. 1985). Conversely, we failed to recover any isolate ascribed to *V. alginolyticus*, a species also common in sharks (Grimes et al. 1993). Similarly, the common marine bacterium *Photobacterium damselae*, which has been isolated from the internal organs of healthy fish (Gomez-Gil et al. 2006; Mylniczenko et al. 2007), was not isolated during our study. These discrepancies could be a factor of host specificity, culture medium used for isolation, culture conditions, and habitat characteristics comprising the geographic locality where the Lesser Electric Rays were captured (Austin 2006). Species of *Pseudoalteromonas* and *Shewanella* were common in the blood of the Lesser Electric Rays studied herein, and isolates representing these genera frequently have been reported from fish; however, only in a few instances have they been isolated from viscera. Some of the lesser-known bacteria isolated in the present study include *Amphritea atlantica*, *Arenibacter nanhaiticus*, and *Zhouia amylolytica.* These species or their closest phylogenetic species were first discovered in marine sediments (Liu et al. 2006; Gartner et al. 2008; Miyazaki et al. 2008; Sun et al. 2010). Species of *Vibrio*, *Pseudoalteromonas*, and *Shewanella* have also been recovered from marine sediments (Urakawa et al. 2000; Holmstrom et al. 2006; Huang et al. 2010). Dean and Motta (2004) theorized that the suction feeding behavior of the Lesser Electric Ray facilitates the ingestion of sediment, and we think it is plausible that many bacteria would also be ingested during this feeding activity; however, we lack adequate behavior observations and microbial data to accept or reject this notion. The interstitial and benthic habitat of the Lesser Electric Ray could drive the taxonomic composition of the microbiota. Regardless, how bacteria enter the blood is unknown and also seemingly exceedingly difficult to test in an open, natural system. A comparison of the present results with those from a pelagic ray that is phylogenetically related to the Lesser Electric Ray may be informative along these lines.

The culture techniques used in this study likely underestimated the bacterial diversity of the tested samples since only 1–10% of all bacteria can be cultured under laboratory conditions (Amann et al. 1995). We chose a culture-based strategy because culture methods are still the "gold standard" in fish disease diagnostics laboratories (AFS–FHS 2014) and because the low cost associated with this approach makes it seemingly more accessible to a broader spectrum of researchers. The type of culture medium, even general media such as MA and BA, inadvertently can select for specific bacterial groups, and we observed this in the present study: species of *Pseudoalteromonas*, *Amphritea*, *Nautella*, *Arenibacter*, *Tenacibaculum*, and *Zhouia* were recovered on MA only; whereas, species of *Ferrimonas*, *Microbacterium*, *Micrococcus*, *Photobacterium*, and *Pseudomonas* were recovered on BA only.

In summary, the present study reported a high prevalence of bacteria in the blood of wild, apparently healthy Lesser Electric Rays. *Vibrio* spp. were found in all but one individual and

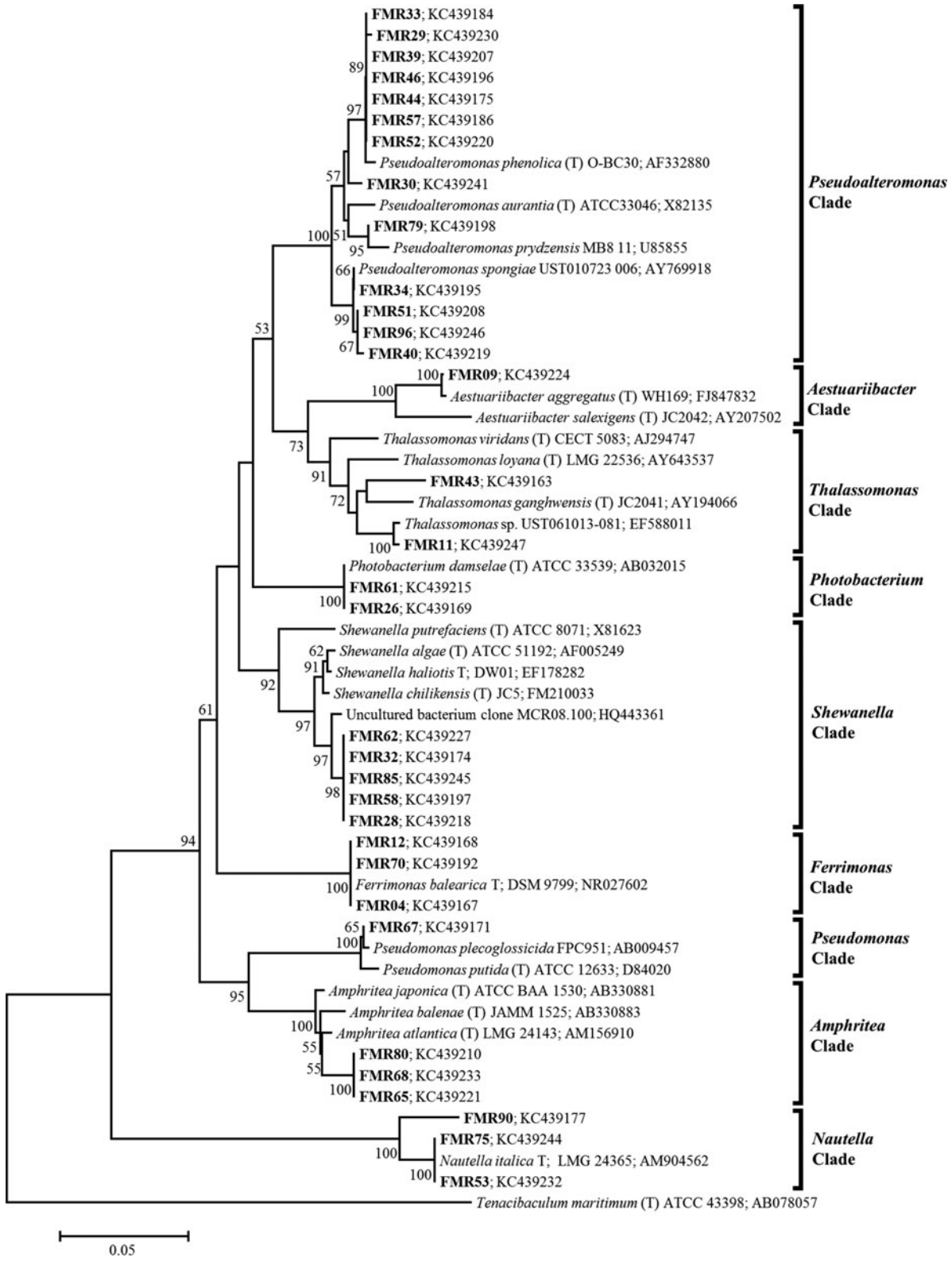

FIGURE 4. Phylogeny (partial 16S rRNA gene sequences) of bacterial isolates from the blood of Lesser Electric Rays and assigned as non-*Vibrio* Gammaproteobacteria species. The isolate number is followed by the GENBANK accession number. Sequences from type strains, or the closest match, were used for comparison. The tree topology was obtained by neighbor-joining methods (Jukes–Cantor correction). Each genus clade is indicated. The numbers at the nodes indicate bootstrap values (1,000 replicates). Scale bar = 5% sequence divergence.

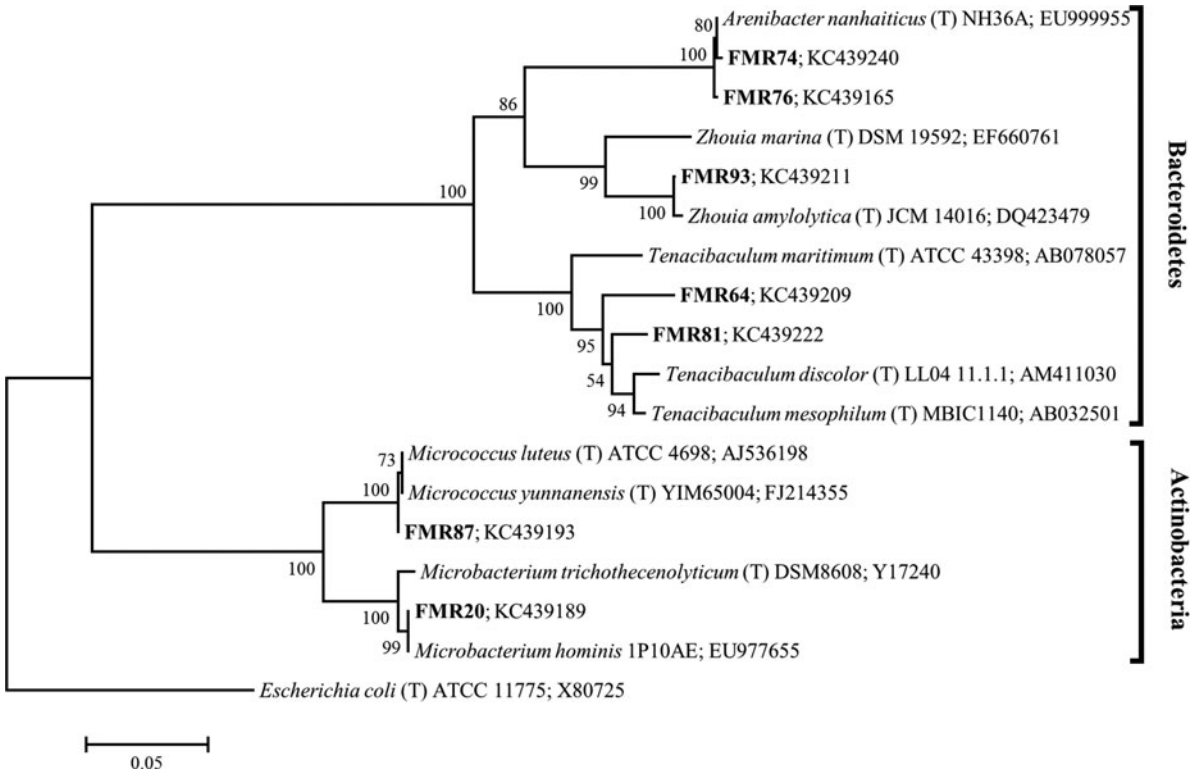

FIGURE 5. Phylogeny (partial 16S rRNA gene sequences) of bacterial isolates from the blood of Lesser Electric Rays and assigned as non-Gammaproteobacteria. The isolate number is followed by the GENBANK accession number. Sequences from type strains, or the closest match, were used for comparison. The tree topology was obtained by neighbor-joining methods (Jukes–Cantor correction). Each genus clade is indicated. The numbers at the nodes indicate bootstrap values (1,000 replicates). The scale bar represents 5% sequence divergence.

included opportunistic fish pathogens (*V. harveyi*) and potentially unnamed species (Figure 3, Clade III). Non-*Vibrio* Proteobacteria were also common and contained putative unnamed species within the *Shewanella* and *Amphritea* clades. These putative new species require further corroboration by full-length sequence of their 16S rRNA gene and additional taxonomic markers. Taken together, these insights on the elasmobranch microbiota are relevant to the fundamental ecology and evolutionary biology of aquatic symbioses. They are also vital to husbandry and veterinary staffers who are employed by the aquarium industry and tasked with keeping exhibited sharks and rays healthy, oftentimes following protocols that use blood culture as a means of assessing the overall health status of the exhibited elasmobranch.

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