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Novel Role for *Aeromonas jandaei* as a Digestive Tract Symbiont of the North American Medicinal Leech▽

Mark E. Siddall,[1]* Paul L. Worthen,[2] Matthew Johnson,[1] and Joerg Graf[2]

Invertebrate Zoology, American Museum of Natural History, New York, New York 10024,[1] and Department of Molecular and Cell Biology, University of Connecticut, Storrs, Connecticut 06268[2]

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The gut bacteria of the North American medicinal leech, *Macrobdella decora*, were characterized. Biochemical tests and DNA sequences indicated that *Aeromonas jandaei* is the dominant culturable symbiont in leeches from a broad geographic area. In this work we identified a new habitat for *A. jandaei*, and here we suggest that there is unexpected specificity between leeches and *Aeromonas* species.

Symbiotic bacteria of leeches recently have been the subject of studies to determine both the specific identities of microbes and the nature of the symbiotic relationships (9). A particularly interesting model symbiosis is the symbiosis between European medicinal leeches and their gut symbiont, *Aeromonas veronii* biovar sobria (8, 9), which was once considered a unique species, *Pseudomonas hirudinis* (4). *A. veronii* biovar sobria is the single cultured symbiont with clinical significance residing in the crop of the digestive tract with an uncultured member of the *Bacteroidetes* (30). It is unclear how widespread *Aeromonas* species are in the guts of different leech species.

The potential for aeromonad infections due to postoperative leech use was quickly recognized (29), and concern for appropriate prophylaxis with third-generation cephalosporins soon followed this recognition (11). Meanwhile, following the use of medicinal leeches, the digestive tract symbiont has been implicated in cellulitis and loss of replanted tissue (7, 15), as well as septicemia and meningitis (6, 19). The incidence of such infections can be reduced by preemptive antibiotic treatment.

European medicinal leeches in the genus *Hirudo* are not the only leeches being used for the relief of venous congestion. In Asia, *Hirudinaria manillensis* is more commonly encountered, whereas *Aliolimnatis michaelseni* is the leech of choice in South Africa (2, 27). The dominant gut symbiont of leeches was reported by Mackay et al. to be *Aeromonas caviae*, not *A. veronii* biovar sobria (16), but considering the difficulty of accurately identifying environmental *Aeromonas* isolates to the species level with biochemical tests, this result should be considered preliminary. Here, we investigated the aeromonad gut flora of the common North American medicinal leech, *Macrobdella decora*, a species often encountered in freshwater environments by swimmers and anglers across North America (14).

Isolates. Leeches (*M. decora*) were collected from four localities using the traditional method of wading into water bare legged and retrieving them either with a dip net as they approached or after they attached to bare skin but prior to the onset of blood feeding. These localities were Broadwing Lake, Ontario, Canada (45°35′50″N, 78°31′42″W); Douglas Lake, MI (45°34′49″ N, 84°40′12″W); a pond in Storrs, CT (41°49′2.80″N, 72°15′32.12″W); and Horseshoe Pond in Chester, VT (43°14′19.27″N, 72 34′22.39″W). European medicinal leeches were obtained from LeechesUSA (Westbury, NY). Leeches were rinsed with distilled water and washed with bleach. A longitudinal incision was made in the ventral surface, and intraluminal fluid of the crop was collected and serially diluted in saline (0.85% NaCl). Intraluminal fluid dilutions were streaked onto sheep blood agar (Becton-Dickinson, Sparks, MD) using sterile swabs and incubated aerobically at 30°C. Multiple colonies were subcultured on blood agar for isolation.

Amplification, sequencing, and phylogenetic analyses. DNA was isolated from luminal contents of the crop ceca of leeches using a DNeasy tissue kit (QIAGEN Inc., Valencia, CA). A portion of the 16S rRNA gene was amplified using universal primers AGAGTTTGATCCTGGCTCAG and ATTACCGC GGCTGCTGGC and a cycling program consisting of 94°C for 4 min and then 35 cycles of 94°C for 15 s, 57°C for 15 s, and 72°C for 30 s, followed by 72°C for 7 min. A portion of the *gyrB* locus was amplified using specific primers TGTTGCTGACC ATTCGTCGTAAC and TTGGCATCGCTCGGGTTTTC and a cycling program consisting of 94°C for 4 min and then 35 cycles of 94°C for 15 s, 50°C for 15 s, and 72°C for 30 s, followed by 72°C for 7 min. For all amplifications we used Ready-To-Go PCR beads (Amersham Pharmacia Biotech, Piscataway, NJ), 0.5 μl of each primer at a concentration of 10 μM, 1 μl DNA template, and 23 μl RNase-free H_2O. Products were sequenced in both directions. Each sequencing reaction mixture contained 1 μl BigDye (Applied Biosystems, Perkin-Elmer Corporation), 1 μl of primer at a concentration of 1 μM (a single primer was used for each direction), and 3 μl of DNA template, and the reaction was performed by using 40 cycles consisting of 96°C for 15 s, 50°C for 30 s, and 60°C for 4 min. Sequences were purified by ethanol precipitation and electrophoresed in an ABI Prism 3730 sequencer (Applied Biosystems). Sequences of complementary strands were edited and reconciled using CodonCode Aligner (CodonCode, Dedham, MA). In addition to sequences obtained from direct sequencing of DNA from freshly collected specimens, *gyrB* data were obtained from GenBank for taxa as described in previous

* Corresponding author. Mailing address: Division of Invertebrate Zoology, American Museum of Natural History, Central Park West at 79th Street, New York, NY 10024. Phone: (212) 769-5638. Fax: (212) 769-5277. E-mail: siddall@amnh.org.

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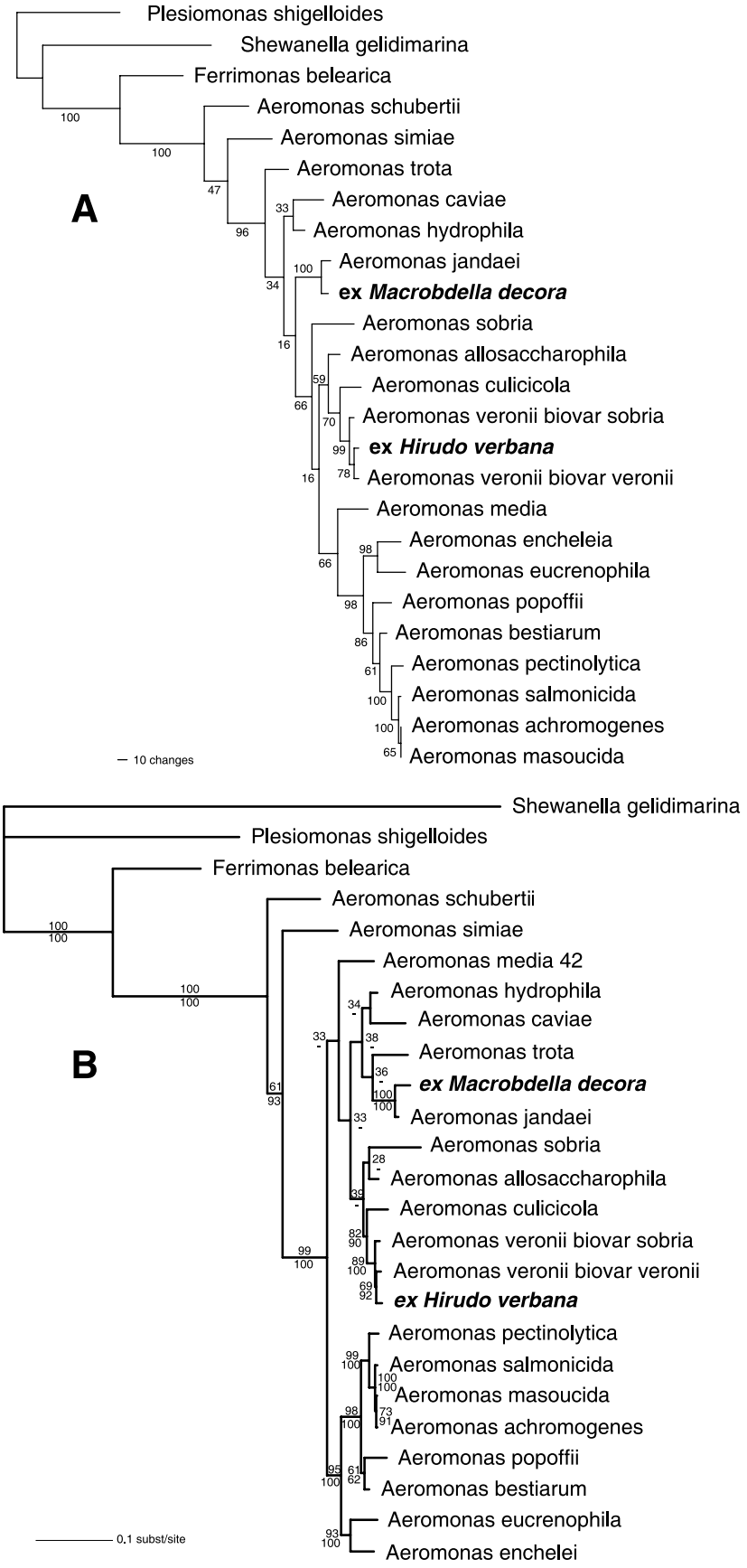

FIG. 1. Results of phylogenetic analyses. (A) Tree with bootstrap support values resulting from parsimony analysis. (B) Tree with the highest likelihood, including both bootstrap support values (upper numbers at the nodes) and Bayesian clade credibility values (lower numbers at the nodes).

TABLE 1. Biochemical tests distinguishing *M. decora* crop isolates from selected *Aeromonas* spp.

Test	*M. decora* isolates	*Aeromonas hydrophila*[a]	*Aeromonas trota*[a]	*Aeromonas eucrenophila*[a]	*Aeromonas jandaei*[a]	*Aeromonas veronii* bv. sobria[a]	*Aeromonas caviae*[a]
Voges-Proskauer	−[b]	+	−	−	d	d	−
Lysine decarboxylase	−	+	+	−	+	+	−
Orthinine decarboxlyase	−	−	−	−	−	−	−
Arginine dehydrolase	+	+	+	d	+	+	+
Esculin hydrolysis	+	+	−	d	−	−	d
Gas from D-glucose	+	+	d	d	+	+	−
L-Arabanose fermentation	+	d	−	d	−	−	+
Sucrose fermentation	−	+	d	d	−	+	+
D-Mannitol fermentation	+	+	d	+	+	+	+
Citrate utilization	−	d	+	−	d	d	d
Hemolysis	+	+	d	d	+	+	d

[a] Biochemical tests for *Aeromonas* species (1).
[b] −, <10% positive; +, >90% positive; d, 11 to 89% positive.

analyses (25, 31) and for three outgroup taxa. The *gyrB* sequences required 12-nucleotide insertion/deletion sites. These sites corresponded to one amino acid insertion for *Aeromonas simiae*, another amino acid insertion shared by nine *Aeromonas* species, and two amino acid indels for only the outgroup taxa.

Parsimony analyses were conducted with PAUP* (26). ModelTest (21) suggested a GTR+I+Γ nucleotide substitution model for *gyrB*. Maximum likelihood analyses were conducted with the separate data sets using PhyML (10). The Bayesian method was employed with MrBayes (12) for 1,000,000 generations (the last 500,000 generations of which were used for clade credibility values).

Amplification of the 16S rRNA gene and *gyrB* generated sequenced fragments up to 603 and 717 bp long, respectively. PCR amplicons obtained from *M. decora* were identical to each other at both loci regardless of the geographic origin. The 16S rRNA genes of the isolates were identical to 16S rRNA gene of *Aeromonas jandaei* strain ATCC 49568 (GenBank accession no. X74678). The *gyrB* sequence obtained from the crop contents of *M. decora* corroborated this identification by most closely matching the *A. jandaei* sequence (GenBank accession no. AJ868391). In contrast, the *gyrB* sequence obtained for isolates from European medicinal leeches most closely matched that obtained for *A. veronii* strain MTCC 3249 substrain SH (GenBank accession no. AY130993; originally described as *Aeromonas culicicola* before more recent phylogenetic work [22]).

Parsimony analysis of *gyrB* yielded one tree with a length of 1,136 steps and a retention index of 0.591 (Fig. 1A). Maximum likelihood analysis also generated a single tree with a log(L) value of −6397.199 for *gyrB* (Fig. 1B). Notably, the isolates from *M. decora* grouped together with *A. jandaei* with high support values in all analyses. As expected, the isolates from the European medicinal leech were identified as *A. veronii*.

Phenotypic tests. Bacteria were successfully cultured from two *M. decora* individuals, and five isolates were characterized further using biochemical tests as described previously (1, 8). Only colonies resembling *Aeromonas* colonies were observed after 48 h. The sensitivities of three isolates to antibiotics were evaluated using Sensi-Discs (Becton-Dickinson and Company, Sparks, MD). All isolates were sensitive to cefotaxime (30 μg), cefuroxime (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), nalidixic acid (30 μg), and trimethoprim-sulfamethoxazole (1.25/23.75 μg). One of the isolates was resistant to cephalothin (30 μg), and another isolate exhibited intermediate resistance, indicating that cephalothins are not an appropriate choice for antibiotic therapy. The results of biochemical tests for five isolates were identical. Consistent results (positive or negative for >90% of colonies) favored identification as either *Aeromonas hydrophila*, *A. jandaei*, or *A. caviae* (Table 1). Overall, the inability to utilize citrate was shared only with *Aeromonas eucrenophila*. Overall, the failure to ferment sucrose was shared only with *A. jandaei*. Together, our data are consistent with the hypothesis that *A. jandaei* is the dominant culturable symbiont of the North American medicinal leech.

It seems remarkable that two ecologically similar leech species contain distinct *Aeromonas* species as the dominant culturable bacterial symbionts in the gut lumen, with consistency across the geographic ranges of the leeches. Both *A. jandaei* and *A. veronii* are ubiquitous and global in terms of their known distributions (5, 28) and even have been found coinfecting the same wound (13). There seems to be little reason to contemplate that there is a geographic or ecological barrier excluding either species of symbiont from either species of leech. Like the European leech symbiont, *A. jandaei* has been implicated in several pathological cases, usually involving the exposure of wounds to a freshwater environment (13, 23, 28), although its involvement is less common than the involvement of other aeromonads.

The phylogenetic results obtained here are in agreement (where support values are strong) with those obtained previously for this group of bacteria (25, 31). Whereas genetic and phylogenetic characterization of the gut symbiont of *M. decora* was clear, like the previous characterization of *Aeromonas* from *Hirudo* species, the biochemical results did not agree unambiguously with the previously published biochemical results for any single *Aeromonas* species. Such difficulties in identifying environmental *Aeromonas* strains have been reported previously (18) and may reflect the source of most of the characterized isolates or perhaps the possibility that a different subset of strains inhabits leeches.

M. decora, although not yet used clinically, produces a useful platelet aggregation inhibitor, decorsin (24), and it belongs to

an evolutionary lineage that is distinct from the Old World medicinal leeches (3). Moreover, it is a widespread, commonly encountered species in freshwater environments, where its typical hosts, besides the occasional human, are frogs and fish. Different species of *Aeromonas* have different susceptibilities to available antimicrobial agents (17, 28). Notably, *A. jandaei* may be the most resistant species of *Aeromonas* both in terms of the extent of multiple-antibiotic resistance and in terms of the frequency with which such resistance is found in clinical isolates (20). The spectrum of *Aeromonas* species dominating the gut lumen of various leeches commonly encountered around the world deserves closer scrutiny, particularly for the leech species that are used locally for the relief of venous congestion or simple hematomas.

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