Legionella saoudiensis sp. nov., isolated from a sewage water sample

Leena Hussein Bajrai,1,2 Esam Ibraheem Azhar,3 Muhammad Yasin,3 Priscilla Jardot,2 Lina Barrassi,2 Didier Raoult,2 Bernard La Scola2 and Isabelle Pagnier2

1Department of Biochemistry, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia
2Unité des Rickettssies, URMITE UMR CNRS 7278 IRD 198 INSERM U1095, Facultés de Médecine et de Pharmacie, Aix-Marseille Université, 27 Boulevard Jean Moulin, 13385 Marseille Cedex 05, France
3Department of Medical Laboratory Technology, Special Infectious Agents Unit, King Fahd Medical Research Center, Faculty of Applied Medical Sciences, King Abdulaziz University, Jeddah, Saudi Arabia

A Gram-stain-negative, bacilli-shaped bacterial strain, LS-1T, was isolated from a sewage water sample collected in Jeddah, Saudi Arabia. The taxonomic position of strain LS-1T was investigated using a polyphasic taxonomic approach. Phylogenetic analysis based on 16S rRNA gene sequences and those of four other genes indicated that 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 strain LS-1T showed 94 % sequence similarity with that of L. lytica L2T and 93 % similarity with that of L. rowbothamii LLAP-6T. Strain LS-1T grew optimally at a temperature of 32 °C on a buffered charcoal yeast extract (BCYE) agar plate in a 5 % CO2 atmosphere and had a flagellum. The combined phylogenetic, phenotypic and genomic sequence data suggest that 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=CSUR P2101T).

Legionellosis is considered to be a major health problem and is 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 Legionellaceae (Brenner et al., 1979). Species of the genus Legionella are facultatively intracellular bacteria and are able to use and exploit amoebae both as natural hosts and as reservoirs in freshwater environments (Campocasso et al., 2012; La Scola et al., 2001). Of the 59 characterized type strains of species of the genus Legionella, at least 24 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, novel species of the genus Legionella are increasingly being reported from environmental and clinical samples (Greub & Raoult, 2004; La Scola et al., 2001). In our laboratory, an improved isolation technique, based on cocultivation with amoeba, led to the identification of numerous amoebae-resistant bacteria (Pagnier et al., 2008) and two novel species of the genus Legionella (Campocasso et al., 2012). Here we report the taxonomic characterization of the newly isolated bacterial strain LS-1T, which represents a novel species within the genus Legionella for which we propose the name Legionella saoudiensis sp. nov.

Strain LS-1T 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 strains: *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 25 °C for *D. discoideum* to select amoeba-resistant micro-organisms, as previously described (La Scola et al., 2001; Pagnier et al., 2008). After 7 days, when amoebal lysis was observed, 50 µl of subculture was used to inoculate buffered charcoal yeast extract agar plates, containing alpha-ketoglutarate (BCYE; Oxoid), and Columbia agar plates enriched with 5% sheep blood (Cos; Bio Mérieux). The plates were incubated for 1 month, in a 5% CO₂-enriched atmosphere, at the temperatures corresponding to the amoebal co-culture, i.e. 32 °C for *A. polyphaga* and *A. castellanii*, 30 °C for *V. vermiformis* and 25 °C for *D. discoideum*. The plates were checked daily for growth. Electron microscopy was used to study the cell morphology of the bacteria isolated, and to look for the presence of a flagellum. The novel bacterial strain was grown in co-culture with *D. discoideum* in starvation medium (Reteno et al., 2015) and on BCYE agar plates. Cells were negatively stained with a 3% ammonium molybdate solution and observed with a Tecnai G2 Cryo (FEI) electron microscope. The co-cultures were also analysed with Gram and Gimenez staining. Auto-fluorescence was evaluated by illuminating the strain on BCYE plates with a Wood’s lamp. The presence of β-lactamase and oxidase was assessed using Cefinase and Oxidase discs (Becton Dickinson), respectively. API 20E and campylobacter galleries (BioMerieux) were used to determine hippurate and gelatinase reactions, and sugar metabolism. For API galleries, heavy bacterial inoculums resuspended in normal saline buffer were dropped into the wells of the strip tests, following the manufacturer’s guide instructions. Strips were incubated at 37 °C for up to 72 h. Normal saline buffer was used as a negative control, and two strains representing the genus *Legionella* were used as positive controls (*Legionella massiliensis* CSUR P146T and *Legionella tunisiensis* CSUR P145T). The growth of the strain was tested on BYCE agar plates at the following temperatures: 25, 30, 32, 35 and 37 °C. L-Cysteine auxotrophy was determined using BCYE agar without l-cysteine (Oxoid).

Cells of strain LS-1T were Gram-stain-negative and Gimenez-stain-positive bacilli, appearing red after both staining procedures. Colonies were able to grow and become visible after 3 days at the optimum temperature (32 °C) on BCYE agar plates in a 5% CO₂ atmosphere. However, the strain was neither productive without CO₂ nor able to grow on Colombia agar with 5% sheep blood. At the other temperatures tested, growth was slow and resulted in fewer colonies. The strain grew at 42 °C with 5% CO₂, but formed very small colonies. The colonies were circular, with an approximate diameter of 0.8 mm, white in colour with a transparent appearance and showed blue auto-fluorescence under the Wood’s lamp. The colonies showed the typical opal-like appearance with reflected light. Cefinase and oxidase tests were positive, while hippurate and gelatinase reactions were negative. The electron microscopy images revealed a mean size of 1.87 µm in length and 0.61 µm in width when the bacteria were grown on BCYE agar plates. Flagella could be distinctly observed on electron microscopic pictures, one per bacteria, with mainly a polar position. Phenotypical characteristics of *L. saoudiensis* sp. nov. LS-1T are provided in Table 1, in comparison with those of the most closely related species of the genus *Legionella*. For most characteristics, strain LS-1T corresponds to the definition of the family *Legionellaceae*, and the genus *Legionella*, except for gelatin hydrolysis, which is negative for strain LS-1T. Strain LS-1T was primarily isolated from the co-culture with the amoeba *D. discoideum*. However, in further co-culture with other amoebal species, it could grow slowly within *A. polyphaga*, but did not grow within *A. castellanii* or *V. vermiformis* in starvation medium, at 32 and 30 °C, respectively. The size of the bacterial cells was different when grown in co-culture with the amoeba *D. discoideum*, in a starvation medium, showing a mean size of 1.19 µm in length and 0.701 µm in width. Bacterial motility showed a high speed in amoebae, especially in *D. discoideum*, but was lower with *A. polyphaga*.

The genome of strain LS-1T was sequenced using MiSeq Technology (Illumina) with the mate pair strategy. The minimum coverage was 163 and the quality score was 30. Automated cluster generation and sequencing runs were performed using a 2×300 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 Space and Opera software v1.2 (Bankevich et al., 2012; Boetzer et al., 2011; Gao et al., 2011). Multiple alignments were performed using the MUSCLE method, and the phylogenetic tree was reconstructed using a neighbour-joining method in FastTree, with bootstrap values based on 1000 replications. Pair-wise sequence similarity values between *L. saoudiensis* sp. nov. strain LS-1T and related taxa were computed using the EzTaxon server (http://www.ezbiocloud.net/eztaxon). Sequences of related taxa were obtained from the GenBank database.

After 3 days of 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 was negative for l-cysteine auxotrophy, as it did not grow on BCYE agar without l-cysteine.
Strain LS-1<sup>T</sup> exhibited 16S rRNA gene sequence similarity values of 98.6% and 98.5% with the type strains *Legionella rowbothamii* LLAP-6<sup>T</sup> and *Legionella lytica* L2<sup>T</sup>, 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<sup>T</sup> showed 94% similarity with that of *L. lytica* L2<sup>T</sup> and 93% similarity with that of *L. rowbothamii* strain LLAP-6<sup>T</sup>. The topology of
the phylogenetic tree based on the neighbour-joining method using 16S rRNA gene sequences supported the result that *L. saoudiensis* sp. nov. is a novel member of the genus *Legionella* (Fig. S1).

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

The *L. saoudiensis* sp. nov. genome is 3 847 980 bp long, containing one plasmid, with 39.42 mol% DNA G+C content, which is consistent with the DNA G+C contents of members of the genus *Legionella* (Brenner et al., 1979). It consists of 10 scaffolds (composed of 12 contigs). Of the 3387 predicted genes, 3341 are protein-coding genes, and 46 are 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 2288 genes (68.48%) were assigned a putative function (by cogs or by NR blast), and 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**

*Legionella saoudiensis* (sa.ou.di.en′sis N.L.fem. adj. saoudien-sis pertaining to Saudi Arabia, the country where the type strain originates).

Corresponds to the definition of the family *Legionellaceae* and to the definition of the genus *Legionella* (Brenner et al., 1979). Cells are Gram-stain-negative bacilli, which grow 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. Possesses β-lactamase activity, is oxidase-positive, hippurate-negative, and in contrast with other members of the genus *Legionella*, is unable to hydrolyse gelatin.

The type strain is LS-1\(^T\) (=DSM 101682\(^T\)=CSUR P2101\(^T\)), isolated from a sewage sample collected from Jeddah in the western region of Saudi Arabia. The DNA G+C content of the type strain is 39.42% and is within the range of that reported for the genus.

The CSUR culture collection website is available via the following link: http://www.mediterranean-infection.com/article.php?laref=14&title=collection-de-souches.

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**References**


