Members of the phylum Bacteroidetes are commonly found in the digestive tracts of humans and a wide range of animals, and have been shown to influence host nutrition and the development and regulation of host immune responses (An et al., 2014; Comstock, 2009; Leser & Molbak, 2009). The family Rikenellaceae is a member of the order Bacteroidales, phylum Bacteroidetes, for which only three genera are currently recognized, namely Rikenella, Anaerocella and Alistipes (http://www.bacterio.net/; Graf, 2014). The type species of the type genus of the family, Rikenella microfusus, was isolated from a Japanese quail and initially identified as a member of the genus Bacteroides before being reclassified within its own genus and family on the basis of later phenotypic and molecular biological data (Collins et al., 1985; Kaneuchi & Mitsuoka, 1978). Similarly, the type species of the genus Alistipes, Alistipes putredinis, was initially identified as a member of the genus Bacteroides before later being reclassified within its own genus (Rautio et al., 2003). The reclassification of the type strains of the genera Rikenella and Alistipes as representatives of unique genera was necessary due to an historical artefact whereby all anaerobic Gram-stain-negative bacteria were assigned to the genera Bacteroides or Fusobacterium when these strains were first isolated and characterized. To date, only eight species belonging to the family Rikenellaceae have been described and formally recognized. An additional genus, ‘Ruminofilibacter’, has been reported in various studies to belong to the family Rikenellaceae (Kröber et al., 2009; Liu et al., 2009; Siddall et al., 2011; Weiss et al., 2011), and is listed as such within the NCBI Taxonomy database (http://www.ncbi.nlm.nih.gov/taxonomy), but no strains from this genus have been formally described and the name has no current official standing within prokaryotic taxonomy.

The medicinal leech Hirudo verbana is an obligate blood-feeding annelid with a digestive tract divided into two main compartments (Sawyer, 1986). Consumed blood meals are stored within the crop, which comprises the majority of the digestive tract, and erythrocytes are slowly passed into the much smaller intestinum for digestion and nutrient absorption (Graf et al., 2006; Nelson & Graf, 2012; Sawyer, ...

Mucinivorans hirudinis gen. nov., sp. nov., an anaerobic, mucin-degrading bacterium isolated from the digestive tract of the medicinal leech Hirudo verbana

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Three anaerobic bacterial strains were isolated from the digestive tract of the medicinal leech Hirudo verbana, using mucin as the primary carbon and energy source. These strains, designated M3T, M4 and M6, were Gram-stain-negative, non-spore-forming and non-motile. Cells were elongated bacilli approximately 2.4 µm long and 0.6 µm wide. Growth only occurred anaerobically under mesophilic and neutral pH conditions. All three strains could utilize multiple simple and complex sugars as carbon sources, with glucose fermented to acid by-products. The DNA G+C contents of strains M3T, M4 and M6 were 44.9, 44.8 and 44.8 mol%, respectively. The major cellular fatty acid of strain M3T was iso-C15:0. Phylogenetic analysis of full-length 16S rRNA gene sequences revealed that the three strains shared >99 % similarity with each other and represent a new lineage within the family Rikenellaceae of the order Bacteroidales, phylum Bacteroidetes. The most closely related bacteria to strain M3T based on 16S rRNA gene sequences were Rikenella microfusus DSM 15922T (87.3 % similarity) and Alistipes finegoldii AHN 2437T (87.4 %). On the basis of phenotypic, genotypic and physiological evidence, strains M3T, M4 and M6 are proposed as representing a novel species of a new genus within the family Rikenellaceae, for which the name Mucinivorans hirudinis gen. nov., sp. nov. is proposed. The type strain of Mucinivorans hirudinis is M3T (=ATCC BAA-2553T=DSM 27344T).
1986). Initial non-culture-based studies found that the *H. verbana* crop and intestine are inhabited by two primary bacterial symbionts, a species of the genus *Aeromonas* and a previously uncultured member of the phylum *Bacteroidetes* related to *R. microfusus* identified as clone PW3 (Maltz et al., 2014; Worthen et al., 2006). Our initial attempts to isolate the *Rikenella*-like bacterium anaerobically using culture media designed for the enrichment and isolation of other members of the phylum *Bacteroidetes* failed to recover the *Rikenella*-like symbiont. A meta-transcriptomic analysis of the leech crop suggested that 42 h after host feeding, the *Rikenella*-like bacterium was both highly abundant in the crop and possibly foraging on host mucus as a primary nutrient and energy source (Bomar et al., 2011). Based on this observation, a modification of standard Eggerth–Gagnon (EG) agar was used, replacing glucose with bovine submaxillary gland mucin (2 g l\(^{-1}\)) as the primary carbon and energy source.

To test the effectiveness of this medium in recovering the *Rikenella*-like symbiont, a single *H. verbana* was fed sterile sheep’s blood 42 h prior to being killed and immediate dissection in an anaerobic chamber with a gas atmosphere composition of 85 % N\(_2\), 10 % CO\(_2\), and 5 % H\(_2\). The intraluminal fluid of the crop was serially diluted in anaerobic 0.85 % NaCl and plated on the modified EG agar plates. The plates were then incubated anaerobically at 25 °C for up to 14 days, after which isolated colonies were subcultured onto fresh plates for further identification and characterization. One abundant colony type, small and grey with whitish centres, was positively identified as being the *Rikenella*-like bacterium through diagnostic PCR and sequencing of the full-length 16S rRNA gene (Bomar et al., 2011). Cryopreservation of isolates using traditional glycerol stocks failed, but isolates could be preserved and revived from 15 % skimmed milk stocks according to the procedure of Bacic & Smith (2005). After confirmation of the identity of strain M3\(^{T}\) as the *Rikenella*-like bacterium, two additional strains, M4 and M6, were isolated from the crop of a second leech on similarly modified EG agar, with porcine gastric mucin used in place of bovine submaxillary gland mucin (referred to as EG-PM medium).

Cell morphology and the Gram reaction were determined from one-week-old cells growing on EG-PM agar by bright-field microscopy. All three strains were Gram-stain-negative and exhibited elongated bacillus-shaped cell morphologies. Cells of strain M3\(^{T}\) from a slightly turbid, one-week-old 5 ml liquid culture in API 20A medium were used for negative-stain transmission electron microscopy imaging by the University of Connecticut’s Bioscience Electron Microscopy Laboratory for the calculation of mean cell size. Briefly, 3 μl aliquots of culture were fixed onto carbon-coated grids and stained with 0.25 or 0.5 % uranyl acetate before imaging with an FEI Tecnai G2 Biotwin transmission electron microscope. Cells were 1.4–3.4 μm (mean 2.4 μm) in length and 0.4–1.1 μm (mean 0.6 μm) in width (Fig. S1, available in the online Supplementary Material).

To test for carbon substrate utilization, a further modification of the culture medium was required as none of the strains grew reliably or sufficiently in liquid media for testing with commercial kits, and all three strains were able to grow on EG agar containing blood but with no additional carbon source added. None of the strains was able to grow on EG-PM agar without the addition of sheep’s blood, although growth did occur after replacement of the blood with a haemin–vitamin K1 solution [5 and 2.5 mg l\(^{-1}\)] respective final concentrations, prior to carbon source addition (referred to as EG-H medium). Growth of all three strains was tested in duplicate on EG-H agar containing various carbon substrates added at 0.8 % final concentration in place of mucin. Fermentation of glucose and mucin to acid by-products was tested by the addition of bromocresol purple to the medium at a final concentration of 0.04 % and visual observation of changes in colour of the medium from purple to yellow indicating a drop in pH. Sensitivity to bile was determined by the addition of 2 % bile salts (Fisher Scientific) to modified EG-H agar containing glucose as the carbon source. Antibiotic resistance to rifamycin (10 μg ml\(^{-1}\)) and kanamycin (1 mg ml\(^{-1}\)) was tested on modified EG-H agar containing glucose. The range of temperatures capable of supporting cell growth was tested on EG-H agar containing glucose prepared as slants in sealed 50 ml serum vials with the headspace gas composition as for the anaerobic chamber as described above.

All three strains were able to grow on modified EG-H agar containing glucose, sucrose, maltose, cellobiose, raffinose, sorbitol, inulin, glycerol, chitin and N-acetylglucosamine as the primary carbon substrates. Growth was not observed for any of the strains on modified EG-H agar containing arabinose. None of the three strains produced acid by-products after 2 weeks when grown on EG-H agar containing mucin, but acid by-products were produced when cells were grown on EG-H agar containing glucose. None of the three strains was resistant to 2 % bile acids and all three were sensitive to rifamycin and kanamycin. Growth occurred at temperatures ranging from 17 to 30 °C, but not at 4 or 35 °C and above.

The cellular fatty acid composition of strain M3\(^{T}\) strain was determined by the Identification Service of the DSMZ, Braunschweig, Germany. According to the DSMZ’s standard protocol, fatty acid methyl esters were obtained from 40 mg of cells scraped from a 48 h lawn culture growing on EG-PM agar by saponification, followed by methylation and extraction using modifications of the protocols of Miller (1982) and Kuykendall et al. (1988). Analysis and detection of fatty acid methyl esters was conducted by GC using the Sherlock Microbial Identification System (MIDI) with automated peak calling, integration and identification. The major cellular fatty acids identified were iso-C\(_{15:0}\) (49.9 %) and iso-C\(_{17:0}\) 3-OH (20.2 %). The complete cellular fatty acid profile of strain M3\(^{T}\), as well as those of related species, is given in Table S1.
Total genomic DNA was isolated from all three strains using the Epicentre MasterPure DNA Purification kit. The nearly full-length 16S rRNA gene was PCR-amplified using primers 27F/1492R (Lane, 1991) and sequenced on an ABI 3330xl Genetic Analyzer. Whole genome sequencing and assembly for strain M3T was conducted by Pacific Biosciences to produce the complete, circularized genome (Nelson et al., 2015). Whole genome shotgun libraries for strains M4 and M6 were prepared using a Nextera XT DNA sample preparation kit, sequenced for 250 bp paired-end on an Illumina MiSeq yielding >80× coverage, and assembled de novo using CLC Genomics Workbench (version 6).

Analysis of the Sanger-sequenced 16S rRNA gene from each of the three strains revealed >99% nucleotide similarity when compared with each other, and 92.5% similarity with the sequence of the PW3 clone. For phylogenetic analysis, the full-length 16S rRNA gene for all three strains, as well as the type strains of the seven recognized species of the family Rikenellaceae and selected type strains of other species within the order Bacteroidales were identified from the available genome assemblies by BLAST searches (Altschul et al., 1990), except for Anaerocella delicata WN081T, for which the reported 16S rRNA gene sequence in GenBank was used. The sequences collected along with that of the PW3 clone were aligned using the program MUSCLE (Edgar, 2004) and the alignment was then manually trimmed to approximately 1400 nt, corresponding to the regions between the 27F and 1492R primer sites. A consensus phylogenetic tree was reconstructed using PHYML with 1000 bootstrap replicates to determine the phylogenetic relationships of the three strains within the family Rikenellaceae and the order Bacteroidales overall. The resulting consensus tree showed that strains M3T, M4 and M6 formed a monophyletic group with the PW3 clone within the family Rikenellaceae, distinct from the other three recognized genera (Fig. 1). The DNA G+C contents of strains M3T, M4 and M6 as determined from the genome assemblies were 44.9, 44.9 and 44.8 mol%, respectively (Nelson et al., 2015).

**Fig. 1.** Maximum-likelihood tree showing the phylogenetic position of strains M3T, M4, and M6 in relation to known strains of the family Rikenellaceae and selected members of the order Bacteroidales based on 16S rRNA gene sequence data. Bootstrap values from 1000 replicates are shown at node branch points. Accession numbers for reference genomes or GenBank records from which sequences were obtained are given in parentheses. Bar, 0.07 changes per nucleotide position.
**Table 1.** Differential characteristics between strains M3\(^T\), M4 and M6 and related members of the family Rikenellaceae

Taxa: 1, strain M3\(^T\); 2, strain M4; 3, strain M6; 4, *Rikenella* (data from Goodfellow et al., 2011); 5, *Anaerocella* (Abe et al., 2012); 6, *Alistipes* (Nagai et al., 2010; Song et al., 2006). S, Sensitive; R, Resistant; NT, not tested.

<table>
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<th>Characteristic</th>
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<td><em>H. verbana</em> crop</td>
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<td><em>Elongated rods</em></td>
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<td><strong>Cell morphology</strong></td>
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<td>Resistance to:</td>
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<td><em>Bile salts (2%)</em></td>
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<td><em>Rifamycin (10 µg ml(^{-1}))</em></td>
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<td>NT</td>
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<td><em>Kanamycin (1 mg ml(^{-1}))</em></td>
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<td>s</td>
<td>s</td>
<td>NT</td>
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<td>r</td>
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<tr>
<td><strong>DNA G+C content (mol%)</strong></td>
<td>44.9</td>
<td>44.9</td>
<td>44.8</td>
<td>59.5-60.7</td>
<td>32.3</td>
<td>52-58</td>
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<td><strong>Major fatty acid (% of total)</strong></td>
<td>iso-C(_{15:0}) (50%)</td>
<td>iso-C(_{15:0}) (36%)</td>
<td>iso-C(_{15:0}) (70%)</td>
<td>iso-C(_{15:0}) (23-56%)</td>
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</table>

*Alistipes putredinis* is negative; all other species are positive.

†*Alistipes indistinctus* is negative; all other species are positive.

§*A. putredinis* and *A. indistinctus* are positive; all other species are negative.

§*A. putredinis* and *A. indistinctus* are susceptible; all other species are resistant.
This report describes three isolates obtained from the crop of the medicinal leech H. verbana. Phylogenetic analysis of the 16S rRNA gene placed these strains within the family Rikenellaceae but in a separate clade from the current genera Rikenella, Anaerocella and Alistipes. The DNA G+C contents for the three strains were significantly different from those of other strains within the family Rikenellaceae (Table 1). Phenotypic testing showed that all three strains were widely saccharolytic and able to ferment glucose to acid by-products, as with other members of the order Bacteroidales. On the basis of phenotypic and, in particular, phylogenetic differences, strains M3T, M4 and M6 are considered to represent a novel species of a new genus within the family Rikenellaceae, for which the name Mucinivorans hirudinis gen. nov., sp. nov. is proposed.

**Description of Mucinivorans gen. nov.**

*Mucinivorans* (Mu.ci.ni.vor’ans. N.L. neut. n. mucinum mucin; L. v. vorare to devour; N.L. masc. n. Mucinivorans mucin consumer).

Cells are Gram-stain-negative, rod-shaped, non-spore-forming and non-motile. Obligately anaerobic, mesophilic and neutrophilic. Growth is saccharolytic. Non-haemolytic. Catalase-negative. Does not produce indole. Cellular fatty acids are primarily iso-branched chains, with iso-C₁₅ : ₀ the primary component. The DNA G+C content is 44.8–44.9 mol%. The type species is *Mucinivorans hirudinis*.

**Description of Mucinivorans hirudinis sp. nov.**

*Mucinivorans hirudinis* (hi.ru’di.nis. L. n. hirudinis of the leech).

Exhibits the following characteristics in addition to those given for the genus. Cells are 1.4–3.4 µm (mean 2.4 µm) in length and 0.4–1.1 µm (mean 0.6 µm) in width. Growth occurs only on media supplemented with blood or a haemin-vitamin K₁ solution. Growth in liquid media is absent to minimal. Cells are able to utilize D-glucose, sucrose, maltose, D-mannose, D-galactose, cellobiose, raffinose, sorbitol, inulin, glycerol, chitin, N-acetylglucosamine and mucin as carbon sources. L-Arabinose is not used. Glucose is readily fermented to acid by-products, but mucin is not. Cells are sensitive to bile acids, rifamycin (10 µg ml⁻¹) and kanamycin (1 mg ml⁻¹). The cellular fatty acid profile consists of iso-C₁₅ : ₀, iso-C₁₇ : ₀ 3-OH, iso-C₁₅ : ₀ 3-OH, C₁₈:1ω9c,isoC₁₂G, iso-C₁₅ : ₀ aldehyde, iso-C₁₅ : ₀, C₁₈:1ω9c and C₁₆ : ₀. The type strain, M3T (=ATCC BAA-2553=DSM 27344ᵀ), was isolated from the crop of the medicinal leech *Hirudo verbana*. The DNA G+C content of the type strain is 44.9 mol%.

**Acknowledgements**

We thank Stephen Daniels of the University of Connecticut Bioscience Electron Microscopy Laboratory for his consultation and transmission electron microscopy. Sequencing and initial assembly of the M3T genome was conducted by Pacific Biosciences as part of their 2013 ASM SMRT Sequencing Solutions Grant Contest. This research was supported by NSF Career Award MCB 0448052 (J.G.) and NIH R01 GM095390 (J.G., Peter Visscher and Hilary G. Morrison).

**References**


