Novel role for *Aeromonas jandaei* as digestive-tract symbiont of North American medicinal leeches.

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The gut bacteria of 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 range. This identifies a new habitat for *A. jandaei* and suggests an unexpected specificity between leeches and *Aeromonas* species.

Symbiotic bacteria of leeches recently have been the subject of studies characterizing both the specific identities of microbes as well as the nature of the symbiotic relationships (9). A particularly interesting model symbiosis exists between European medicinal leeches and their gut symbiont, *Aeromonas veronii* biovar sobria (8, 9), once considered a unique species. *Pseudomonas hirudinis* (4). *Aeromonas veronii* biovar sobria constitutes 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 widely spread the presence of *Aeromonas* species is in the gut of different leech species.

The potential for aeromonad infections pursuant to postoperative leech use was quickly recognized (29) and concern for appropriate prophylaxis with third generation cephalosporins soon followed (11). Meanwhile, following use of medicinal leeches, the digestive-tract symbiont has been implicated in cellulitis, loss of replanted tissue (7, 15) as well as septicaemia and meningitis (6, 19). The incidence of such infections can be reduced with a preemptive antibiotic treatment.
European medicinal leeches in the genus *Hirudo*, are not alone in 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 was reported 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, these results should be considered preliminary. Here, we investigate 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 by dip-net as they approach or after they attach to bare skin but prior to the onset of bloodfeeding. These localities were: Broadwing Lake, Ontario, Canada (45 35' 50"N, 78 31' 42"W), Douglas Lake, Michigan (45 34' 49" N, 84 40' 12" W), a pond in Storrs, Connecticut (41 49' 2.80" N, 72 15' 32.12" W) and Horseshoe Pond, Chester, Vermont (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, intraluminal fluid (ILF) of the crop was collected and serially diluted in saline (0.85% NaCl). ILF dilutions were streaked onto Sheep Blood Agar (Benton-Dickenson, 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 the DNeasy Tissue Kit (QIAGEN Inc. Valencia, California). A portion of the 16S rDNA was amplified using universal primers AGAGTTTGATCCTGGCTCAG and ATTACCGCGGCTGCTGGC and a cycling program of 94° for 4 min, 35 cycles of 94° for 15 sec, 57° for 15 sec, and 72° for 30 sec, followed by 72° for 7 min. A portion of the gyrB locus was amplified using specific primers TGTTGCTGACCATTGCTGTAAC and TTGGCATCGCTCGGGTTTTC and a cycling program of 94° for 4 min, 35 cycles of 94° for 15 sec, 50° for 15 sec, and 72° for 30 sec, followed by 72° for 7 min. All amplification used Ready-To-Go™ PCR Beads (Amersham Pharmacia Biotech, Piscataway, NJ), 0.5 µL of each 10 µM primer, 1 µL DNA template and 23 µL RNase-free H₂O. Products were sequenced in both directions. Each sequencing reaction, including 1 µL BigDye (Applied Biosystems, Perkin-Elmer Corporation), 1 µL of 1 µM primer (single primer for each direction), and 3 µL of DNA template, ran for 40 cycles of 96˚C (15 sec), 50˚C (30 sec), and 60˚C (4 min). Sequences were purified by ethanol precipitation and electrophoresed in an ABI Prism 3730 sequencer (Applied Biosystems). Sequences of complimentary strands were edited and reconciled using CodonCode Aligner (CodonCode, Dedham, Massachusetts, USA). In addition to sequences obtained from direct DNA sequencing from freshly collected specimens, gyrB data were obtained from GenBank for taxa as detailed in previous analyses (25, 31), and for 3 outgroup taxa. The gyrB sequences required 12 nt insertion/deletion sites. These corresponded to one amino acid insertion for *Aeromonas simiae*, another amino acid insertion shared by 9 *Aeromonas* species and two amino acid indels concerning only the outgroup taxa.
Parsimony analyses were conducted with PAUP* (26). ModelTest (21) suggested a GTR+I+Γ nt substitution model for gyrB. Maximum likelihood analyses were conducted on the separate data sets with PhyML (10). The bayesian method was employed with MrBayes (12) for 1,000,000 generations (of which the last 500,000 generations were used for clade credibility values).

Amplification of 16S rDNA and gyrB generated sequenced fragments of up to 603 and 717 bp respectively. PCR amplicons obtained from *M. decora* were identical to each other at both loci regardless of geographic origin. The 16S rDNA from these isolates was identical to that from *Aeromonas jandaei* strain ATCC 49568 (GenBank X74678). The gyrB sequence obtained from the crop contents of *M. decora* corroborated this identification by most closely matching *A. jandaei* (GenBank AJ868391). In contrast, the gyrB sequence obtained from isolates of European medicinal leeches most closely matched that for *Aeromonas veronii* strain MTCC 3249 substrain SH [GenBank AY130993 – originally described as *A. culicicola* in advance of 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 sister to *A. jandaei* with high support values in all analyses. As expected, the isolates from the European medicinal leech indicated were identified as *A. veronii*.

**Phenotypic Tests.** Bacteria were successfully cultured from two *M. decora* and five isolates were further characterized using biochemical tests as described previously (1, 8). Only colonies resembling *Aeromonas* were observed after 48 hr. The sensitivity of
three isolates to antibiotics was 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), gentamycin (10 µg), naladixic acid (30 µg) and trimethoprim/sulfamethoxazole (1.25 / 23.75 µg). One of the isolates was resistant to cephalothin (30 µg) and another one exhibited intermediate resistance, indicating that cephalothins would not be an appropriate choice for antibiotic therapy.

Results of biochemical tests of five isolates were identical. Consistent results (+ or – for > 90% of colonies) favored identification as either A. hydrophila, A. jandaei or A. caviae (Table 1). Overall inability to utilize citrate was shared only with A. eucrenophila. Overall failure to ferment sucrose was shared only with A. jandaei. Together our data are consistent with A. jandaei as the dominant culturable symbiont of the North American medicinal leech.

It seems remarkable that two ecologically similar leech species retain distinct Aeromonas species as the dominant culturable bacterial symbiont in their gut lumen, and with such consistency across the geographic range of the leeches. Aeromonas jandaei and A. veronii each are ubiquitous and global in terms of their known distributions (5, 28) even having been found co-infecting the same wound (13). There would seem to be little reason to contemplate 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), though its involvement is less common than other aeromonads.
The phylogenetic result obtained here is in agreement (where support values are strong) with those found previously for this group of bacteria (25, 31). Whereas genetic and phylogenetic characterization of the gut symbiont of *M. decora* was clear, like previous characterization of *Aeromonas* from *Hirudo* species, biochemical results did not agree unambiguously with the 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 that a different subset of strains inhabits leeches.

*Macrobdella decora*, though not yet clinically employed, produces a useful platelet aggregation inhibitor, decorсин (24), and 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 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, in particular in those species of leech that are used locally for the relief of venous congestion or simple haematomas.

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Table 1: Biochemical Tests distinguishing *Macrobdella decora* crop isolates from selected *Aeromonas* spp.

<table>
<thead>
<tr>
<th>Test</th>
<th><em>M. decora</em> isolates</th>
<th><em>Aeromonas hydrophila</em>&lt;sup&gt;a&lt;/sup&gt;</th>
<th><em>Aeromonas trota</em>&lt;sup&gt;a&lt;/sup&gt;</th>
<th><em>Aeromonas eucrenophila</em>&lt;sup&gt;a&lt;/sup&gt;</th>
<th><em>Aeromonas jandaei</em>&lt;sup&gt;a&lt;/sup&gt;</th>
<th><em>Aeromonas veronii</em> bv. <em>sobria</em>&lt;sup&gt;a&lt;/sup&gt;</th>
<th><em>Aeromonas caviae</em>&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Voges-Proskauer</td>
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<tr>
<td>Lysine decarboxylase</td>
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<td>+</td>
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<td>Orthinine decarboxylase</td>
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<td>Arginine Dehydrolyase</td>
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<td>d</td>
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<td>Esculin hydrolysis</td>
<td>+</td>
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<td>-</td>
<td>d</td>
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<td>Gas from D-glucose</td>
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<td>L-Arabanose Fermentation</td>
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<td>Sucrose Fermentation</td>
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<tr>
<td>D-Mannitol Fermentation</td>
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<td>Citrate Utilization</td>
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<td>Hemolysis</td>
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Symbols: +, >90% positive results; -, <10% positive results; d, 11-89% positive results.

<sup>a</sup> Biochemical tests for *Aeromonas* species (1).
Figure legend

Figure 1. The results of phylogenetic analyses are illustrated (A) with bootstrap support values from parsimony analysis as well as (B) the tree with the highest likelihood including both bootstrap support values (upper number on nodes) and Bayesian clade credibility values (lower number on nodes).