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NOTE

Legionella thermalis sp. nov., isolated from hot spring water in Tokyo, Japan

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ABSTRACT

Strain L-47^T of a novel bacterial species belonging to the genus *Legionella* was isolated from a sample of hot spring water from Tokyo, Japan. The 16S rRNA gene sequences (1477 bp) of this strain (accession number AB899895) had less than 95.0% identity with other *Legionella* species. The dominant fatty acids of strain L-47^T were a15:0 (29.6%) and the major ubiquinone was Q-12 (71.1%). It had a guanine-plus-cytosine content of 41.5 mol%. The taxonomic description of *Legionella thermalis* sp. nov. is proposed to be type strain L-47^T (JCM 30970^T = KCTC 42799^T).

Key words hot spring water, *Legionella thermalis* sp. nov, taxonomy.

The genus *Legionella* was first identified in 1977 following an epidemic of acute pneumonia in Philadelphia (1). At the time of writing this report, 59 species had been described (2). In 2002, a mass outbreak of legionellosis, transmitted through hot spring water in Miyazaki Prefecture, Japan, highlighted the importance of the hygienic management of bath water (3). Extensive information is available on *L. pneumophila*, a well-known causative agent of legionellosis, which is a respiratory disease (4–8). We have been investigating the distribution of *Legionella* spp. in the water of all of the hot springs in Japan for several years (9, 10). Because strain L-47^T was not included in the existing species of *Legionella* spp. when we identified strains that had been isolated from hot spring water by a 16S rRNA gene sequence analysis, we thought that this strain might represent a novel species and thus examined its taxonomy. In this report, we describe the phenotypic and phylogenetic characteristics of *Legionella* strain L-47^T and propose that it should indeed be classified as a novel species.

Strain L-47^T was isolated from hot spring water (spring quality; salt, 41.3°C, pH 7.3) in Tokyo, Japan, in May 2011, by a plating method. Five hundred milliliters of hot spring water was concentrated to 5 mL by filtration (pore size, 0.45 μm; cellulose acetate; Advantec, Tokyo, Japan). The concentrate was mixed with an equal volume of 0.2 M acid-phosphate buffer (pH 2.2) for preprocessing and the mixture incubated at room temperature for 10 min; 0.1 mL of the mixture was then spread over the surface of glycine vancomycin polymyxin-B cycloheximide and α-ketoglutarate medium (GVPCα) (Merck, Tokyo, Japan) with a Conradi stick and cultured at 36°C for 7 days. Several colonies of each isolate, which were suspected to be of the genus *Legionella*, were selected, smeared onto both BCYEα and blood agar mediums and subjected to pure culture and testing for cysteine requirement. The strains that failed to grow on the blood agar medium but grew on the BCYEα medium were presumed to belong to the genus *Legionella*. The isolated strain was preserved in 20% (w/v) glycerol and stored at −80°C.

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List of Abbreviations: BCYEα, buffered charcoal yeast extract α; CHCA, α-cyano-4-hydroxycinnamic acid; G + C, guanine-plus-cytosine; JCTC, Korean Collection for Type Cultures; MALDI-TOFMS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MIC, minimum inhibitory concentration.

The near-complete 16S rRNA gene sequence of strain L-47^T was determined as follows. Genomic DNA was extracted by the alkaline boiling method described by Beige *et al.* (11). The 16S rRNA genes were amplified using the 10F primer (5′-AGTTTGATCCTGGCTCAG-3′, corresponding to positions 10–27 of *Escherichia coli* 16S rRNA) and the 1541R primer (5′-AAGGAGGTG ATCCAGCCG-3′, positions 1524–1541) in a Thermal Cycler SP (Takara Bio, Otsu, Japan).

The nucleotide sequences were determined with a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) using 10F primer, 800F primer (5′-ATTAGATACCCTGGTA-3′, positions 800–816), 786R primer (5′-GACTACCA-GGGTATCTAATC-3′, positions 767–786) and 1541R primer according to the manufacturer's instructions and the results read on an Applied Biosystems 3130*xl* genetic analyzer.

A multiple sequence alignment analysis was performed using the CLUSTAL W software program (12) and gaps and unidentified base positions were deleted using a BioEdit (13) software program. Evolutionary distances were calculated using Kimura's two-parameter model (14). A phylogenetic tree was constructed using the neighbor-joining method (15), bootstrap values being calculated based on 1000 replications (16).

The near-complete 16S rRNA gene sequence (1477 bp) of strain L-47^T was determined (accession number, AB899895). A neighbor-joining tree (Fig. 1) showed that strain L-47^T was closely related to the 29 recognized species of the genus *Legionella* and one outgroup and that four strains formed a distinct cluster in the phylogenetic tree with 77% of bootstrap. The 16S rRNA gene in strain L-47 showed 91–95% similarity to the other species of *Legionella* and was most closely related to *L. busanensis* K9951 ^{T} (17) and *L. gresilensis* Gréoux 11 D13 ^{T} (18).

Gram staining was performed using a Gram stain kit (Nissui Pharmaceutical, Tokyo, Japan) according to the manufacturer's instructions. The shape and motility of the bacterial cells were observed under a phase-contrast microscope (×1000) with cell suspensions made from cultures grown on BCYEα agar (Merck Japan, Tokyo, Japan) at 36°C for 3 days. The cultures were plated on BCYEα agar and Müeller–Hinton agar (Becton Dickinson, Sparks, MD, USA) with and without the addition of 5% sheep blood at 36°C for seven days. Biochemical tests for gelatinase, urease and catalase, as well as for hippurate hydrolysis and nitrate reduction, were performed as described previously (19). Oxidase and β-lactamase activities were examined using test paper containing tetramethylphenylenediamine dihydrochloride (Nissui Pharmaceutical) and nitrocefin discs (Becton Dickinson), respectively.

In Table 1, we present the important phenotypic characteristics of strain L-47^T and compare them with those of the most closely related strain, *L. beliardensis* ATCC700512^T. The cells of strain L-47^T are gram-negative, motile, non-spore-forming rods. At 0.3–0.5 × 2.6–3.9 μm, these cells are relatively small and no longer cells were observed. The colonies of strain L-47^T aee gray-pigmented and measure approximately 1.0 mm in diameter on BCYEα agar after seven days of incubation at 36°C. No autofluorescence was observed when the colonies were exposed to UV light. No colony growth of strain L-47^T was observed on sheep blood or Müeller–Hinton agar after 7 days of incubation at 36°C. Strain L-47^T is positive for oxidase, catalase and β-lactamase and negative for urease and gelatinase activities. The characteristics of strain L-47^T are identical to those of *L. beliardensis* ATCC700512^T.

An API ZYM system (SYSMEX; bioMérieux, Tokyo, Japan) was used to test the cultures of legionellae for 18 different enzyme activities in accordance with the recommendations of the manufacturer regarding the preparation of the cell suspensions and conducting, incubating and reading the tests. The cell suspensions were made from cultures that had been grown on BCYEα agar at 36°C for 5 days.

In the 18 API ZYM tests, strain L-47^T was positive for alkaline phosphatase, acid phosphatase and leucine arylamidase activities (Table 1). The results were negative for esterase, esterase lipase, lipase, valine arylamidase, cysteine arylamidase, trypsin, chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. *L. beliardensis* ATCC700512^T is positive for alkaline phosphatase, acid phosphatase and leucine arylamidase activities; thus, evaluation of the enzyme activities was not useful for identifying the species.

Antibiotic susceptibility was determined using an Etest (SYSMEX; bioMérieux) according to the manufacturer's technical guidelines. The drugs tested consisted of piperacillin, imipenem, gentamicin, amikacin, erythromycin, clarithromycin, azithromycin, tetracycline, minocycline, vancomycin, ofloxacin, ciprofloxacin, levofloxacin, fosfomycin and rifampicin (a total of 15 drugs). A bacterial cell suspension (0.5 mL) was dripped onto 60 ml of BCYEα agar in a 150 mm dish (Corning, Cambridge, MA, USA), smeared over the surface using a Conradi stick and Etest strips were securely attached to the medium. The plates were cultured at 36°C for 5 days, and the growth inhibition zones that formed around the strips read. The MIC was judged by macroscopically reading the gradation at which the end of the growth inhibition zone and the strip crossed.

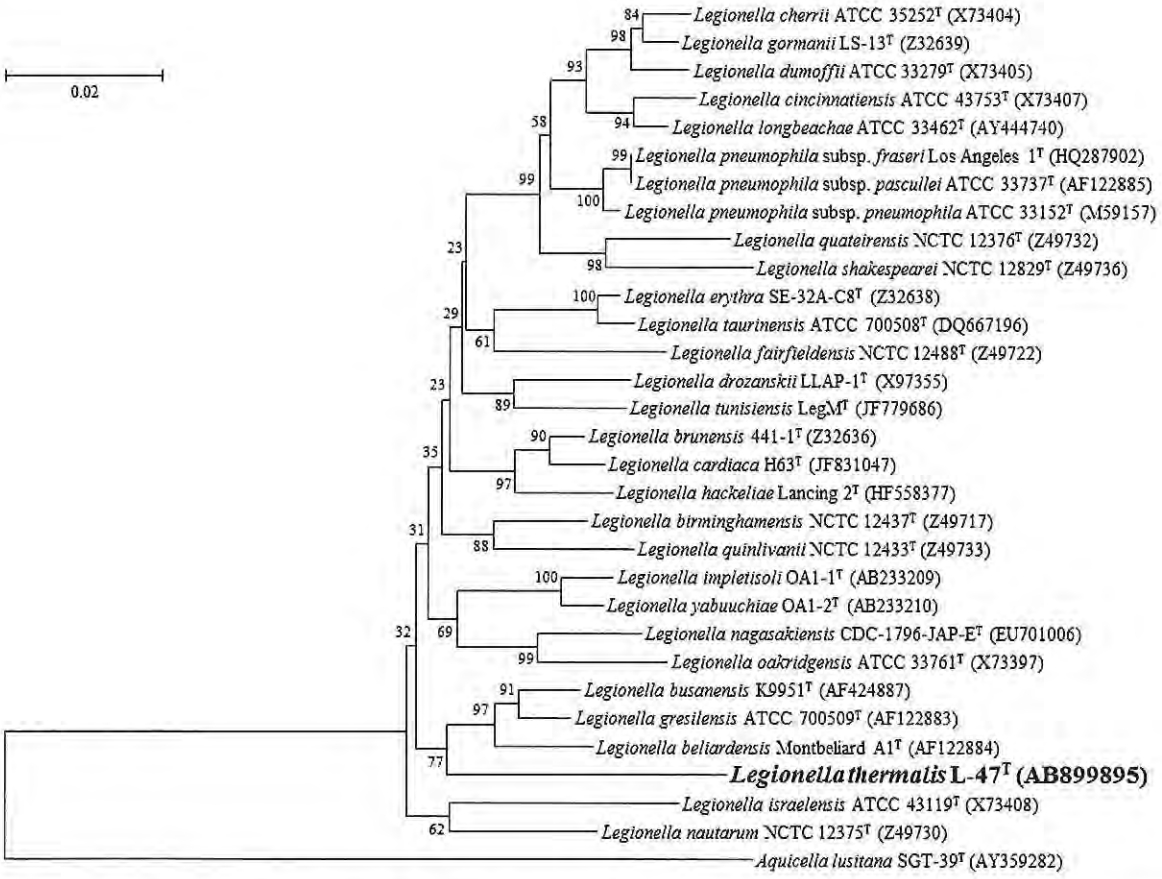

Fig. 1. Phylogenetic tree constructed using the neighbor-joining method based on the partial sequences of the 16S rRNA region (1477 bp) of strain L-47^T (accession number: AB899895) and related bacteria. The scale bar indicates the number of substitutions per nucleotide position.

Strain L-47^T was susceptible to piperacillin (MIC = 0.016 μg/mL), imipenem (MIC = 0.064 μg/mL), gentamicin (MIC = 2 μg/mL), amikacin (MIC = 4 μg/mL), erythromycin (MIC = 0.25 μg/mL), clarithromycin (MIC = 0.125 μg/mL), azithromycin (MIC = 0.064 μg/mL), tetracycline (MIC = 0.5 μg/mL), minocycline (MIC = 2 μg/mL), vancomycin (MIC = 4 μg/mL), ofloxacin (MIC = 0.25 μg/mL), ciprofloxacin (MIC = 0.25 μg/mL), levofloxacin (MIC = 0.125 μg/mL), fosfomycin (MIC = 32 μg/mL) and RF (MIC = 0.5 μg/mL). As shown in Table 1, the susceptibility of strain L-47^T resembled that of the other legionellae.

Analyses of the cellular fatty acid composition, quinones and DNA G + C content were performed by the TechnoSuruga Laboratory (Shizuoka, Japan). For these analyses, strain L-47^T was cultured at 36°C for 5 days in BCYEα agar medium. The cellular fatty acid composition was determined using a Sherlock Microbial Identification System (Version 6.0) (Midi, Newark, DE, USA) and the cellular fatty acid profile compared with the known profiles of Library CLIN6 6.20. Quinones were extracted from freeze-dried cells and analyzed using ultra performance liquid chromatography (ACQUITY UPLC system; Waters, Milford, MA, USA) using a modified version of a previously described method (20, 21). The G + C content of DNA from the isolate was determined using the method proposed by Katayama *et al.* (22). Genomic DNA was extracted from cultured cells by a phenol extraction method described by Marmur (23) and nucleosides obtained from the DNA by nuclease hydrolysis. The various peaks of the nucleosides were isolated and detected using the ACQUITY UPLC system and the G + C content determined.

Strain L-47^T was found to contain methyl branched fatty acids, predominantly a15:0 (29.6%), followed by i16:0 (18.1%) and a17:0 (11.9%) fatty acids (Table 2). The other detected fatty acids were as follows: 16:1ω7c

Table 1. Major phenotypic characteristics of two *Legionella* species

Characteristics	L-47^T	*L. beliardensis* ATCC 700512^T
Gram staining	−	−
Spore	−	−
Cell shape	rod	rod
Size (μm)	0.3–0.5 × 2.6–3.9	0.5–0.6 × 3.4–3.9
Motility	+	+
Autofluorescence	−	−
Growth on:		
BCYEα agar	+	+
Sheep blood agar	−	−
Müeller–Hinton agar	−	−
Biochemical reactions		
Oxidase	+	+
Catalase	+	+
Urease	−	−
Gelatinase	−	−
β-lactamase	+	+
Nitrate reduction	−	−
Hippurate hydrolysis	−	−
Enzyme activity (API ZYM test)		
Alkaline phosphatase	+	+
Acid phosphatase	+	+
Leucine arylamidase	+	+
Susceptibility (MIC, μg/mL)		
Amikacin	2	0.5
Tetracycline	2	8
Vancomycin	2	32
Fosfomycin	0.5	8
Major ubiquinone	Q-12	Q-12†
DNA G+C content (mol%)	41.5	38†
Isolation source	Hot spring water	Water†

†, Data from Presti *et al.* (18).

Table 2. Major cellular fatty acid composition (%) of *Legionella* species

Fatty acid	L-47^T	*L. beliardensis* ATCC 700512^T	Frequency in strain (%)
Saturated			
16:0	5.99	2.1	100
Saturated hydroxy			
15:0 2OH	0.73	0.22	16.4
Unsaturated			
15:1 ω6c	4.68	2.21	100
i16:1 H	0.14	0.52	56.7
16:1 ω7c	15.47	30.17	100
Methyl branched			
a15:0	29.55	26.99	100
i16:0	18.08	11.78	100
i17:0	0.29	0.47	73.1
a17:0	11.89	17.15	100
Branched-chain hydroxy			
i14:0 3OH	1.31	0.8	57.8
Total	88.13	92.41	

(15.5%), 16:0 (6.0%), 15:1ω6c (4.7%) and i14:0 3OH (1.3%). Strain L-47^T differs from *L. beliardensis* ATCC 700512^T in that it has a low 16:1ω7c content (15.5%). The fatty acid composition analysis of strain L-47^T does not correspond to that of any previously described *Legionella* species (24), most closely resembling that of *L. birminghamensis*, the similarity index which is 0.842 in the CLIN6 6.20 library. The fatty acid profile further supported that strain L-47^T differs from the other *Legionella* species.

As with other members of the genus *Legionella* (18), the predominant quinone of strain L-47^T is ubiquinone-12 (Q-12), which was found to be present at a rate of 71.1%, followed by Q-13 (25.0%) and Q-11 (3.9%). The DNA G+C content of strain L-47^T is 41.5 mol% (Table 1).

Microflex (Bruker Daltonics, Boston, MA, USA) was used to perform MALDI-TOFMS. The cells of a colony that had been cultured on BCYEα medium were smeared on a target plate (Bruker Daltonics), 1 μL of 1% CHCA matrix solution(Bruker Daltonics), which is a saturated solution of CHCA in 50% acetonitrile–2.5% trifluoroacetic acid added to the smeared cells and the measurement performed once it had dried. The acquired spectrum of each bacterial strain was imported into the BioTyper software program (version 2.0, Bruker Daltonik GmbH), and the spectra analyzed by standard pattern matching (with default parameter settings) against the spectra of the 5627 species in the BioTyper reference database (these spectra are an integrated part of the BioTyper version 2.0 software program, as updated in June 2015). The strain is defined when the score is ≥2.000.

The spectra acquired by MALDI-TOFMS analysis are presented in Figure 2. When these spectra were compared with the database, *L. beliardensis* ATCC700512 corresponded to *L. beliardensis* (score: 2.002). However, no spectra coincided with strain L-47^T (score: 1.246). The MALDI-TOFMS analysis further supported the notion that strain L-47^T differs from other *Legionella* species.

The properties of strain L-47^T were further examined. Some of the properties that differentiate it from *L. beliardensis* ATCC700512^T are shown in Table 1. According to comparison of the 16S rRNA gene sequences (1477 bp) and the data from the phylogenetic analysis (Fig. 1), strain L-47^T was classified as a member of the genus *Legionella* but, with similarity values of <97%, it was clearly separate from previously-described

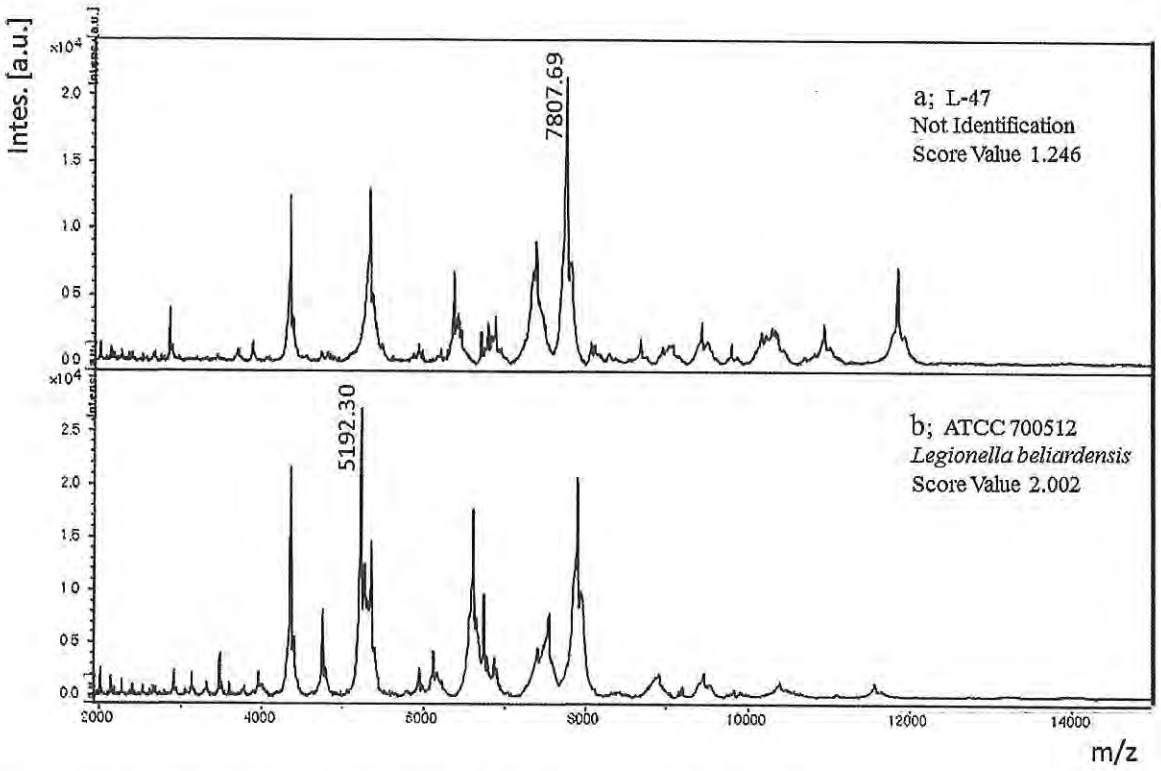

Fig. 2. The MALDI spectra of (a) *Legionella* sp. L-47 and (b) *Legionella beliardensis* ATCC700512.

species. It has been suggested that bacterial strains with <97% 16S rRNA gene sequence identity are members of different genomic species (25). Based on the phylogenetic data, it is therefore evident that strain L-47^T represents a novel species of the genus *Legionella*. Based on the above-described phylogenetic, chemotaxonomic and phenotypic results, it was concluded that strain L-47^T represents a novel species of the genus *Legionella*, *Legionella thermalis* sp. nov.

DESCRIPTION OF *LEGIONELLA THERMALIS* SP. NOV.

Legionella thermalis (ther'ma.lis. N.L. fem. adj. *thermalis*, of a hot spring, indicating the site from which the type strain was isolated).

The cells are gram-negative, non-spore-forming, motile, 0.3–0.5 × 2.6–3.9 μm rods. The colonies are not irregular, with a diameter of approximately 1.0 mm on BCYEα agar after 7 days of incubation at 36°C. No autofluorescent reaction was observed. The cells are positive for oxidase, catalase and β-lactamase activity and negative for urease and gelatinase activity, nitrate reduction and hippurate hydrolysis. Alkaline phosphatase and acid phosphatase are present at high levels, and there are moderately strong leucine arylamidase activities. The cells are susceptible to piperacillin (MIC = 0.016 μg/mL), imipenem and azithromycin (MIC = 0.064 μg/mL), clarithromycin and levofloxacin (MIC = 0.125 μg/mL) and erythromycin, ofloxacin and ciprofloxacin (MIC = 0.25 μg/mL). The major cellular fatty acids of strain L-47^T are a15:0 (29.6%), followed by i16:0 (18.1%), 16:1ω7c (15.5%) and a17:0 (11.9%). The major ubiquinone is Q-12 (71.1%), followed by Q-13 (25.0%) and Q-11 (3.9%). The type strain, L-47^T (= JCM 30970^T = KCTC 42799^T), was isolated from hot spring water in Tokyo, Japan. It has a G + C content of 41.5 mol%.

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DISCLOSURE

No authors declare any conflicts of interest in association with the present study.

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