Listeria floridensis sp. nov., Listeria aquatica sp. nov., Listeria cornellensis sp. nov., Listeria riparia sp. nov. and Listeria grandensis sp. nov., from agricultural and natural environments

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Sampling of agricultural and natural environments in two US states (Colorado and Florida) yielded 18 Listeria-like isolates that could not be assigned to previously described species using traditional methods. Using whole-genome sequencing and traditional phenotypic methods, we identified five novel species, each with a genome-wide average BLAST nucleotide identity (ANIb) of less than 85% to currently described species. Phylogenetic analysis based on 16S rRNA gene sequences and amino acid sequences of 31 conserved loci showed the existence of four well-supported clades within the genus Listeria; (i) a clade representing Listeria monocytogenes, L. marthii, L. innocua, L. welshimeri and L. ivanovii, which we refer to as Listeria sensu stricto, (ii) a clade consisting of Listeria fleischmannii and two newly described species, Listeria aquatica sp. nov. (type strain FSL S10-1188T = DSM 26686T = LMG 28120T = BEI NR-42633T) and Listeria floridensis sp. nov. (type strain FSL S10-1187T = DSM 26687T = LMG 28121T = BEI NR-42632T), (iii) a clade consisting of Listeria rocourtiae, L. weihenstephanensis and three novel species, Listeria cornellensis sp. nov. (type strain TTU A1-0210T = FSL F6-0969T = DSM 26689T = LMG 28123T = BEI NR-42630T), Listeria grandensis sp. nov. (type strain TTU A1-0212T = FSL F6-0971T = DSM 26688T = LMG 28122T = BEI NR-42631T) and Listeria riparia sp. nov. (type strain FSL S10-1204T = DSM 26685T = LMG 28119T = BEI NR-42634T) and (iv) a clade containing Listeria grayi. Genomic and phenotypic data suggest that the novel species are non-pathogenic.

Abbreviations: AAI, average amino acid identity; ANIb, BLAST average nucleotide identity; MR, methyl red; PI-PLC, phosphoinositide phospholipase C; VP, Voges–Proskauer.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains TTU A1-0210T, TTU A1-0212T, FSL S10-1188T, FSL S10-1189T and FSL S10-1204T are JX961634, JX961635, JX961636, JX961637 and JX961638, respectively. The accession numbers for the genome sequences of strains TTU A1-0210T, TTU A1-0212T, FSL S10-1188T, FSL S10-1189T and FSL S10-1204T are AODE00000000, AODD00000000, AODF00000000, AOCG00000000 and AODL00000000, respectively; the versions described in this paper are the first versions, AODE01000000, AODD01000000, AODF01000000, AOCG01000000 and AODL01000000. Five supplementary figures and five supplementary tables are available with the online version of this paper.
The genus *Listeria* was described by Pirie (1940), and at present comprises 10 recognized species, *Listeria monocytogenes* (Pirie, 1940), *Listeria grayi* (Errebo Larsen & Seeliger, 1966), *Listeria innocua* (Seeliger, 1981), *Listeria welshimeri* (Rocourt & Grimont, 1983), *Listeria seeligeri* (Rocourt & Grimont, 1983), *Listeria ivanovii* (Seeliger et al., 1984), *Listeria marthii* (Graves et al., 2010), *Listeria rocourtiae* (Leclercq et al., 2010), *Listeria fleischmannii* (Bertsch et al., 2013) and *Listeria weihenstephanensis* (Lang Halter et al., 2013). Additionally, two subspecies have been recognized within *L. ivanovii* (subsp. *ivanovii* and subsp. *londoniensis*; Boerlin et al., 1992), *L. grayi* (subsp. *grayi* and subsp. *murrayi* (Stuart & Welchimer, 1973), though these names are not validly published] and *L. fleischmannii* (subsp. *fleischmannii* and subsp. *coloradensis*; den Bakker et al., 2013).

During sampling projects of agricultural and natural environments in the US states of Florida and Colorado, we isolated 70 isolates, predominantly from water, with a colony morphology on *Listeria monocytogenes* plating medium (LMPM) reminiscent of strains of *Listeria*. These isolates could be placed in the family *Listeriaceae* by phylogenetic analysis of partial sequences of the 16S rRNA gene, and 52 isolates could be assigned to *L. fleischmannii* (32 isolates from Florida and 20 isolates from Colorado); however, 18 isolates did not cluster phylogenetically within previously described species and therefore could not be classified as members of any of the previously described species. Based on further genotypic characterization of these isolates, including by sigB sequencing (Saunders et al., 2012), one or two isolates per putative novel taxon were selected for further phenotypic and genomic characterization (see Fig. S1, available in the online Supplementary Material, for more information).

Draft genome sequences were assembled as detailed by den Bakker et al. (2013) from Ion PGM instrument reads obtained for (i) *Brochotrichia thermophilica* ATCC 11509T and *B. campestris* ATCC 43754T, (ii) the type strains *L. monocytogenes* ATCC 15313T, *L. weihenstephanensis* DSM 24698T and *L. rocourtiae* CIP 109804T and *L. grayi* ATCC 25401 (the type strain of *Listeria murrayi*), (iii) an additional strain of *L. fleischmannii* (FSL S10-1203) and (iv) five isolates that showed *Listeria*-like characteristics, but could not be classified to species by traditional approaches. Briefly, *de novo* assembly was performed using the MIRA assembler (Chevreux, 1999), and homologues were corrected using a homopolymer correction script (available at http://tinyurl.com/oke6xaa); this script uses output from VarScan (Koboldt et al., 2012) and BWA (Li & Durbin, 2009) to obtain a consensus about the homopolymer length, which is then used to correct the homopolymers. *De novo* assemblies (draft genomes) of Ion Torrent reads ranged in total size from 2.37 (B. campestris) to 3.37 (L. weihenstephanensis) Mbp (see Table S1 for total draft genome lengths and additional genome assembly statistics). Comparison of the draft genome obtained here for *L. monocytogenes* ATCC 15313T and a recently finished genome of *L. monocytogenes* SLCC5850 (Kuenne et al., 2013), a strain closely related to the type strain (McLauchlin & Rees, 2009), showed that the draft genome covers 97.3 % of the finished genome. We therefore conclude that the draft genomes obtained in this study are of high quality and suitable for comparative analysis. These draft genomes will also provide a starting point for future in-depth genomic and evolutionary analyses of the genus *Listeria*.

**BLAST** average nucleotide identities (ANIb) were calculated based on draft genome sequences and publicly available genome sequences using JSpecies (Richter & Rosselló-Móra, 2009). Additionally, **BLAST** average amino acid identities (AAI) (Konstantinidis & Tiedje, 2005) were calculated using the AAI.rb script from the Enve-omics package (http://enve-omics.ce.gatech.edu/). Pairwise comparisons between currently recognized species in *Listeria sensu stricto* yielded ANIb and AAI values <95 % for comparisons between different species (Fig. 1; Table S2); an ANIb value of 95–96 % generally corresponds to 70 % DNA–DNA hybridization and is typically used as a cut-off value for species delineation in bacterial taxonomy (Richter & Rosselló-Móra, 2009). Our data also support conclusions from previous studies that ANIb values are robust even with fragmented unfinished draft assemblies. This robustness is demonstrated by an ANIb value of 99.94 % for the comparison between the draft genome for the *L. monocytogenes* type strain, ATCC 15313T (a draft genome obtained in this study), and the finished genome for *L. monocytogenes* SLCC5850. For all genomes representing previously reported species, ANIb values supported prior species classification, despite the mixed use of finished and draft genomes (Fig. 1). Most importantly, our analyses showed that all isolates representing novel species proposed in this study show ANIb values <84 % and AAI values <87.9 % (Table S2) when their genomes are compared to each other and to phylogenetically related, previously described species (*L. fleischmannii, L. rocourtiae* and *L. weihenstephanensis*). While the high 16S rRNA gene sequence similarity (>99 %) between *L. rocourtiae* CIP 109804T, *L. weihenstephanensis* DSM 24998T, *L. cornellensis* sp. nov. TTU A1-0210T, *L. grandensis* sp. nov. TTU A1-0212T and *L. riparia* sp. nov. FSL S10-1204T could be interpreted as suggesting that these taxa represent ectotypes of a single species (Cohan, 2001; Konstantinidis et al., 2006), ANIb and AAI values and shared gene content do not support this hypothesis. AAI and ANIb values between these species range from 83.2 to 88.6 % and from 77.7 to 83.2 %, respectively, and the proportion of shared gene content between each species pair ranges from 63 to 75 %, justifying recognition as individual species (Fig. S2).

Phylogenetic analysis of (i) 16S rRNA gene sequences (Fig. S2) and (ii) concatenated sequences of 31 amino acid sequences (Wu & Eisen, 2008) (Fig. 2) was performed as described previously (den Bakker et al., 2013), with the exception that Gblocks version 0.91b (Castresana, 2000) was used to remove ambiguous sites from the amino acid sequence alignment. Maximum-parsimony analyses were performed in *PAUP* version 4.0b10 (Wilgenbusch &
Swofford, 2003). Phylogenies obtained from the maximum-parsimony analysis (data not shown) did not differ from those found by maximum-likelihood-based analyses, and hence the results of the maximum-likelihood analyses are discussed here. Both 16S rRNA gene and amino acid sequence phylogenies confirmed the placement of the novel taxa in the genus *Listeria*, and revealed four well-supported clades [100 % bootstrap support (BS)] within the genus *Listeria*: (i) a clade containing *L. monocytogenes* and related species (*Listeria sensu stricto*; *L. marthii*, *L. innocua*, *L. weihenstephanensis*, *L. seeligeri* and *L. ivanovii*), (ii) a clade consisting of *L. rocourtiae*, *L. weihenstephanensis*, *L. cornellensis* sp. nov., *L. grandensis* sp. nov. and *L. riparia* sp. nov., (iii) a clade consisting of *L. fleischmannii*, *L. floridensis* sp. nov. and *L. aquatica* sp. nov., and (iv) a clade consisting of *L. grayi*. The 16S rRNA gene sequence analysis (Fig. S3) also indicates that a previously unidentified *Listeria*-like bacterium (referred to as ‘unidentified bacterium isolate MB405’ in Fig. S3) from Belgium (Rijpens et al., 1998) represents a putative sister species of *L. aquatica* sp. nov. Phylogenetic analysis of individual genes, such as *iap* (Fig. S4), also reveal the subdivision of *Listeria* into four distinct clades. The phylogenetic relationships between the four clades are unresolved in the 31 amino acid loci tree (Fig. 1; BS values < 60 %); however, the 16S rRNA gene sequence analysis suggests that the *L. rocourtiae* clade and the *L. grayi* clade are sister groups (Fig. S3; 85 % BS). AAI values between members of each phylogenetically distinct clade range from 67 to 70 % (Fig. S2). While Konstantinidis & Tiedje (2005, 2007) show that AAI between 65 and 72 % coincides with traditional genus delimitations in bacterial taxonomy, we do not propose classification of these clades into separate genera at this point, in part because reclassification of *L. grayi* into a new genus has previously been unsuccessful and controversial (McLauchlin & Rees, 2009).

For methyl red (MR) and Voges–Proskauer (VP) tests, all isolates were cultured aerobically on BHI agar (Becton Dickinson) for 18 h at 30 °C. A single colony was suspended in 5 ml MR-VP broth (pH 6.9 ± 0.2; Becton Dickinson); inoculated tubes were incubated aerobically for 5 days at 35 °C. MR and VP tests were carried out according to the manufacturer’s guidelines (Becton Dickinson; procedure L007474). Results are listed in the species descriptions and Table 1.

Testing for catalase activity was performed according to *Bacteriological Analytical Manual* (BAM) protocol R12 (US Food and Drug Administration, 2013). An isolated colony from BHI agar was emulsified in a drop of 3 % hydrogen peroxide on a glass slide. Bubbling within 15 s was interpreted as a positive result for the catalase test. Results were scored independently by two individuals and are listed in the species descriptions and Table 1.

To test for nitrate reduction, isolates were grown in nitrate broth (BAM medium M108; US Food and Drug Administration, 2013) with Durham tubes for 48 h at 30 °C without shaking. Durham tubes allowed detection of gas production at 24 and 48 h, which is indicative of N₂ production. Reduction of nitrate to nitrite was tested by adding 50 μl bacterial culture grown in nitrate broth to 0.9 ml phosphate buffer (50 mM, pH 7.4), followed by the addition of 1 ml sulfanilic acid/HCl (5.0 g sulfanilic acid, 100 ml concentrated HCl, 400 ml water; final pH 0.38) and 1 ml 0.58 % N-(1-naphthyl) ethylenediamine. An
immediate colour change to deep pink to purple indicated the production of nitrite. For all isolates, nitrite reduction was determined using a protocol comparable to the nitrate reduction protocol described above, except that KNO₂ was used instead of KNO₃ in BAM medium M108. Nitrite reduction was negative if the reaction solution produced magenta to purple coloration. If an isolate reduces nitrate to an oxidation state lower than nitrite, the nitrate reduction test will produce a false negative. Therefore, separately testing nitrite reduction and the generation of N₂ (Durham tubes) measures the reduction of nitrate to nitrite. All non-motile species were found to be able to reduce nitrate, with the exception of L. floridensis sp. nov. None of the novel species reported here was able to reduce nitrite.

Haemolysis for each isolate was determined using the CAMP test as described in BAM (US Food and Drug Administration, 2013) and performed in duplicate. To test for phosphatidylinositol-specific phospholipase C (PI-PLC) activity, single colonies from BHI agar plates were subcultured to LMPM (R&F Laboratories). PI-PLC activity-positive strains colour blue on LMPM medium. None of the novel species described here showed haemolytic activity or PI-PLC activity, suggesting that these species lack the genes (i.e. hly and plcA) associated with virulence in L. monocytogenes and L. ivanovii. Virulence genes (i.e. genes found in the prfA cluster; Schmid et al., 2005) or LIPI-2 (Domínguez-Bernal et al., 2006) were absent from the draft genomes, further supporting the conclusion that none of the novel species described here are pathogenic.

API Listeria and API 50 CH (with API 50 CHB/E medium) kits (bioMérieux) were utilized for further phenotypic characterization, using bacterial colonies taken from BHI agar plates. API Listeria tests were incubated for 24 h at 34 °C for all isolates (within the manufacturer’s recommended temperature range of 36 ± 2 °C). API 50 CH tests were incubated for 48 h at 37 °C for all isolates. A mineral oil cover was not used, since all species studied here are facultative anaerobes. Results were recorded at 48 h, unless there were positive or variable results at 24 h with a corresponding negative result at 48 h, in which case 24 h results were reported (as instructed in the API 50 CH/E manual). Results are summarized in the species descriptions and Table 1. Results for individual isolates are reported in Table S3.

To test growth characteristics, isolates were grown in BHI broth at 4, 7, 22.5, 30, 37 and 41 °C. A Synergy H1 hybrid multi-mode microplate reader (BioTek Instruments) was employed to test for growth at 22.5 ± 0.5, 30 ± 0.1, 37 ± 0.1

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**Fig. 2.** Maximum-likelihood phylogeny based on concatenated amino acid sequences of 31 core genes. Values on branches represent bootstrap values based on 1000 replicates. Bootstrap values <70% are not indicated. Novel species are shown in bold.
Table 1. Physiological characteristics of members of the genus *Listeria* based on observations made in this study and the current literature

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*Motility was reported for these species in the original descriptions (Lang Halter et al., 2013; Leclercq et al., 2010). We did not observe motility in the type strains of these species, nor did we find genes encoding a flagellar apparatus in the genomes of the type strains (Table S4). In agreement with our observations, Bertsch et al. (2013) did not observe motility in the type strain of *L. rocourtiae*.

and $41 \pm 0.1$ °C (temperature tolerances as provided by manufacturer). Colony-inoculated cultures were grown for 24 h in BHI broth; an aliquot of these cultures (20 μl) was used to inoculate Costar 96-well flat-bottom plates (clear polystyrene) prefilled with 180 μl BHI. Inoculated plates were incubated at 22.5 °C for 48 h, 30 °C for 41 h, 37 °C
Light microscopic observations were performed on isolates were found in the novel species described here.

Motility was determined in motility test medium (MTM) (prepared according to BAM medium M103; US Food and Drug Administration, 2013). For each isolate, 13 mm tubes containing MTM were inoculated, 1 cm under the surface, with a stab from a colony on BHI agar. Isolates were incubated aerobically for 7 days at 22, 30 or 37 °C or for 10 days at 4 °C. An isolate was considered motile if cloudy growth beyond the stab was observed. Results are reported in Table 1 and the species descriptions. No growth at 4, 22, 30 or 37 °C. Voges–Proskauer-negative; nitrite reduction-negative. Other characteristics are reported in Table 1. Currently, the only member of the genus Listeria that both lacks motility and is unable to reduce nitrate. A unique API Listeria numerical profile (2 710) is observed for the type strain.

The type strain, FSL S10-1188T (=DSM 26687T=LMG 28120T=BEI NR-42632T), was isolated from running water in Florida, USA. The DNA G+C content of the type strain is 41.8 mol% (determined by genome sequencing).

**Description of Listeria aquatica sp. nov.**

Listeria aquatica (a.qua’ti.ca. L. fem. adj. aquatica found in water, aquatic).

Cells are 0.6–0.7 × 1.5–2.4 μm; mean length/width ratio 3.2. Gram-stain-positive, straight rods with rounded ends. No growth at 7 °C or below. Optimum growth temperature 37–41 °C. No motility at 4, 22, 30 or 37 °C. Voges–Proskauer-negative for type strain; positive for strain FSL S10-1181. Catalase-positive. CAMP-negative. Nitrate reduction-positive; nitrite reduction-negative. Other characteristics are reported in Table 1. Currently, it is unique in its ability to ferment D-tagatose. A unique API Listeria numerical profile (6 731) is observed for the type strain and FSL S10-1181.

The type strain, FSL S10-1188T (=DSM 26687T=LMG 28120T=BEI NR-42632T), was isolated from running water in Florida, USA. The DNA G+C content of the type strain is 40.9 mol% (determined by genome sequencing).

**Description of Listeria cornellensis sp. nov.**

Listeria cornellensis (cor.nel.len’sis. N.L. fem. adj. cornellensis named after Cornell, the university where most of the research was performed that led to the discovery of the species described in this study).

Cells are 0.4–0.7 × 2.4–3.8 μm; mean length/width ratio 5.3. Gram-stain-positive, straight rods with rounded ends. Optimal growth at 30–37 °C. No motility at 4, 22, 30 or 37 °C. Voges–Proskauer-negative, catalase-positive. CAMP-negative. Nitrate reduction-positive, nitrite reduction-negative. Other characteristics are indicated in Table 1. While phylogenetically and genomically distinct, this species resembles L. grandensis for the phenotypic characteristics described here.
recorded here. Among the non-motile species of the genus Listeria, L. cornellensis and L. grandensis are the only ones that are L-rhamnose-negative in the API 50 CH test (performed at 37 °C); however, the API Listeria test strip (incubated at 34 °C) shows acidification of L-rhamnose for L. grandensis and for one of the two L. cornellensis strains tested. An operon involved in rhamnose utilization could be found in the draft genome of L. grandensis TTU A1-0212T, but is absent from the draft genome of the type strain of L. cornellensis, suggesting the absence of rhamnose utilization for the type strain of L. cornellensis and temperature-dependent ability of L. grandensis to acidify L-rhamnose. L. cornellensis can be further distinguished from L. grandensis by weak acidification of lactose. API Listeria numerical profile 2 330 is observed for the two known isolates (TTU 28123T and FSL 09-0970) of this species; this numerical profile is associated with L. ivanovii according to the API Listeria manual (bioMérieux; version 04/2007).

The type strain, TTU A1-0210T ( = DSM 26689T = LMG 28123T = BEI NR-42630T), was isolated from water in Colorado, USA. The DNA G + C content of the type strain is 42.5 mol% (determined by genome sequencing).

**Description of Listeria grandensis sp. nov.**

Listeria grandensis (gran.den sis. N.L. fem. adj. grandensis of or belonging to Grand, the county where the type strain was isolated).

Cells are 0.6–0.7 × 2.0–3.1 μm; mean length/width ratio 4.0. Gram-stain-positive, straight rods with rounded ends. Optimal growth temperature 30–37 °C. No motility at 4, 22, 30 or 37 °C. Voges–Proskauer-negative, catalase-positive. CAMP-negative. Nitrite reduction-negative and nitrate reduction-positive. Other characteristics are reported in Table 1. See the description of L. cornellensis for differentiation of L. grandensis from this species. A unique API Listeria numerical profile (2 730) is observed for the type strain.

The type strain, TTU A1-0212T ( = DSM 26685T = LMG 28119T = BEI NR-42634T), was isolated from running water in Florida, USA. The DNA G + C content of the type strain is 41.9 mol% (determined by genome sequencing).

**Description of Listeria riparia sp. nov.**

Listeria riparia (ri па`ri a. L. fem. adj. riparia of the bank of a river or stream).

Cells are 0.5–0.7 × 2.3–3.7 μm; mean length/width ratio 4.8. Gram-stain-positive, straight rods with rounded ends. Optimum growth temperature 37–41 °C. No motility at 4, 22, 30 or 37 °C. Voges–Proskauer-negative and catalase-positive. CAMP-negative. Nitrite reduction-negative and nitrate reduction-positive. Other characteristics are reported in Table 1. Can be differentiated from other non-motile species of the genus Listeria by a combination of x- mannosidase activity and the ability to acidify L-rhamnose, D-galactose and L-arabinose. A unique API Listeria numerical profile (6 710) is observed for the isolates tested (FSL S10-1204T and FSL S10-1219).

The type strain, FSL S10-1204T ( = DSM 26685T = LMG 28119T = BEI NR-42634T), was isolated from running water in Florida, USA. The DNA G + C content of the type strain is 41.9 mol% (determined by genome sequencing).

**Acknowledgements**

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**Reference**


