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***Listeria costaricensis* sp. nov.**

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Abstract

A bacterial strain isolated from a food processing drainage system in Costa Rica fulfilled the criteria to belong to the genus *Listeria*, but could not be assigned to any of the known species. Phylogenetic analysis based on the 16S rRNA gene showed highest similarity with *L. floridensis* (98.7%). Phylogenetic analysis based on *Listeria* core genome placed the novel taxon within the *L. fleishmannii*, *L. floridensis* and *L. aquatica* clade (*Listeria sensu lato*). Whole-genome sequence analyses based on the average nucleotide BLAST identity (ANI<80%), indicated that this isolate belonged to a new species. Results on the pairwise amino acid identity (AAI>70%) and on the percentage of conserved proteins (POCP>68%) with currently known *Listeria* species, as well as on biochemical characterization, confirmed that the strain constituted a new species within the *Listeria* genus. The name *Listeria costaricensis* sp. nov. is proposed for the novel species, and is represented by the type strain CLIP 2016/00682^T (=CIP 111400^T=DSM 105474^T).

The GenBank/EMBL/DDBJ accession numbers for the draft genome sequence of strain CLIP 2016/00682^T are FXUT01000001-FXUT01000044.

Main text

With the rapid development of whole genome sequencing technologies, the genus *Listeria* has expanded since 2009 to reach 17 species with validly published names [1]. Recently, the subdivision of *Listeria* genus in two major groups has been proposed [1-5]: (i) *Listeria sensu stricto*, constituted by the species *Listeria monocytogenes* [6], *L. innocua* [7], *L. welshimeri* [8], *L. seeligeri* [8] and *L. ivanovii* [9], and (ii) *Listeria sensu lato*, presumably non-pathogenic and constituted by the species *L. grayi* [10, 11], *L. marthii* [12], *L. rocourtiae* [13], *L. fleischmannii* [3, 14], *L. weihenstephanensis* [15], *L. floridensis*, *L. aquatica*, *L. cornellensis*, *L. riparia*, *L. grandensis* [4], *L. booriae* and *L. newyorkensis* [16]. The two species *L. monocytogenes* and *L. ivanovii* are pathogenic for human and animals [17]. Due to the rarity of *L. ivanovii* infection cases, only *L. monocytogenes* represents a worldwide public health concern [18, 19].

In 2016, the World Health Organization Collaborating Centre for *Listeria* (WHOCCL), Paris, France, received the isolate CLIP 2016/00682 (originally designated ES106) from the Institute of Technology of Costa Rica. The isolate was collected from a food processing drainage system in the province of Alajuela, Costa Rica, in August 2015, and characterized using the method described in the Bacteriological Analytical Manual (BAM) [20]. Briefly, selective enrichment was performed using buffered *Listeria* enrichment broth (BLEB, Oxoid, Basingstoke, United Kingdom) with subsequent selective plating in Modified Oxford *Listeria* selective agar (MOX, Oxoid). Typical *Listeria* species colonies growing on MOX agar were characterized by PCR using the primers *List-univ* 1 and *List-univ* 2, as described [21]. Positive PCR amplification was assumed as *Listeria* spp. and the isolate was sent to WHOCCL for further identification. Species identification of the isolate was attempted using MALDI-TOF mass spectrometry, based on the mass spectra obtained from full protein extraction protocol analyzed on a MicroFlex LT system (Bruker Daltonics, Bremen, Germany), according to the manufacturer's instructions [22]. Spectra analyses against the MBT library (DB-5989 MS; Bruker Daltonics), which is limited to 8 *Listeria* species [22], were inconclusive. The genome of isolate CLIP 2016/00682^T was sequenced after DNA extraction (DNeasy Blood & Tissue kit, Qiagen, København, Denmark), library preparation (Nextera XT DNA Sample kit, Illumina, California, USA), using NextSeq 500 (2 x 150 bp) platform (Illumina), according to the manufacturer's protocol, as described [23]. The data obtained followed the quality standards for its use for taxonomic purposes [24]. The draft genome assembly was obtained from high-quality reads (final

coverage of 108 X) using CLC Assembly Cell 4.3.0 (Qiagen, Hilden, Germany) as described [23], and annotated with Prokka v.1.9 [25]. The draft assembly contained 44 contigs, with a total length of 3.18 Mb and a N50 length of 224,949 bp.

Phylogenetic analyses were performed based on the 16S rRNA gene sequence comparisons and on the concatenated deduced amino acid sequences of 243 core genes present in all *Listeria* species, defined in this study using Roary v.3.11 [26] and a BLASTP identity cut-off of 80%. Sequences were aligned using MUSCLE v.3.8 [27] and maximum likelihood phylogenetic trees were inferred by using IQ-Tree v.1.5 [28]. 16S rRNA gene phylogenetic analysis showed highest similarity with *L. floridensis* (98.71%; Fig. 1), i.e. at the lower cut-off value previously proposed for species delineation based on 16S sequence similarity (98.7-99.0%; [29, 30]). Maximum likelihood phylogeny based on the amino acid sequences of *Listeria* core genes (Fig. 2) placed the novel taxon within the *L. fleishmannii*, *L. floridensis* and *L. aquatica* clade (*Listeria sensu lato*).

Species identification was determined by whole-genome pairwise average nucleotide and amino acid BLAST identity comparisons (ANI and AAI, respectively) against *Listeria* species reference genomes deposited at NCBI database, using the enveomics package [31]. The percentage of conserved proteins (POCP) was calculated as described by Qin *et al.* [32]. ANI analysis revealed that CLIP 2016/00682^T shared less than 80% genome sequence identity with all known *Listeria* species (Fig. 3), thus lower than the proposed genomic species cut-off of 95% [33]. Further analysis based on the deduced proteomes showed the highest AAI with *L. fleischmannii* subsp. *fleischmannii* (two-way AAI of 70.40%, based on 2041 orthologous proteins) and *L. fleischmannii* subsp. *cornellensis* (two-way AAI of 70.09%, based on 2056 orthologous proteins), above the 60% cut-off proposed for genus delineation [34]. The pairwise POCP, which takes also into account the size of deduced proteomes [32], between CLIP 2016/00682^T and *L. fleischmannii* subspecies (POCP=68%), was also higher than the genus boundary cut-off of 50%. These results confirmed that the unusual isolate represented a novel *Listeria* taxon.

In silico PCR-serogrouping [35] was positive for the *prs* gene (serogroup L, typical of *Listeria* species with the exception for *L. monocytogenes*), although negative by multiplex PCR due to primers mismatches.

The isolate CLIP 2016/00682^T was also characterized at phenotypic and biochemical levels Table 1 (adapted from [16]).

CLIP 2016/00682^T was grown on BHI agar plates, Agar *Listeria* according to Ottaviani and Agosti (ALOA; bioMérieux, Craaponne, France) and on MOX (Oxoid) agar plates after incubation for 24h at 30 or 37°C. Colonies on BHI were opaque, flat and yellow pigmented, with a diameter of 0.5 to 1.0 mm. The production of a yellow pigment in CLIP 2016/00682^T (Supplementary Fig. S1) is atypical in *Listeria* species. On MOX agar, colonies were dimpled, round and pewter coloured with black haloes. On ALOA Agar, colonies were blue, due to β -glucosidase activity, and not surrounded by a white halo, denoting the absence of phosphatidylinositol-specific phospholipase C (PI-PLC) activity (Table 1).

CLIP 2016/00682^T did not show the presence of capsule after performing the India ink test as described by Hughes and Smith (2013) [36]. Gram staining, catalase and oxidase activities, respiratory characteristics and endospore formation were tested as described in the BAM [18]. The isolate was a Gram-stain-positive, rod-shaped bacterium, that was oxidase negative, non-spore-forming and facultative anaerobic. CLIP 2016/00682^T was catalase negative. The organism was catalase negative, in contrast to all other 17 *Listeria* species. Expression of catalase is actually a major phenotypic characteristic of the *Listeria* genus, although there are reports of catalase negative *Listeria monocytogenes* strains [37, 38]. The lack of catalase activity in CLIP 2016/00682^T was consistent with the absence of catalase gene in the draft genome. To determine growth characteristics, isolates were grown on BHI agar and broth at 4 °C for 10 days and at 22, 30, 37 and 42 °C for 7 days. Growth was considered positive if there was an increase in cell number of at least 1.0 log (cfu.ml⁻¹) over 14 days. CLIP 2016/00682^T showed growth between 22 °C and 42 °C but not at 4°C.

Motility was tested by stab-inoculation in mannitol-mobility semi-solid agar (Bio-Rad, Marnes-La-Coquette, France) and incubation at different growth temperatures (4 °C, 22 °C and 37 °C) for 10 days in aerobic conditions. Strains *L. monocytogenes* ATCC 35152^T and *L. booriae* CIP 111022^T were used as positive and negative controls, respectively. Contrarily to other *Listeria sensu lato* species [4, 16], CLIP 2016/00682^T was motile (only at 37 °C), giving a typical umbrella-like growth pattern, due to the

presence of the flagella biosynthesis regulon typical of *Listeria sensu stricto*. Nitrate reduction and Voges-Proskauer tests were performed as described in the BAM [18]. Nitrate reductase tests were positive (Table 1), whereas nitrite reduction was negative. Voges-Proskauer were positive (Table 1).

No haemolysis was detected using either Columbia agar plates containing 5% defibrinated horse blood (bioMérieux) or the Christie, Atkins, Munch-Petersen (CAMP) test on Columbia agar containing 5% defibrinated sheep blood (bioMérieux) as described in the BAM [18]. The lack of haemolysis was consistent with the absence of *Listeria* pathogenicity islands (LIPI-1 to -4) [39, 40] within the draft genome of CLIP 2016/00682^T, suggesting that this novel taxon is not pathogenic.

Biochemical tests performed with API *Listeria* strips (bioMérieux) and the API50CH system (bioMérieux) as recommended by the manufacturer [41]. API *Listeria* tests were recorded after incubation at 37 °C for 24h. API50CH tests were recorded after 2, 5, 10 and 15 days of incubation at 30 °C. Results are summarized in Table 1. CLIP 2016/00682^T fit the API *Listeria* numerical profile 2730 of *Listeria ivanovii* (identification at 99.7%; bioMérieux API web database, version 04/2017) with divergent tests for D-arylamidase and glucose-1-phosphate.

Interestingly, according to its phenotypic profile (Table 1), isolate CLIP 2016/00682^T clustered together with *Listeria grayi* and the subgroup of *Listeria sensu stricto* (Fig. 4). Susceptibility to a wide range of antibacterial agents was determined with the disk diffusion method on Mueller–Hinton agar plates (Bio-Rad), using the interpretative criteria and recommendations from the French Microbiology Society and the European Committee on Antibiotic Susceptibility Testing [42, 43]. CLIP 2016/00682^T was sensitive to penicillin G, ampicillin, amoxicillin, imipenem, kanamycin, streptomycin, gentamicin, fosfomicin, rifampicin, erythromycin, levofloxacin, moxifloxacin, clindamycin, tetracycline, chloramphenicol, sulphonamides, trimethoprim and vancomycin. However, CLIP 2016/00682^T showed resistance to nalidixic acid and cefotaxime, and intermediate resistance to ciprofloxacin and fusidic acid, as other *Listeria* species [44].

Thus, on the basis of the molecular findings described above as well as the phenotypic distinctiveness of strain CLIP 2016/00682^T, we propose that this strain should be

classified as a member of a novel species of the genus *Listeria* for which the name *Listeria costaricensis* sp. nov. is proposed.

DESCRIPTION OF *LISTERIA COSTARICENSIS* SP. NOV.

Listeria costaricensis (cos.ta.ri.cen'sis N.L. fem. adj. *costaricensis*, "from Costa Rica", the country where the type strain was isolated).

Cells are straight, Gram-stain-positive, straight rods. Capsule is not formed. Spores are not produced. Colonies are opaque, yellow pigmented (atypical in *Listeria* species), with a flat shape and entire margin on BHI. On ALOA, colonies are blue centred without white halo, typical of *Listeria* species. On MOX, colonies are convex and pewter. Growth occurs at 22-42 °C, with optimal growth at 30-37 °C. Motile at 37°C. Negative for catalase, haemolysis and nitrite reduction. Positive for Voges-Proskauer and nitrate reduction tests. Acid was produced from aesculin, N-acetylglucosamine, amygdalin, D-arabitol, arbutin, D-cellobiose, L-fucose, D-fructose, D-galactose, gentiobiose, D-glucose, glycerol, D-lactose, D-maltose, D-mannose, methyl α -D-glucopyranoside, methyl α -D-mannopyranoside, potassium 5-ketogluconate, L-rhamnose, D-ribose, D-saccharose, salicin, starch, D-trehalose, xylitol and D-xylose. Acid was not produced from D-adonitol, D-arabinose, L-arabinose, D-arabitol, dulcitol, erythritol, D-fucose, glycogen, inositol, inulin, D-lyxose, D-mannitol, D-melezitose, D-melibiose, methyl β -D-xylopyranoside, potassium gluconate, potassium 2-ketogluconate, D-raffinose, D-sorbitol, L-sorbose, D-tagatose, D-turanose, L-xylose. It can be differentiated from other species of the genus *Listeria* by the absence of catalase reaction, the production of acid from potassium 5-ketogluconate, and the production of a yellow pigment on BHI.

The type strain CLIP 2016/00682^T was isolated in August 2015 from the drainage system of a food-processing plant in the province of Alajuela, North of Costa Rica. CLIP 2016/00682^T is deposited in CIP (CIP 111400^T) and DSMZ (DSM 105474^T) culture collections. The genomic DNA G+C content of the type strain is 43.7 mol%, as determined by genome sequencing.

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Conflicts of interest

The authors declare no conflict of interests.

Abbreviations:

AAI	average amino acid BLAST identity
ANI	average nucleotide BLAST identity
BAM	Bacteriological Analytical Manual
BHI	Brain Heart Infusion
BLAST	Basic Local Alignment Search Tool
BLEB	Buffered <i>Listeria</i> Enrichment Broth
MOX	Modified Oxford Agar
N50	minimum contig length covering 50% of the genome
POCP	percentage of conserved proteins
WHOCCL	World Health Organization Collaborating Centre for <i>Listeria</i>

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Table 1. Biochemical characteristics of species of the genus *Listeria* based on observations made in this study and on previously published studies [16].

Characteristics	Lco	Lmo	Lin	Lse	Liv	Lws	Lma	Lgy	Lro	Lwp	Lcn	Lri	Lgd	Lfc	Laq	Lfo	Lny	Lbo
Motility	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	+	-	-	-	-	-	-	v	+	+	+	+	+	+	+	-	+	+
Voges–Proskauer	+	+	+	+	+	+	+	+	-	-	-	-	-	-	v	-	-	-
Catalase	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Haemolysis	-	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Arylamidase	-	-	+	+	v	v	-	+	-	-	-	-	-	-	-	-	-	-
α-Mannosidase	-	+	+	-	-	+	+	v	+	-	-	+	-	-	+	-	-	+
Phosphatidylinositol-specific phospholipase C (PI-PLC)	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Acidification of:																		
D-Arabitol	+	+	+	+	+	+	+	+	-	+	-	-	v	+	-	-	-	+
D-Galactose	+	v	-	-	v	-	-	+	+	-	-	+	-	-	-	+	+	+
D-Glucose	+	v!	v!	+	v!	+	v!	+	+	+	+	+	+	+	+	+	+	+
Glycerol	+	v	+	+	+	+	-	v	+	+	v	v	-	+	v	-	+	+
L-Fucose	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Lactose	+	+	+	+	+	+	+	+	+	v!	(+)	+	-	+	-	+	+	+
D-Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
L-Rhamnose	+	+	v	-	-	v	-	-	+	+	-	+	-	+	+	+	v	+
D-Ribose	+	-	-	-	+	-	-	+	+	-	+	v	+	+	+	-	+	V
D-Saccharose	+	+	+	+	+	+	-	-	-	-	-	-	-	v	-	-	-	-
α-Methyl D-glucoside	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
Methyl α-D-mannose	+	-	-	nd	-	nd	-	+	-	-	-	-	-	v	-	-	-	-
Potassium 5-ketogluconate	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Xylose	+	-	-	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
L-Arabinose	-	-	-	-	-	-	-	-	-	-	v	+	-	-	+	+	+	+
Glucose 1-phosphate	-	-	-	-	v	-	-	-	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-	-	v	-	v	v	-	-	-

Inulin	-	v!	v!	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Lyxose	-	v	v	-	-	v	-	v	-	-	-	-	-	-	v	+	-	-
D-Mannitol	-	-	-	-	-	-	-	+	+	+	-	v	-	v	-	-	+	+
D-Melezitose	-	v	v	v	v	v	-	-	-	-	-	-	-	v	-	-	-	-
D-Melibiose	-	v!	v	-	-	-	v	-	+	-	-	v	-	v	-	-	-	+
L-Sorbose	-	v!	v!	-	v!	-	v!	v!	-	-	-	-	-	v	-	-	-	-
D-Tagatose	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-
D-Turanose	-	-	v	-	-	-	+	-	-	-	-	-	-	v	-	-	-	-

Notation: +, positive; (+), weakly positive; -, negative; v, variable (between replicates and/or between strains); v!, variable between studies (possibly due to differences in incubation times and temperatures between studies); nd, not determined or not recorded.

Strains: Lco, *L. costaricensis* sp. nov. strain CLIP 2016/00682^T (this study); Lmo, *L. monocytogenes* strain 10403S (data from McLauchlin & Rees, 2009 and Bertsch et al., 2013); Lin, *L. innocua* strain FSL S4-378 (data from McLauchlin & Rees, 2009 and Bertsch et al., 2013); Lse, *L. seeligeri* (data from McLauchlin & Rees, 2009 and Bertsch et al., 2013); Liv, *L. ivanovii* strain ATCC BAA-678 (data from McLauchlin & Rees, 2009; Bertsch et al., 2013); Lws, *L. welshimeri* (data from Bille et al., 1992; McLauchlin & Rees, 2009; Bertsch et al., 2013); Lma, *L. marthii* strain FSL S4-120^T (data from den Bakker et al., 2014); Lgy, *L. grayi* strains ATCC 19120^T, ATCC 25401^T (data from den Bakker et al., 2014); Lro, *L. rocourtiae* strain CIP 109804^T (data from den Bakker et al., 2014); Lwp, *L. weihenstephanensis* strain DSM 24698^T (data from den Bakker et al., 2014); Lcn, *L. cornellensis* strains TTU A1-0210^T, FSL F6-0970 (data from den Bakker et al., 2014); Lri, *L. riparia* strains FSL S10-1204^T, FSL S10-1219 (data from den Bakker et al., 2014); Lgd, *L. grandensis* strain TTU A1-0212^T (data from den Bakker et al., 2014); Lfc, *L. fleischmannii* strains DSM 24998^T, ATCC BAA-2414^T, FSL F6-1019, FSL S10-1186, FSL S10-1203 and FSL S10-1220 (data from den Bakker et al., 2014); Laq, *L. aquatica* strains FSL S10-1188^T and FSL S10-1181 (data from den Bakker et al., 2014); Lfo, *L. floridensis* strain FSL S10-1187^T (data from den Bakker et al., 2014); Lny, *L. newyorkensis* strains FSL M6-0635^T and A5-0209; Lbo, *L. booriae* strains FSL A5-0279^T and FSL A5-0281.

All species/strains are positive for aesculine and acid production from N-acetylglucosamine, amygdalin, arbutin, D-cellobiose, D-fructose, D-mannose and salicin.

All species/strains are negative for nitrite reduction and acid production from D-adonitol, D-arabinose, glycogen, methyl β -D-xylopyranoside, potassium 2-ketogluconate, and D-raffinose.

Legends to figure

FIGURE 1. Phylogenetic analysis of the 16S rRNA gene based on the maximum likelihood method. Distance estimation was obtained by the model of Kimura 2-Parameter [45]. Selected members of *Brochothrix* genus were used as outgroup. Positions containing gaps and missing data were eliminated, resulting in a total of 1071 positions. Branch lengths represent the number of nucleotide substitutions per site and bootstrap percentages of 1,000 replicates are shown. GenBank accession numbers are provided in brackets. The newly *Listeria costaricensis* isolate is highlighted in bold.

FIGURE 2. Maximum likelihood phylogenetic analysis based on the concatenated amino acid sequences of 243 core genes present in all *Listeria* species. Distance estimation was obtained by the model of WAG [46]. Branch lengths represent the number of amino acid substitutions per site and bootstrap percentages of 1,000 replicates are shown. GenBank accession numbers are provided in brackets. The newly *Listeria costaricensis* isolate is highlighted in bold.

FIGURE 3. Unweighted pair group method with arithmetic mean (UPGMA) clustering based on the genomic average nucleotide difference (ANI). The vertical dashed bar represents the proposed 95% ANI species cut-off that correlates with the 70% DNA-DNA hybridization threshold [33]. Scale bar represents the percentage of similarity. The newly *Listeria costaricensis* isolate is highlighted in bold.

FIGURE 4. Unweighted pair group method with arithmetic mean (UPGMA) analysis based on the 33 biochemical characteristics of *Listeria* species shown in Table 1. Unknown data and traits with variations between different isolates and/or studies were ignored in the clustering analysis. Scale bar represents the percentage of similarity of biochemical profiles.

FIGURE 1.

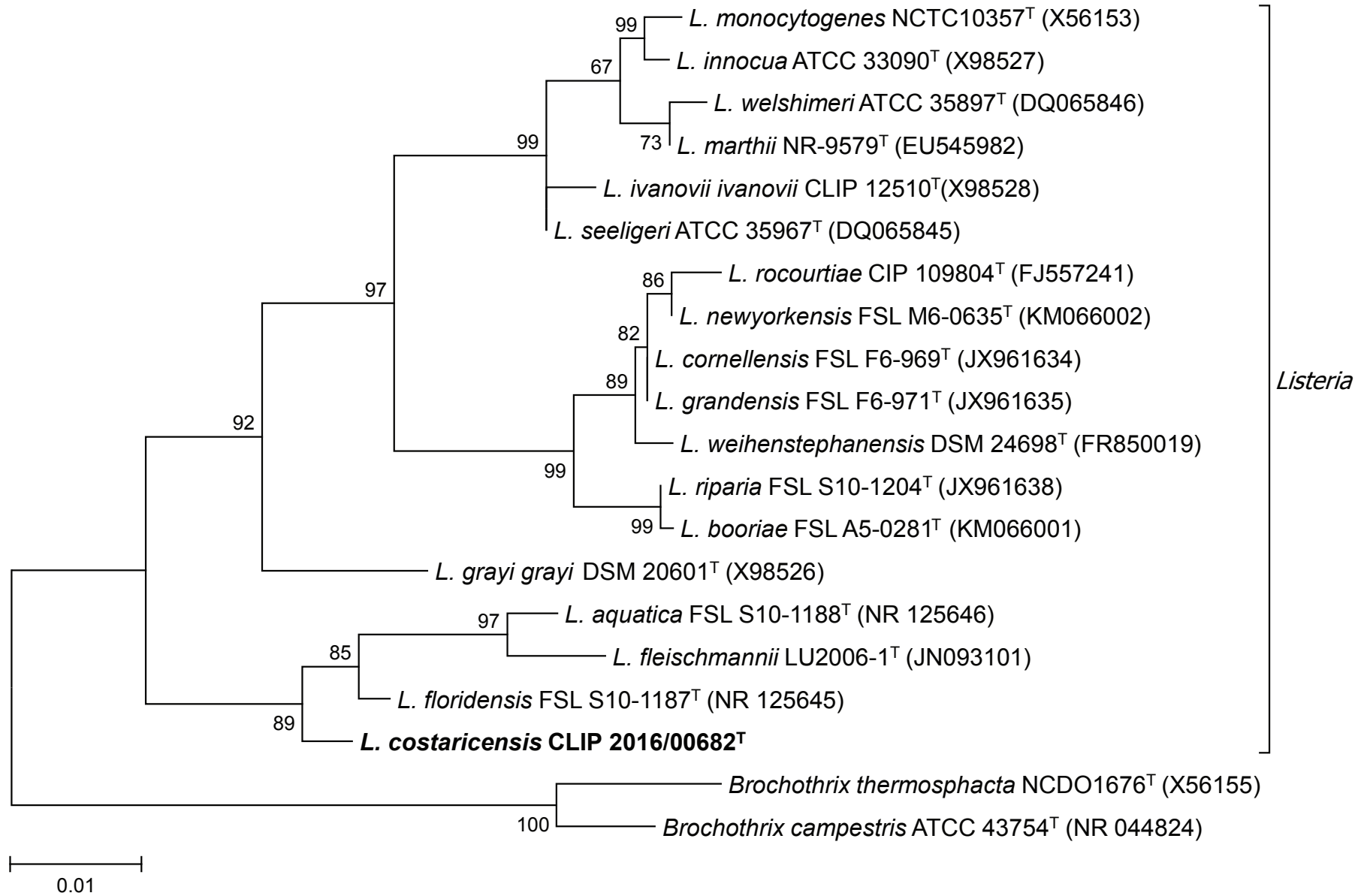


FIGURE 2.

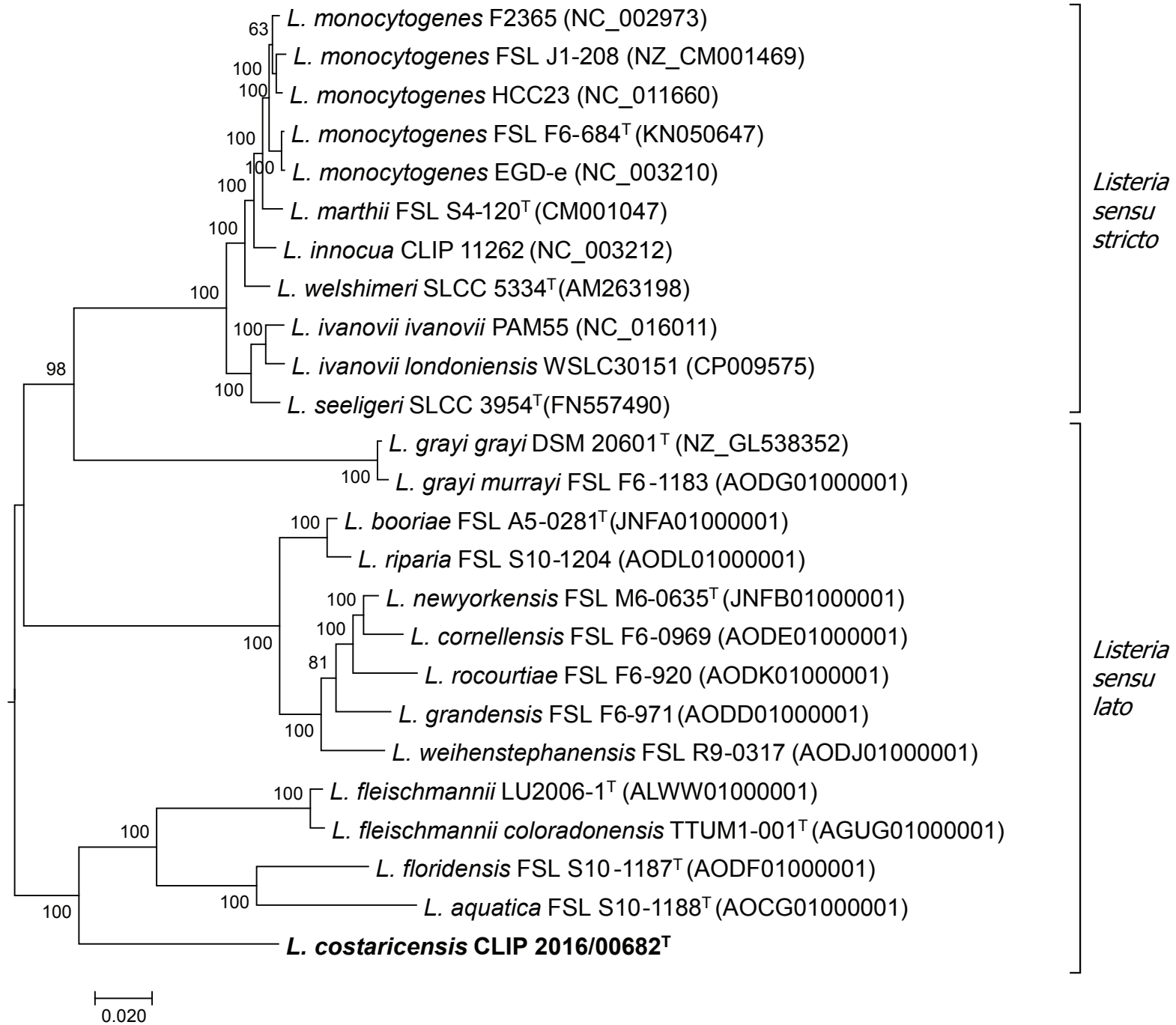


FIGURE 3.

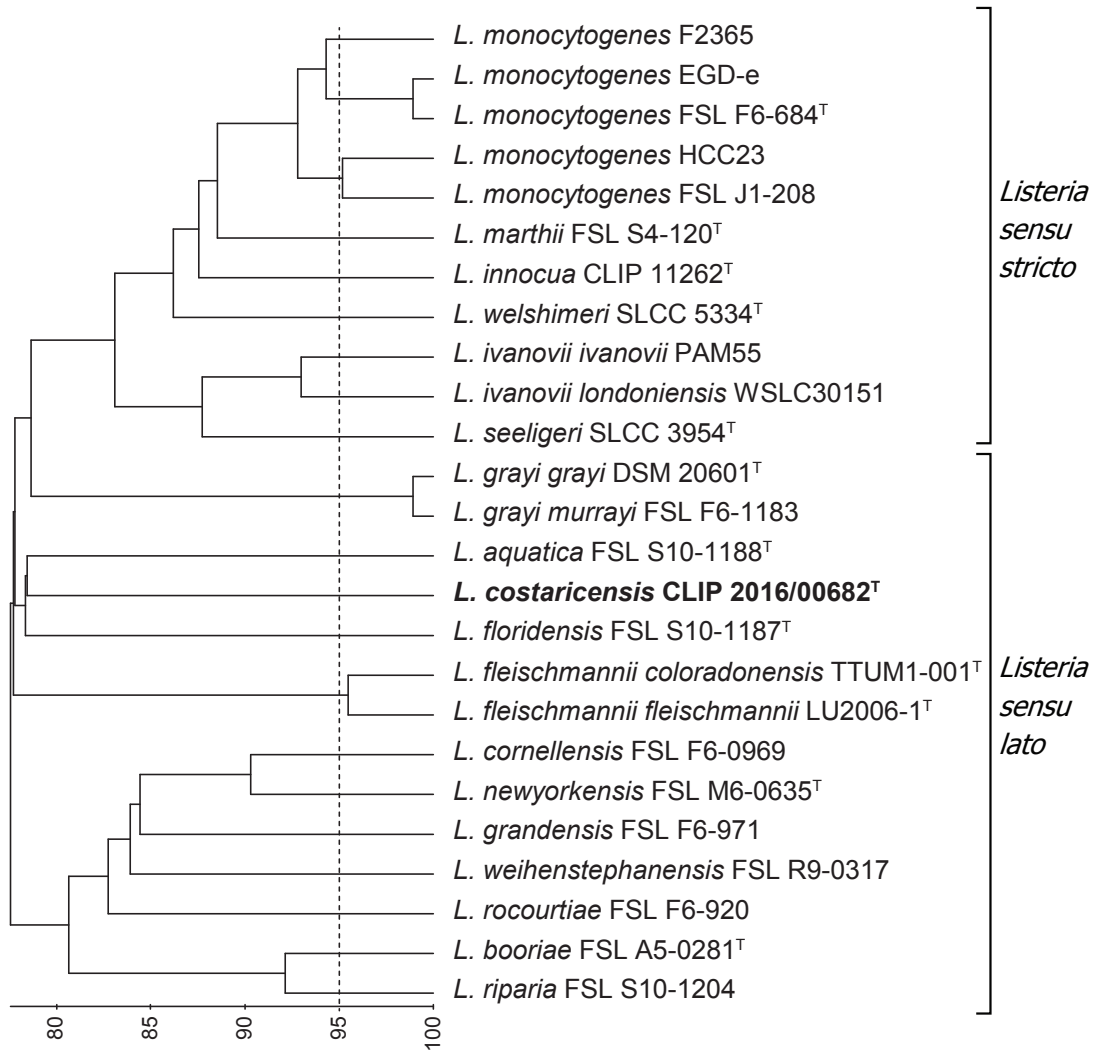
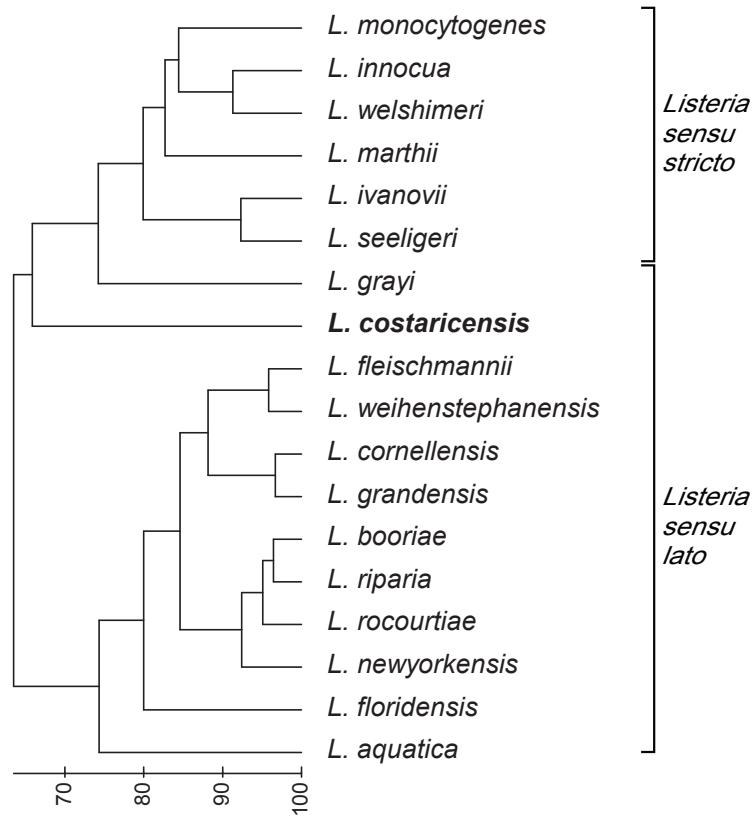
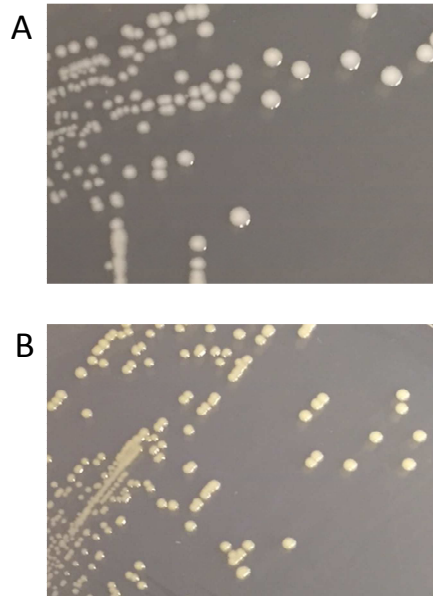


FIGURE 4.





SUPPLEMENTARY FIGURE S1. Pigment production on Brain Heart Infusion agar after 24 hours at 37 °C. (A) white colonies of *L. monocytogenes* ATCC 19114; (B) yellow pigmented colonies of *L. costaricensis* CLIP 2016/00682^T.