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--Manuscript Draft--

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Abstract:	A Gram-negative, bacilli shaped bacterial strain, LS-1T, was isolated from a sewage water sample collected in Jeddah, Saudi Arabia. The taxonomic position of the strain LS-1T was investigated using a polyphasic taxonomic approach. Phylogenetic analysis based on 16S rRNA gene sequences and 4 other genes indicated that the strain LS-1T belongs to the genus Legionella in the family Legionellaceae. Regarding the 16S rRNA gene, the most closely related species are Legionella rowbothamii LLAP-6T (98.6 %) and Legionella lytica L2T (98.5 %). The mip gene sequence of the strain LS-1T showed 94% sequence similarity with the L. lytica L2T and 93% with L. rowbothamii LLAP-6T. The strain LS-1T grows optimally at a temperature of 32°C on a buffered charcoal yeast extract (BCYE) agar plate in a 5% CO2 atmosphere and has a flagellum. The combined phylogenetic, phenotypic and genomic sequence data suggest that the strain LS-1T represents a novel species of the genus Legionella, for which the name Legionella saoudiensis sp. nov. is proposed. The type strain is LS-1T (= DSM 101682T = CSURP2101T).

1 ***Legionella saoudiensis* sp. nov., isolated from sewage water sample**

2 **Running title:** *Legionella saoudiensis* sp. nov.

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19

20 The GenBank/EMBL/DDBJ accession number for the 16S rRNA of the strain

21 *Legionella saoudiensis* LS-1^T is LN899829.

22 The GenBank/ DDBJ/EMBL accession number for the whole genome shotgun project

23 of the strain *Legionella saoudiensis* LS-1^T is CZVG01000000 (CZVG01000001-

24 CZVG010000012).

25

26 **Abstract**

27 A Gram-negative, bacilli shaped bacterial strain, LS-1^T, was isolated from a sewage
28 water sample collected in Jeddah, Saudi Arabia. The taxonomic position of the strain
29 LS-1^T was investigated using a polyphasic taxonomic approach. Phylogenetic
30 analysis based on 16S rRNA gene sequences and 4 other genes indicated that the
31 strain LS-1^T belongs to the genus *Legionella* in the family Legionellaceae. Regarding
32 the 16S rRNA gene, the most closely related species are *Legionella rowbothamii*
33 LLAP-6^T (98.6 %) and *Legionella lytica* L2^T (98.5 %). The *mip* gene sequence of the
34 strain LS-1^T showed 94% sequence similarity with the *L. lytica* L2^T and 93% with *L.*
35 *rowbothamii* LLAP-6^T. The strain LS-1^T grows optimally at a temperature of 32°C
36 on a buffered charcoal yeast extract (BCYE) agar plate in a 5% CO₂ atmosphere and
37 has a flagellum. The combined phylogenetic, phenotypic and genomic sequence data
38 suggest that the strain LS-1^T represents a novel species of the genus *Legionella*, for
39 which the name *Legionella saoudiensis* sp. nov. is proposed. The type strain is LS-1^T
40 (= DSM 101682^T = CSURP2101^T).

41

Legionellosis is considered to be a major health problem associated with water-related outbreaks, mainly caused by inhalation of aerosols generated from water sources such as engineered water systems and cooling towers (Viswanathan *et al.*, 2012). This infection is mainly caused by the different species of the genus *Legionella*, which are Gram-negative bacilli and are grouped in the family of Legionellaceae (Brenner *et al.*, 1979). Species of *Legionella* are facultative intracellular bacteria, and are able to use and exploit amoebae both as natural hosts and reservoir, in freshwater environment (Campocasso *et al.*, 2012; La Scola *et al.*, 2001). Of the 59 characterized type strains of *Legionella*, at least 24 species are associated with human infections (Newton *et al.*, 2010; Parte, 2014). The main causative agent of legionellosis is *Legionella pneumophila*, which is responsible for severe pneumonia (Brenner *et al.*, 1979). As culture techniques improve, new species of *Legionella* are increasingly reported from environmental and clinical samples (Greub & Raoult, 2004; La Scola *et al.*, 2001). In our laboratory, an improved isolation technique, based on co-cultivation with amoeba, lead to the identification of numerous amoebae-resistant bacteria (Pagnier *et al.*, 2008) and two novel species of *Legionella* (Campocasso *et al.*, 2012). Here we report the taxonomic characterization of the isolated bacterial strain LS-1^T, which represents a novel species within the genus *Legionella* and we propose the name *Legionella saoudiensis* LS-1^T sp. nov.

The strain LS-1^T was isolated from a water sample collected from a sewage filtration plant located in Jeddah, Saudi Arabia. About 50 µl of sewage water was used to inoculate an amoebal monolayer of the following species: *Acanthamoeba polyphaga* Linc AP-1, *Acanthamoeba castellanii* Neff (= ATCC 30010), *Vermamoeba vermiformis* CDC-19, and *Dictyostelium discoideum* ATCC 44841. Co-cultures were incubated at 32°C for *A. polyphaga* and *A. castellanii*, 30°C for *V. vermiformis*, and

67 25°C for *D. discoideum*, to select amoeba-resistant microorganisms, as previously
68 described (La Scola *et al.*, 2001; Pagnier *et al.*, 2008). After seven days, when
69 amoebal lysis was observed, 50 µl of subculture was used to inoculate buffered
70 charcoal yeast extract agar plates, containing alpha-ketoglutarate (BCYE; Oxoid), and
71 Columbia agar plates enriched with 5% sheep blood (Cos, Bio Mérieux). The plates
72 were incubated for one month, in a 5% CO₂ enriched atmosphere, at the temperatures
73 corresponding to the amoebal co-culture, i.e. 32°C for *A. polyphaga* and *A. castellanii*,
74 30°C for *V. vermiformis*, and 25°C for *D. discoideum*. The plates were checked daily
75 for growth. Electron microscopy was used to study the cell morphology of the isolated
76 bacteria, and to look for the presence of a flagellum. The bacterial strain was grown in
77 co-culture with *D. discoideum* in starvation medium (Reteno *et al.*, 2015) and on
78 BCYE agar plates. Cells were negative stained with a 3% ammonium molybdate
79 solution and observed with a Tecnai G20 Cryo (FEI, France) electron microscope.
80 The co-cultures were also analyzed with Gram and Gimenez staining. Auto-
81 fluorescence was evaluated by illuminating the strain on BCYE plates with a Wood's
82 lamp. The presence of β-lactamase and oxidase was assessed using Cefinase and
83 Oxidase discs (Becton Dickinson, USA) respectively. API 20E and campylobacter
84 galleries (BioMérieux, Marcy-l'Etoile) were used to determine hippurate and
85 gelatinase reactions, and sugar metabolization. For API galleries, heavy bacterial
86 inoculums resuspended in normal saline buffer were dropped into the wells of the
87 strip tests; following the manufacturer's guide instructions. Strips were incubated at
88 37°C for up to 72 h. Normal saline buffer was used as negative control, and two
89 *Legionella* strains were used for positive control (*L. massiliensis* CSURP0146 and *L.*
90 *tunisiensis* CSURP0145). The growth of the strain was tested on BYCE agar plates at
91 the following temperatures: 25, 30, 32, 35, 37°C. L-Cystein auxotrophy was

determined using BCYE Agar without L-cysteine (Oxoid).

The genome of the strain LS-1^T was sequenced using MiSeq Technology (Illumina Inc, San Diego, CA, USA) with the mate pair strategy. The minimum coverage is 163 and the quality score is 30. Automated cluster generation and sequencing runs were performed using 2x300-bp library. Illumina reads were trimmed using Trimmomatic (Guindon *et al.*, 2010), then assembled using Spades software (Lohse *et al.*, 2012; Nurk *et al.*, 2013). Contigs obtained were combined using SSpace and Opera software v1.2 (Bankevich *et al.*, 2012; Boetzer *et al.*, 2011; [Gao et al., 2011](http://www.ezbiocloud.net/eztaxon)). Multiple alignments were performed using the MUSCLE method and the phylogenetic tree was constructed using a neighbor-joining method in FastTree, with bootstrap values based on 1,000 replications. Pair-wise sequence similarity values between the *L. saoudiensis* strain LS-1^T and related taxa were computed using the EzTaxon server (<http://www.ezbiocloud.net/eztaxon>). The whole genome shotgun project of the strain LS-1^T has been deposited at DDBJ/EMBL/GenBank under the accession CZVG01000000 (CZVG01000001-CZVG01000012). Sequences of related taxa were obtained from the GenBank database.

After three days incubating the agar plates originating from the amoebal co-cultures, very small colonies appeared on the BCYE plates inoculated with the *D. discoideum* co-culture, but no growth was detected on the corresponding Cos agar plates. No growth occurred for the co-cultures of the three other amoeba, either on BCYE or on Cos agar plates. The strain is negative for L-cystein auxotrophy, as it did not grow on BCYE Agar without L-cysteine.

This strain LS-1^T was Gram negative and Gimenez positive bacilli appeared in red after both staining. It was able to grow and become visible after three days at the optimum temperature (32°C) on BCYE agar plate in a 5% CO₂ atmosphere. However,

117 the strain was neither productive without CO₂, nor able to grow on Colombia agar
118 with 5% sheep blood. At the other tested temperatures, growth was slow and resulted
119 in fewer colonies. The strain grew at 42°C with 5% CO₂, but forming very small
120 colonies. The colonies were circular shaped, with an approximate diameter of 0.8 mm,
121 white in color with a transparent appearance, and showed blue auto-fluorescence
122 under the Wood's lamp. The colonies showed the typical opal-like appearance with
123 the reflected light. Cefinase and oxidase tests were positive, while hippurate and
124 gelatinase reactions were negative. The electron microscopy images revealed a mean
125 size of 1.87 µm in length and 0.61 µm in width when the bacteria were grown on
126 BYCE agar plates. Flagella could be distinctly observed on electron microscopic
127 pictures, on per bacteria, with mainly a polar position. Phenotypical characteristics of
128 *L. saoudiensis* LS-1^T are provided in Table 1, in comparison with those of the closest
129 related *Legionella* species. For most of them, the strain LS-1^T responds to the
130 definition of the family *Legionellaceae*, and the genus *Legionella*, except for the
131 gelatin hydrolysis, which is negative for strain LS-1^T. The strain LS-1^T was primarily
132 isolated from the co-culture with the amoeba *D. discoideum*. However, in further co-
133 culture with other amoebal species, it could grow slowly within *A. polyphaga*, but did
134 not grow within *A. castellanii*, and *V. vermiformis* in starvation medium, at 32 and
135 30°C respectively. The size of the bacterial cells was different when grown in co-
136 culture with the amoeba *D. discoideum*, in a starvation medium, showing a mean size
137 of 1.19 µm in length and 0.701 µm in width. Bacterial motility showed a high speed
138 in amoebae, especially in *D. discoideum*, but lower with *A. polyphaga*.
139 The following gene sequences were extracted from the whole genome sequence of
140 strain LS-1^T, and compared to the closest related *Legionella* species: 16S rRNA (1530
141 bp), *mip* (729 bp), *rpoB* (302 bp) and *rnpB* (288 bp) genes and the 23S-5S region (115

bp) (Table S1). The strain LS-1^T exhibited 16S rRNA gene sequence similarity values of 98.6% and 98.5 % with the type strains of *L. rowbothamii* LLAP-6^T and *L. lytica* L2^T, respectively, and less than 98 % sequence similarity with respect to other taxa used in the phylogenetic analysis. The macrophage infectivity potentiator (*mip*) gene of strain LS-1^T showed 94% similarity with *L. lytica* L2^T and 93% with *L. rowbothamii* strain LLAP-6^T. The topologies of phylogenetic tree based on neighbor-joining tree using 16S rRNA support the result that *L. saoudiensis* is the novel member of the genus *Legionella* (Fig S1).

In order to confirm that LS-1^T is a new species within the genus *Legionella*, phylogenetic analysis of a cluster of the five extracted genes was done. It showed the LS-1^T strain joined specifically the cluster of *Legionella drancourtii* but is a separate species, regardless of the gene used for comparison (Fig. 1). The similarity between *L. saoudiensis* and *L. drancourtii* was 97.5 % for the 16S rRNA gene and 89.2 % for the *mip* gene. However, the three other genes, 23S-5S, *rnpB* and *rpoB*, sequences of strain LS-1^T showed 96.7, 95.1, 85.4 % similarity, respectively, with the sequences of *L. drancourtii*.

The *L. saoudiensis* genome is 3,847,980 bp long, containing one plasmid, with 39.42 % G+C content, which is consistent with the G+C content of the genus *Legionella* (Brenner *et al.*, 1979). It consists of 10 scaffolds (composed of 12 contigs). Of the 3,387 predicted genes, 3,341 were protein-coding genes, and 46 were RNAs (three genes are 5S rRNA, four genes are 16S rRNA, one gene is 23S rRNA, 38 genes are tRNA genes). Also, a total of 2,288 genes (68.48 %) were assigned a putative function (by cogs or by NR blast). 294 genes were identified as ORFans (8.80%). The remaining genes were annotated as hypothetical proteins (634 genes => 18.98%).

Moreover, 99 genes were associated to a toxin/antitoxin system.

Description of *Legionella saoudiensis* sp. nov.

Legionella saoudiensis (sa.ou.di.en'sis N. L. fem. adj. saoudiensis, pertaining to Saudi Arabia, the country where the strain originates). Strain LS-1^T respond to the definition of the family Legionellaceae and to the definition of the genus *Legionella* (Brenner *et al.*, 1979). It is Gram-negative bacilli, it grows optimally on BCYE agar at 32°C with a 5 % CO₂ atmosphere, as small bluish colonies, positive for autofluorescence. It does not grow on blood agar. It is motile with the presence of flagella. The type strain is 0.87 µm in length and 0.61 µm in width when grown on BYCE medium. The strain has β-lactamase activity, is oxidase positive, hippurate negative, and in contrast with other members of the genus *Legionella*, this strain is unable to hydrolyze gelatin. The G+C content is 39.42% and is within the range of the genus.

The type strain is LS-1^T (= DSM 101682^T = CSURP2101^T), isolated from a sewage sample collected from Jeddah in the western region of Saudi Arabia.

The CSUR culture collection is available following the link:

<http://www.mediterranee-infection.com/article.php?leref=14&titre=collection-de-souches>

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258

259

260 **Table 1:** Phenotypic characterization of *L. saoudiensis* LS-1^T, in comparison with the closest related *Legionella* species

	<i>L. saoudiensis</i> LS-1 ^T	<i>L. tucsonensis</i>	<i>L. rowbothami</i>	<i>L. drancourtii</i>	<i>L. anisa</i>	<i>L. lytica</i>
Origin	Sewage water	Pleural fluid	Potable water	Environmental water	Potable and environmental water	Soil
Optimal growth temperature on BCYE	32°C	na	30°C	32°C Strictly intracellular	na	30°C
Auto fluorescence	Blue	Blue white	Blue white	x	Blue-white	Blue-white
Colony shape/color	Circular white, 0.8mm diameter	Small grey	Grey/Blue-grey, convexe	x	na	Grey/Blue-grey
Cell size	1.87µm length 0.61µm width	na	na	na	na	1.9µm length 0.6µm width

Flagella	Yes	Yes	na	Yes	Yes	Yes
Oxidase	+	-	-	na	+	-
Hippurate	-	-	na	na	-	na
Gelatinase	-	+	na	na	+	na
Beta lactamase	+	+	na	na	+	na
G + C content	39.42	44	na	39.4	42	43
Reference		(Thacker <i>et al.</i> , 1989)	(Adeleke <i>et al.</i> , 2001)	(La Scola <i>et al.</i> , 2004)	(Gorman <i>et al.</i> , 1985)	(Adeleke <i>et al.</i> , 2001)

261

262

Figure Legends:

Fig.1: Phylogenetic tree based on the concatenation of five sequences (16S rRNA, *mip*, *rpoB*, *rnpB*, and 23S-5S) showing the relationship of *Legionella saoudiensis* LS-1^T with closely related species of the genus *Legionella*. The phylogenetic tree was constructed using the maximum-likelihood method. Bootstrap values (expressed as percentage of 1,000 replications). Bar, 0.02 substitutions per site.

Fig.S1: Phylogenetic tree showing the relationship of *Legionella saoudiensis* LS-1^T with closely related species of the genus *Legionella*, based on the 16S rRNA gene comparison. The phylogenetic tree was constructed by using the neighbor-joining method and Jukes & Cantor evolutionary distance matrix data obtained from aligned nucleotides. Bootstrap values (expressed as percentage of 1000 replications) greater than 50 % are shown at the branch points. Bar, 1 substitution per 100 nucleotide positions.

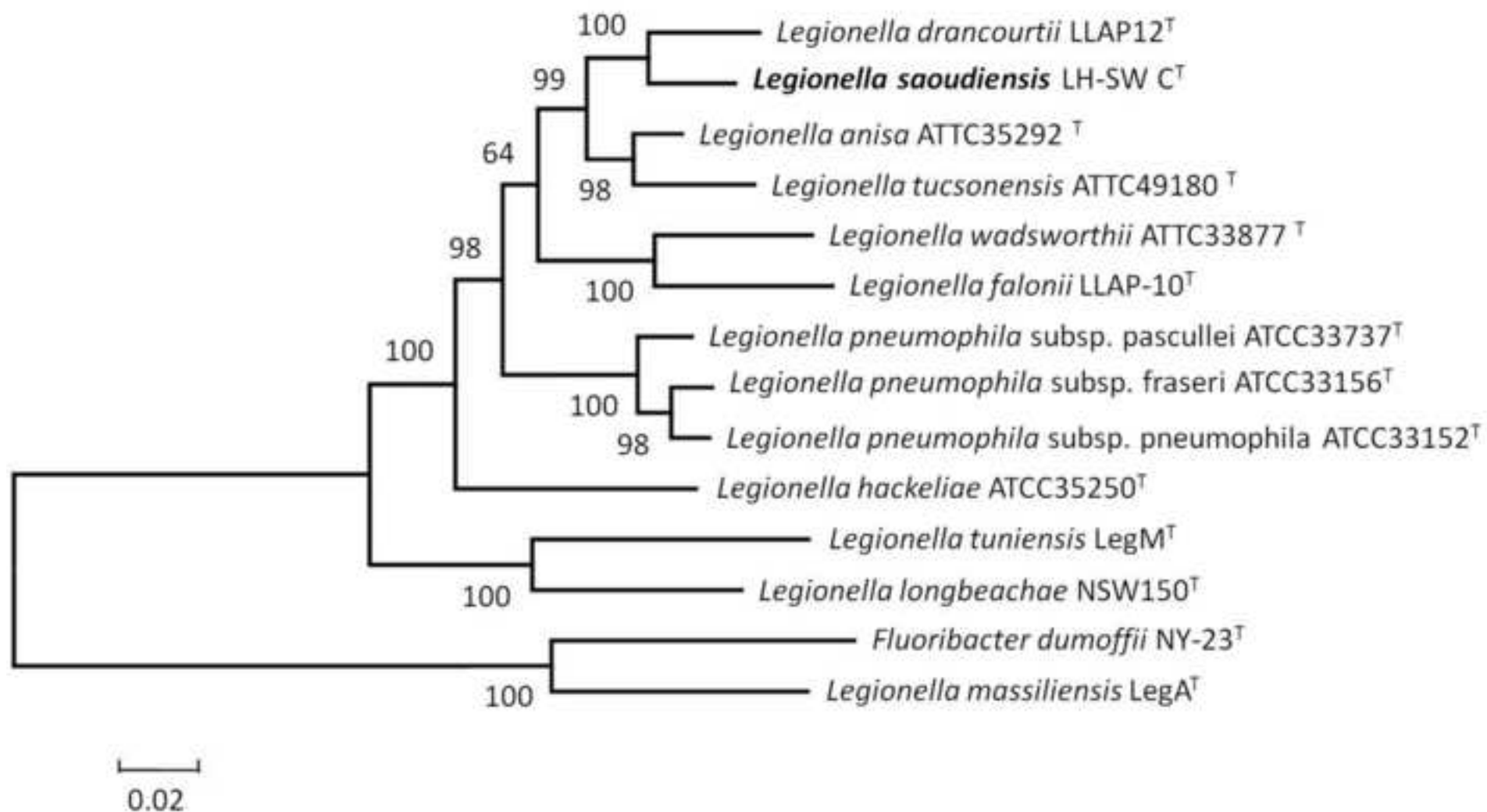


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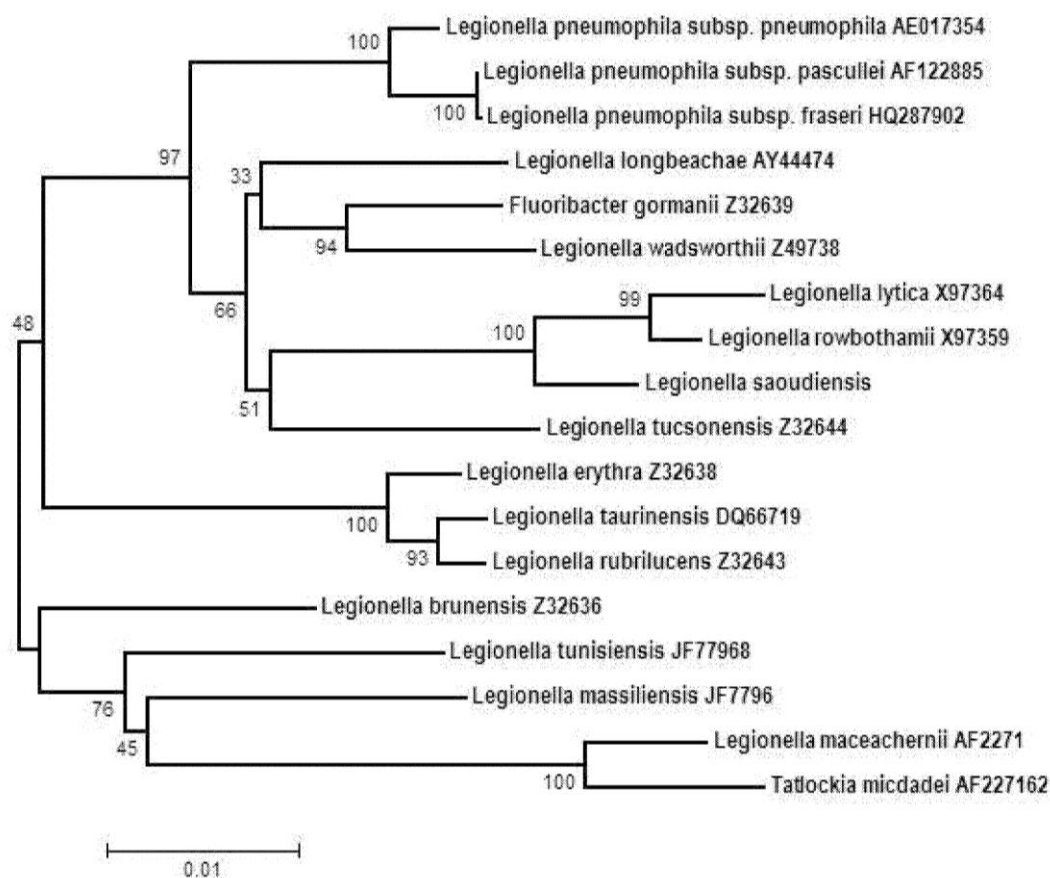


Table S1. Gene sequence similarity of *Legionella saoudiensis* sp. nov., strain LS-1^T, with respect to the 16S rRNA gene and four other genes

Species	<i>Legionella saoudiensis</i> sp. nov.				
	16S rRNA	<i>rnpB</i>	<i>mip</i>	<i>rpoB</i>	23S-5S
Sequence length (bp)	1530	288	729	302	115
<i>Legionella drancourtii</i> (%)	97.5	95.1	89.2	85.4	96.7
<i>Legionella anisa</i> (%)	97.1	94.4	85.9	80.1	96.7
<i>Legionella tucsonensis</i> (%)	96.7	92.7	85.1	79.3	96.7
<i>Legionella wadsworthii</i> (%)	95	92.6	85.2	82.7	96.3
<i>Fluoribacter dumoffii</i> (%)	97.6	90.6	83.5	83.4	96.7
<i>Legionella longbeachae</i> (%)	97.1	92.6	83.8	82.4	96.4
<i>Legionella tunisiensis</i> (%)	95.2	84.4	76.1	79.8	93.5