Legionella nagasakiensis sp. nov., isolated from water samples and from a patient with pneumonia

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A novel *Legionella* species was identified based on analysis of 16S rRNA and *mip* (macrophage infectivity potentiator) gene sequences, cellular fatty acids, isoprenoid quinones, biochemical reactions, antigens and quantitative DNA–DNA hybridization. Strain CDC-1796-JAP-E^T was isolated from well water at the Nagasaki Municipal Medical Center, Japan. Two strains, CDC-3041-AUS-E and CDC-3558-AUS-E, were isolated from water samples during an outbreak of legionellosis in South Australia. Strain CDC-5427-OH-H was isolated from a 66-year-old female patient diagnosed with Legionnaires' disease in the US. Cells from these four strains were Gram-negative, non-fluorescent, rod-shaped, and positive for alkaline phosphatase, esterase, leucine arylamidase, catalase, gelatinase, β -lactamase and tyrosine browning assay. Phylogenetic analysis of 16S rRNA and *mip* genes revealed that the four strains formed a distinct cluster within the genus *Legionella*. The bacteria contained branched-chain fatty acids and quinones that are typical of members of the genus *Legionella*. Slide agglutination tests demonstrated no cross-reaction with 52 previously described members of the *Legionellaceae*. DNA–DNA hybridization studies indicated that DNAs from the four strains were highly related (78–84 %) but they showed 29 % relatedness to *Legionella oakridgensis* ATCC 33761^T and less than 10 % to strains of other *Legionella* species tested. These characterizations suggest that the isolates represent a novel species, for which the name *Legionella nagasakiensis* sp. nov. is proposed; the type strain is CDC-1796-JAP-E^T (=ATCC BAA-1557^T=JCM 15315^T).

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Abbreviations: CFA, cellular fatty acids; LLOs, *Legionella*-like organisms; *mip*, macrophage infectivity potentiator.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains CDC-3558-AUS-E, CDC-3041-AUS-E, CDC-1796-JAP-E^T and CDC-5427-OH-H are FJ236837, FJ236838, EU701006 and EU701007, respectively. The GenBank/EMBL/DDBJ accession numbers for the *mip* gene sequences of strains CDC-3041-AUS-E, CDC-1796-JAP-E^T, CDC-5427-OH-H and CDC-3558-AUS-E are FJ236839, FJ236840, FJ236841 and FJ236842, respectively.

A supplementary figure and two supplementary tables are available with the online version of this paper.

The genus *Legionella* belongs to the family *Legionellaceae*. Fifty-three *Legionella* species containing 73 serogroups have been described (Lück *et al.*, 2010). More than 40 additional isolates have been identified as *Legionella*-like organisms (LLOs) in the Centers for Disease Control and Prevention strain collection (unpublished results). These LLOs resemble members of the *Legionellaceae* morphologically and require L-cysteine for growth. *Legionellaceae* are ubiquitous in soils, and in natural and man-made aquatic environments (Fields *et al.*, 2002). At least 24 species of *Legionella* have been reported as being pathogenic in humans (Newton *et al.*, 2010). The pathogenic species

cause legionellosis, a significant respiratory disease that classically takes two distinct clinical forms: Legionnaires' disease, a severe multisystem disease involving pneumonia; or Pontiac Fever, a milder flu-like illness (Fields *et al.*, 2002). In this paper, the growth, macrophage infectivity potentiator (*mip*) and 16S rRNA gene sequences, physiological characteristics, cellular fatty acid (CFA) composition, serological characteristics, DNA relatedness data of a novel *Legionella* species are described. Strains of this novel species, named *Legionella nagasakiensis* sp. nov., were isolated from well water in Japan, cooling tower water in Australia and a patient with pneumonia in the United States.

Strain CDC-1796-JAP-E^T was isolated from well water in Nagasaki, Japan, using the following method. A 500 ml sample was collected in sterile bottles and centrifuged at 10 000 r.p.m. for 20 min. The supernatant was decanted and 5.0 ml sediment was removed, acid-treated and plated on selective BCYE (buffered charcoal-yeast extract) medium supplemented with PAV (polymyxin B, anisomycin and vancomycin) (Bopp *et al.*, 1981). Plates were incubated at 35 °C and examined for growth of *Legionella* colonies. Colonies resembling *Legionella* were subcultured on BCYE agar. Formalin-fixed cells were stained with direct immunofluorescence conjugates for *Legionella pneumophila* serogroups 1–6, *Legionella micdadei*, *Legionella dumoffii*, *Legionella bozemanii* serogroup 1, *Legionella gormanii* and *Legionella longbeachae* serogroup 1. Strain CDC-1796-JAP-E^T (=82-059^T) failed to react with these antisera and was forwarded to CDC for further studies. Strains IMVS 86 (=CDC-3041-AUS-E) and IMVS 934 (=CDC-3558-AUS-E) were received from the Institute of Medical and Veterinary Science, Adelaide, South Australia. These strains were previously designated 'species B' (Wilkinson *et al.*, 1990). Strain CDC-5427-OH-H was received from Ohio State University Hospital East, Columbus, Ohio, USA, and had been isolated from a bronchial alveolar lavage specimen obtained from a 66-year-old female patient diagnosed with Legionnaires' disease.

All strains were streaked for single colony isolation on BCYE agar. The ACES buffer was omitted for determination of autofluorescence and L-cysteine was omitted for the determination of cysteine requirement. Physiological tests for flagella, autofluorescence and the enzymes listed in Table 1 were performed using API ZYM (bioMérieux) according to the manufacturer's instructions or as described previously (Vesey *et al.*, 1988). All isolates were tested with antisera to previously characterized *Legionella* species available to us ($n=52$) and serogroups ($n=74$) (Benson *et al.*, 1996; Dennis *et al.*, 1993; Thacker *et al.*, 1992; Verma *et al.*, 1992). Antisera to the novel isolates (CDC-1796-JAP-E^T, CDC-3041-AUS-E and CDC-3558-AUS-E) were prepared and tested as described previously (Thacker *et al.*, 1985).

DNA for sequence determination was extracted using a QIAamp DNA Mini kit (Qiagen). 16S rRNA gene was

amplified using the Expand high-fidelity PCR system (Roche) with primers fd1 and rp2 (Weisburg *et al.*, 1991). The *mip* gene was amplified as described previously (Ratcliff *et al.*, 1998). Sequencing reactions were performed on at least two separately generated amplification products to minimize the possibility of sequencing errors. Each set of gene target sequences was phylogenetically compared with the published type strains of each species. Additional sequencing of the 16S rRNA genes from some species was performed to remove ambiguities from published sequences. Sequence assembly, alignment and analysis were performed using Kodon (Applied Maths) and phylogenetic analysis (maximum-likelihood) was performed using PAUP 4 (Sinauer Associates). The best fit model GTR + I + G used for the analysis was assessed using Modeltest 3.7 (Posada & Crandall, 1998) and relationship trees were drawn with FigTree v1.1.2 (A. Rambaut, Institute of Evolutionary Biology, University of Edinburgh, UK).

For CFA and isoprenoid quinone analyses, all strains were suspended in water and diluted to the same concentration (based on optical density readings of 0.9 at 600 nm). A bacterial suspension of 100 µl was streaked on BCYE agar plates and cultured at 35 °C with 2.5 % CO₂ for 48 h. Cells harvested from the second quadrant were saponified and processed and CFA (as methyl esters) were analysed by capillary GLC as described previously (Lambert & Moss, 1989). Isoprenoid quinones were extracted from 100–250 mg lyophilized cells and analysed by reverse-phase HPLC (Moss & Guerrant, 1983). Individual fatty acids and quinones were tentatively identified by retention time comparisons to known standards and were subsequently confirmed by MS (Lambert & Moss, 1989).

Relative binding ratios were determined using the free-resolution hydroxyapatite method performed at 60 °C (Brenner *et al.*, 1982). *In vitro* labelling of DNA was performed enzymically by using [³²P]dCTP provided in a nick-translation reagent kit (Bethesda Research Laboratories), as directed by the manufacturer.

All four isolates (CDC-1796-JAP-E^T, CDC-3558-AUS-E, CDC-3041-AUS-E and CDC-5427-OH-H) required L-cysteine for primary isolation, but they were able to adapt to cysteine-deficient media in serial transfer on BCYE agar. The loss of requirement for L-cysteine has also been observed in *Legionella oakridgensis* and *Legionella jordanis* (Orrison *et al.*, 1983). Cells from these strains were Gram-negative rods with single polar flagella. Colonies of these strains did not exhibit autofluorescence when exposed to UV light.

Analysis of the 16S rRNA and *mip* genes of these strains resulted in a discrete cluster of sequences (0.1 % variant) within the genus *Legionella*. Alignment of the 16S rRNA genes of the isolates produced three sequence variants from two positions, with identity shared between strains CDC-3558-AUS-E and CDC-1796-JAP-E^T (Supplementary Fig. S1a, available in IJSEM Online). Alignment of *mip* genes for the four isolates produced three sequence variants from

Table 1. Biochemical characteristics of *L. nagasakiensis* CDC-1796-JAP-E^T and its most closely related species and controls

Strains: 1, *L. nagasakiensis* CDC-1796-JAP-E^T; 2, *L. oakridgensis* ATCC 33761^T; 3, *L. impletisoli* DSM 18493^T; 4, *L. yabuuchiae* DSM 18492^T; 5, *L. pneumophila* ATCC 33152^T. +, Positive; w, weak positive; —, negative; NA, not available. Tests 1–19 were performed using API ZYM (bioMérieux) following the instructions. Tests 20–27 were performed as described previously (Vesey *et al.*, 1988). All negative controls were performed using uninoculated media unless otherwise indicated. All positive controls were performed using the indicated bacterial strains or purified enzymes.

Test no.	Enzyme/substrate	1	2	3	4	5	Negative control*	Positive control†
1	Alkaline phosphatase	+	+	+	+	+	—	+ ^a
2	Esterase (C4)	+	+	+	+	+	—	+ ^a
3	Esterase lipase (C8)	+	+	+	+	+	—	+ ^a
4	Lipase	—	—	—	—	—	—	+ ^a
5	Leucine arylamidase	+	+	+	+	+	—	+ ^a
6	Valine arylamidase	+	+	+	+	+	—	NA
7	Cystine arylamidase	+	+	+	+	+	—	NA
8	Trypsin arylamidase	—	w	—	—	—	—	+ ^b
9	α-Chymotrypsin	—	—	—	—	—	—	+ ^b
10	Acid phosphatase	+	+	+	+	+	—	+ ^c
11	Naphthol-AS-BI-phosphohydrolase	+	+	w	—	+	—	+ ^c
12	α-Galactosidase	w	—	—	—	—	—	+ ^c
13	β-Galactosidase	—	—	—	—	—	—	+ ^c
14	β-Glucuronidase	—	—	—	—	—	—	NA
15	α-Glucosidase	—	—	—	—	—	—	NA
16	β-Glucosidase	—	—	—	—	—	—	+ ^c
17	N-Acetyl-β-glucosaminidase	—	—	—	—	w	—	+ ^c
18	α-Mannosidase	w	—	—	—	—	—	+ ^c
19	α-Fucosidase	—	—	—	—	—	—	NA
20	Catalase	+	+	—	+	+	—	+ ^d
21	Gelatinase	+	+	—	—	+	— ^a	+ ^e
22	Urease	—	—	—	—	—	—	+ ^f
23	β-Lactamase	+	+	—	—	+	— ^a	+ ^g
24	Hippurate hydrolysis	—	—	—	—	+	— ^a	+ ^d
25	Nitrate	—	—	—	—	—	—	+ ^h
26	D-Glucose	—	—	—	—	—	— ^b	+ ^h
27	Tyrosine‡	+	—	—	—	+	— ^a	+ ^d

*Controls: a, *L. micdadei*; b, *L. pneumophila* sg1.

†Controls: a, *Pseudomonas aeruginosa* ATCC 27853; b, α-chymotrypsin (Sigma); c, β-glucosidase (Sigma); d, *L. pneumophila* sg1 strain Knoxville-1; e, *L. bozemanii* ATCC 33217^T; f, *Providencia rettgeri*; g, *L. micdadei* ATCC 33218^T; h, *E. coli* ATCC 25922.

‡Browning with tyrosine was read twice at 3 days and 5 days post-incubation.

three positions. Identity was shared between strains CDC-5427-OH-H and CDC-1796-JAP-E^T (Supplementary Fig. S1b, available in IJSEM Online). The isolates showed dissimilarities of 3.5 % and 14.4 % for the 16S rRNA and *mip* gene sequences, respectively, to the type strain of the most closely related species, *L. oakridgensis*. Such levels of divergence are, for both gene targets, several fold greater than that for existing recognized species. For example, only 1.7 % dissimilarity in 16S rRNA gene was observed between *L. longbeachae* and *Legionella sainthelensi*, and *mip* gene sequencing revealed less than 7 % dissimilarity between the tight cluster of species comprising *Legionella anisa*, *L. bozemanii*, *Legionella parisiensis* and *Legionella tucsonensis* (data not shown).

The CFA profiles of saponified cells of the four isolates were essentially identical. Like other *Legionella* species (Lambert

& Moss, 1989), they contained significant amounts of branched-chain fatty acids with no or only traces of hydroxyl fatty acids (Supplementary Table S1, available in IJSEM Online). iso-C_{16:0} and C_{16:1}ω7c were the two major fatty acids. In comparison with the most closely related species, *L. oakridgensis*, the isolates contained twice the amounts of anteiso-C_{15:0}, C_{15:1}ω7c, C_{15:0} and C_{17:0} and about half the amounts of iso-C_{16:1}, C_{17:0} and C_{18:0}.

Each of the four isolates contained ubiquinone-10 (Q-10) as the major quinone with small amounts of Q-9 and Q-11. This quinone profile is essentially identical to that of *L. oakridgensis* and *Legionella wadsworthii* that were previously placed together in a separate ubiquinone group (Lambert & Moss, 1989; Wilkinson *et al.*, 1990).

Antisera prepared against three of the isolates (CDC-3558-AUS-E, CDC-3041-AUS-E, CDC-1796-JAP-E^T) did not

react to any of the 52 previously described *Legionella* species or serogroups using the slide agglutination test, including strains of the most closely related species, i.e. *L. oakridgensis* ATCC 33761^T, *Legionella impletisoli* DSM 18493^T and *Legionella yabuuchiae* DSM 18492^T.

Reactions of strain CDC-1796-JAP-E^T in 27 different biochemical tests revealed that the isolate differed from its nearest neighbours and *Legionella pneumophila*, the type species of the genus *Legionella* (Table 1). For example, trypsin arylamidase was positive for *L. oakridgensis* ATCC 33761^T, but negative for CDC-1796-JAP-E^T. On the other hand, CDC-1796-JAP-E^T was positive for α -galactosidase, α -mannosidase and tyrosine browning, whereas *L. oakridgensis* was negative for these reactions. The isolates could be clearly distinguished from *L. impletisoli* DSM 18493^T and *L. yabuuchiae* DSM 18492^T based on tests for naphthol-AS-BI-phosphohydrolase, gelatinase, β -lactamase and tyrosine browning (Table 1).

DNA relatedness, determined by relative binding ratios at 60 °C, showed that labelled DNA from CDC-1796-JAP-E^T was 78.5–83.5 % related to DNA from the other three novel isolates (Supplementary Table S2, available in IJSEM Online). The divergence (%) in these reactions was 0–0.8 %. The levels of relatedness to two strains of *L. oakridgensis*, the most closely related species, were 29 ± 2.8 and 35.5 ± 3.5 % and relatedness was less than 10 % to DNAs of the 32 *Legionella* species and six LLOs tested (Supplementary Table S2).

This study demonstrated that the environmental and clinical isolates (CDC-1796-JAP-E^T, CDC-3558-AUS-E, CDC-3041-AUS-E and CDC-5427-OH-H) represent a novel pathogenic *Legionella* species, *L. nagasakiensis* sp. nov. The morphology, biochemical reactions and CFA profiles of these strains are typical of the genus *Legionella*. They clustered within the family *Legionellaceae* in both 16S rRNA and *mip* gene phylogenetic trees. Their 16S rRNA genes had 96 % sequence similarity to that of the type strain of *L. oakridgensis*, but the less conserved *mip* genes revealed only 85.5 % sequence similarity to the type strain of *L. oakridgensis*. CFA profiles, antiserum agglutination and biochemical reactions of the novel isolates were also different from those of *L. oakridgensis*. Genome-wide DNA–DNA hybridization analysis further proved that the isolates were distinct from *L. oakridgensis* and other *Legionella* species tested. Although the 16S rRNA and *mip* genes of all four isolates were nearly identical, it has been reported that genetic analysis that is based on an individual gene is of limited use in serogroup level differentiation of *Legionellaceae* (Ratcliff *et al.*, 1998). In our studies, we found antisera reacted strongly with their homologous antigens, but presented different patterns for heterologous antigens, suggesting that there are multiple serogroups of *L. nagasakiensis* (data not shown). Indeed, the genome-wide analysis using DNA hybridization demonstrated that the genomes of the *L. nagasakiensis* strains differed by 17–22 % (Supplementary Table S2).

Description of *Legionella nagasakiensis* sp. nov.

Legionella nagasakiensis (na.ga.sa.ki.en'sis. N.L. fem adj. *nagasakiensis* of/from Nagasaki, Japan, where the organism was isolated).

Gram-negative rods, single polar flagella. Grows on BCYE agar and requires L-cysteine for primary isolation. Positive for alkaline phosphatase, esterase, esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, catalase, gelatinase, β -lactamase and tyrosine browning. CFA are predominantly branched-chain; major isoprenoid quinone is Q-10. DNA relatedness data and serological reactions indicate that this species is distinct from other members of the *Legionellaceae*. Associated with human disease as one of the strains was isolated from a pneumonia patient.

The type strain is CDC-1796-JAP-E^T (=ATCC BAA-1557^T=JCM 15315^T).

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References

- Benson, R. F., Thacker, W. L., Daneshvar, M. I. & Brenner, D. J. (1996). *Legionella waltersii* sp. nov. and an unnamed *Legionella* genomospecies isolated from water in Australia. *Int J Syst Bacteriol* **46**, 631–634.
- Bopp, C. A., Sumner, J. W., Morris, G. K. & Wells, J. G. (1981). Isolation of *Legionella* spp. from environmental water samples by low-pH treatment and use of a selective medium. *J Clin Microbiol* **13**, 714–719.
- Brenner, D. J., McWhorter, A. C., Knutson, J. K. & Steigerwalt, A. G. (1982). *Escherichia vulneris*: a new species of *Enterobacteriaceae* associated with human wounds. *J Clin Microbiol* **15**, 1133–1140.
- Dennis, P. J., Brenner, D. J., Thacker, W. L., Wait, R., Vesey, G., Steigerwalt, A. G. & Benson, R. F. (1993). Five new *Legionella* species isolated from water. *Int J Syst Bacteriol* **43**, 329–337.
- Fields, B. S., Benson, R. F. & Besser, R. E. (2002). *Legionella* and Legionnaires' disease: 25 years of investigation. *Clin Microbiol Rev* **15**, 506–526.
- Lambert, M. A. & Moss, C. W. (1989). Cellular fatty acid compositions and isoprenoid quinone contents of 23 *Legionella* species. *J Clin Microbiol* **27**, 465–473.
- Lück, P. C., Jacobs, E., Röske, I., Schröter-Bobsin, U., Dumke, R. & Gronow, S. (2010). *Legionella dresdenensis* sp. nov., isolated from river water. *Int J Syst Evol Microbiol* **60**, 2557–2562.
- Moss, C. W. & Guerrant, G. O. (1983). Separation of bacterial ubiquinones by reverse-phase high-pressure liquid chromatography. *J Clin Microbiol* **18**, 15–17.
- Newton, H. J., Ang, D. K., van Driel, I. R. & Hartland, E. L. (2010). Molecular pathogenesis of infections caused by *Legionella pneumophila*. *Clin Microbiol Rev* **23**, 274–298.
- Orrison, L. H., Cherry, W. B., Tyndall, R. L., Fliermans, C. B., Gough, S. B., Lambert, M. A., McDougal, L. K., Bibb, W. F. & Brenner, D. J. (1983). *Legionella oakridgensis*: unusual new species isolated from cooling tower water. *Appl Environ Microbiol* **45**, 536–545.

- Posada, D. & Crandall, K. A. (1998).** MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14**, 817–818.
- Ratcliff, R. M., Lanser, J. A., Manning, P. A. & Heuzenroeder, M. W. (1998).** Sequence-based classification scheme for the genus *Legionella* targeting the *mip* gene. *J Clin Microbiol* **36**, 1560–1567.
- Thacker, W. L., Plikaytis, B. B. & Wilkinson, H. W. (1985).** Identification of 22 *Legionella* species and 33 serogroups with the slide agglutination test. *J Clin Microbiol* **21**, 779–782.
- Thacker, W. L., Dyke, J. W., Benson, R. F., Havlichek, D. H., Jr, Robinson-Dunn, B., Stiefel, H., Schneider, W., Moss, C. W., Mayberry, W. R. & Brenner, D. J. (1992).** *Legionella lansingensis* sp. nov. isolated from a patient with pneumonia and underlying chronic lymphocytic leukemia. *J Clin Microbiol* **30**, 2398–2401.
- Verma, U. K., Brenner, D. J., Thacker, W. L., Benson, R. F., Vesey, G., Kurtz, J. B., Dennis, P. J., Steigerwalt, A. G., Robinson, J. S. & Moss, C. W. (1992).** *Legionella shakespearei* sp. nov., isolated from cooling tower water. *Int J Syst Bacteriol* **42**, 404–407.
- Vesey, G., Dennis, P. J., Lee, J. V. & Westy, A. A. (1988).** Further development of simple tests to differentiate the legionellas. *J Appl Bacteriol* **65**, 339–345.
- Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991).** 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697–703.
- Wilkinson, I. J., Sangster, N., Ratcliff, R. M., Mugg, P. A., Davos, D. E. & Lanser, J. A. (1990).** Problems associated with identification of *Legionella* species from the environment and isolation of six possible new species. *Appl Environ Microbiol* **56**, 796–802.