Note

Legionella busanensis sp. nov., isolated from cooling tower water in Korea

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Three *Legionella*-like micro-organisms, isolated from cooling tower water of a building in Busan, Korea, were characterized by a variety of biochemical and molecular phylogenetic tests. Analyses of whole-cell fatty acids and results of biochemical tests revealed that these three isolates are distinct from previously described *Legionella* species. Furthermore, results of comparative analyses of 16S rDNA (1476–1488 bp), *mip* (408 bp) and *rpoB* (300 bp) sequences also confirmed that these strains represent a novel species within the genus *Legionella*. The 16S rDNA sequences of the three Korean isolates had similarities of less than 95.8% to other *Legionella* species. Phylogenetic trees formed by analysis of the 16S rRNA, *rpoB* and *mip* genes revealed that the isolates formed a distinct cluster within the genus *Legionella*. Based on the evaluated phenotypic and phylogenetic characteristics, it is proposed that these Korean isolates from water be classified as a novel species, *Legionella busanensis* sp. nov.; the type strain is strain K9951^T (=KCTC 12084^T =ATCC BAA-518^T).

The genus *Legionella* was first identified in 1977 following an epidemic of acute pneumonia in Philadelphia (Fraser *et al.*, 1977). At the time of writing, 47 species have been described (Euzéby, 1997; Lo Presti *et al.*, 2001), including four *Legionella*-like amoebal pathogens (Hookey *et al.*, 1996; Adeleke *et al.*, 2001). Twenty-one species are pathogenic to humans (Winn, 1999). Morphology and biochemical traits are so alike between species or so atypical that they are of no use in the differentiation of species within the genus *Legionella* (Winn, 1999). Thus, the use of fatty acid analysis and molecular tools, such as DNA–DNA relatedness and 16S rRNA gene sequencing, have recently gained prominence for the identification of novel species (Benson *et al.*, 1996; Lo Presti *et al.*, 1999).

Three *Legionella*-like organisms (strains K9951^T, K9952 and K9953) were isolated in 1999 from the water of cooling towers in Busan, Korea, by the Korea National Institute of Health (KNIH). On the basis of their cultural and staining

characteristics, they were tentatively identified as members of *Legionella*. They grew for 3–4 days at 35 ± 1 °C in the presence of 2.5% CO₂ on buffered charcoal/yeast extract (BCYE) agar, but not in the absence of L-cysteine. The organisms were Gram-negative and were observed to form cut-glass colonies on BCYE- α agar (Winn, 1999) under the dissecting microscope, both of which are characteristics of *Legionella*. Buffer and cysteine were respectively omitted for the determination of autofluorescence and requirement for cysteine (Benson *et al.*, 1996). Biochemical tests for gelatinase, urease, catalase, oxidase, peroxidase and β -lactamase activities, as well as hippurate hydrolysis, nitrate reduction, autofluorescence and browning of tyrosine-supplemented agar were performed as described previously (Fox & Brown, 1989; Hebert, 1981; Orrison *et al.*, 1981; Pine *et al.*, 1984; Weaver & Feeley, 1979). The three strains were tested with commercially available antisera (Denka Seiken) and antisera prepared in the KNIH laboratory against the type strains of 13 *Legionella* species including 25 serogroups (*Legionella pneumophila* serogroups 1–15, *Legionella dumoffii*, *Legionella micdadei*, *Legionella bozemanii*, *Legionella gormanii*, *Legionella anisa*, *Legionella feeleyi* serogroup 2, *Legionella longbeachae* serogroups 1 and 2, *Legionella parisiensis*, *Legionella rubrilucens*, *Legionella spiritensis*, *Legionella wadsworthii* and *Legionella jordanis*) by the slide-agglutination test (Benson *et al.*, 1996).

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The GenBank accession numbers for the 16S rDNA, *rpoB* and *mip* sequences of strain K9951^T are AF424887–AF424889.

An *rpoB*-based dendrogram including a wider selection of *Legionella* type strains is available as supplementary material in IJSEM Online (<http://ijs.sgmjournals.org/>).

The three strains were cultivated in BCYE broth for 48 h at 35 ± 1 °C and negatively stained with 2 % uranyl acetate to identify flagella by TEM ($\times 15\,000$; JEOL, JEM-1010). Analysis of cellular fatty acid composition was performed using a Hewlett Packard 6890A GC and the MIDI aerobic method (Chem Station ver. 4.02) at MicroID, Daejeon, Korea. The cellular fatty acid profiles were compared with those of Diogo *et al.* (1999). The G+C contents of DNA from the three strains were determined spectrophotometrically by the thermal denaturation method (Marmur & Doty, 1962).

Genomic DNA was prepared from the three Korean isolates using the bead beater-phenol extraction method (Kim *et al.*, 1999). The 16S rDNA (~ 1.5 kb) was amplified by PCR using primers p246 and pH (Eckloff *et al.*, 1994). The purified PCR product was sequenced directly using an Applied Biosystems model 377 automated sequencer and a BigDye Terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems). Primers pL259 (5'-GGCGACGATCG GTAGCTGGT-3') and pL632 (5'-GTGGAATTTCCGGTG TAGCGG-3') were used as internal primers for sequencing. For the amplification of partial *rpoB* and *mip* DNAs, primers RL1 (5'-GATGATATCGATCAYCTDGG-3')/RL2 (5'-TTC VGGCGTTTCAATNGGAC-3') and ML1 (5'-GATAAGTT GTCTTATAGCATTGG-3')/ML2 (5'-TCTGTCCATCCTG GGATAACTTG-3'), respectively, were used. The determined nucleotide sequences of 16S rDNA (1476–1488 bp), *rpoB* DNA [300 bp; 1407–1436 bp in Nielson *et al.* (2000)] and *mip* DNA [408 bp; 832–1239 bp in Bangsberg *et al.* (1991)] were submitted to GenBank and used for phylogenetic analyses. The 16S rDNA and *mip* sequences of other *Legionella* species and *Coxiella burnetii* retrieved from GenBank were also used. Phylogenetic trees were constructed according to the neighbour-joining method with the PAUP program (Swofford, 1999). The robustness of the groupings was estimated by bootstrap analysis (1000 replications).

The three isolates were thought to belong to the genus *Legionella* in view of their Gram-negative staining and requirement for L-cysteine for growth at 35 °C. Therefore, their biochemical characteristics were compared with those of previously described *Legionella* species. The three strains possessed a single subpolar flagellum. Biochemical tests for gelatinase, hippurate hydrolysis and oxidase were positive. However, tests were negative for β -lactamase and browning of tyrosine-supplemented agar. The results of biochemical tests such as gelatinase, hippurate, oxidase and β -lactamase activities indicated that these strains did not belong to any currently described *Legionella* species (Hookey *et al.*, 1996). No autofluorescence was observed when colonies of the three isolates were exposed to UV light. They did not react with any of the 25 antisera tested.

The three Korean strains contained unsaturated fatty acids, predominantly 16:1 ω 7c (43.58–46.62 %), followed by i-16:0 (16.47–20.07 %) and 16:0 (8.60–10.13 %). Other fatty acids also detected were: 15:1 ω 6c (4.25–5.96 %); 17:0 (2.52–3.25 %); 18:0 (2.91–3.16 %); i-14:0 (2.65–2.98 %);

i-18:0 (1.65–3.05 %); ai-15:0 (1.58–2.18 %); 15:0 (1.32–2.37 %); ai-17:0 (1.30–1.38 %); 14:1 ω 5c (1.06–1.60 %); and 20:0 (0–1.25 %). The fatty acid composition also demonstrated that these strains did not correspond to any previously described *Legionella* species (Diogo *et al.*, 1999). According to Hookey *et al.* (1996) and Diogo *et al.* (1999), *Legionella adelaidensis*, *L. feeleyi*, *Legionella geestiana*, *Legionella moravica*, *Legionella quateirensis*, *Legionella quinlivanii*, *Legionella santicrucis*, *Legionella worsleiensis*, *Legionella* sp. Montbeliard A1 and *Legionella* sp. Greoux 11D13 contain predominantly 16:1 ω 7c. However, all of these species, with the exception of Greoux 11D13, had a 16:1 ω 7c content of below 35 %. In the strain Greoux 11D13, the compositions of the other fatty acids were significantly different from those of the three Korean strains (Diogo *et al.*, 1999), thus supporting the distinction of these three strains from other *Legionella* species.

The sequence similarities of three different genes determined for each of the three Korean isolates were more than 99 %. Sequence similarities of 16S rDNA between K9951^T and other species of *Legionella* ranged from 91.5 to 95.8 %, the highest degree of relatedness being for *Legionella birminghamensis*. Sequence similarities of *rpoB* and *mip* between K9951^T and other *Legionella* species respectively ranged from 76.3 (*L. geestiana*) to 85.0 % (*Legionella hackeliae*) and from 64.7 (*L. worsleiensis*) to 76.5 % (*L. feeleyi*). All phylogenetic trees inferred from the three gene sequences indicated that the three isolates were grouped into a distinct cluster. This cluster was supported robustly with bootstrap values of 100 %, although the relationships with other *Legionella* species showed minor differences depending on the gene (Fig. 1). It has been proposed that members of one genus that share less than 97 % 16S rDNA sequence similarity should be regarded as separate species (Stackebrandt & Goebel, 1994). In this study, nearly complete 16S rDNA sequences (1476–1488 bp) of strains K9951^T, K9952 and K9953 all demonstrated less than 95.8 % sequence similarity with other species in the genus *Legionella*. Coupled with the 16S rDNA sequences, the sequences for *rpoB* and *mip*, which have been suggested as alternative targets for bacterial phylogeny and identification (Mollet *et al.*, 1997; Ratcliff *et al.*, 1998; Kim *et al.*, 1999; Dahllöf *et al.*, 2000; Ko *et al.*, 2002), also showed considerable divergence from other members of *Legionella*. The G+C contents of strains K9951^T, K9952 and K9953 were respectively 37.5, 36.1 and 35.9 mol%.

Thus, in light of the complementary nature of all the results described above, of biochemical tests, cellular fatty acid composition and molecular genetic studies, a novel species of the genus *Legionella*, *Legionella busanensis* sp. nov., is proposed for the Korean isolates.

Description of *Legionella busanensis* sp. nov.

Legionella busanensis (bu.san.en'sis. N.L. adj. *busanensis* from Busan in Korea, where the type strain was isolated).

Fig. 1. Phylogenetic relationships of *L. busanensis* sp. nov. and reference *Legionella* species. The trees were inferred by the neighbour-joining method. Numbers at branching nodes are percentages of 1000 bootstrap replications; only values greater than 50% are indicated. (a) Tree constructed on the basis of 16S rDNA sequences. *Coxiella burnetii* was used as an outgroup, as shown in (b). (b) *rpoB* phylogeny of 15 *Legionella* type strains and the three strains of *L. busanensis* sp. nov. A dendrogram including 38 species is available as supplementary material in IJSEM Online (<http://ijs.sgmjournals.org/>). (c) *mip* phylogeny of *Legionella* species including the three strains of *L. busanensis* sp. nov. Abbreviation: gsp., genomospecies. Bars, 1 (a) or 5 (b, c) substitutions per 100 nt.

Gram-negative rod with a single subpolar flagellum. Grows on BCYE agar, but not on media without L-cysteine. Positive for gelatinase activity, catalase activity, hydrolysis of hippurate, oxidase activity and hydrolysis of starch; negative for urease activity, peroxidase activity, reduction of nitrate to nitrite and browning of tyrosine-supplemented agar. No autofluorescent reaction. Contains the unsaturated fatty acids 16:1 ω 7c, i-16:0 and 16:0 as the major fatty acid components. All three strains of this species were isolated from water samples taken from cooling towers in Busan, Korea. The type strain is strain K9951^T (=KCTC 12084^T =ATCC BAA-518^T). It has a G+C content of 37.5 mol%.

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