**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 K9951T (=KCTC 12084T = ATCC BAA-518T).

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 K9951T, 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 feelei 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).
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 (x15 000; JEOL, JEM-1010). Analysis of cellular fatty acid composition was performed using a Hewlett Packard 6890A GC and the MIDI aerobe 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′-GTGGAATTTCGGTG 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 VGCCGTCTCAATNGGAC-3′) and ML1 (5′-GATAAGTT GTCTTATAGCATTG-3′)/ML2 (5′-TCTGTCATCCTG 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 Coxella 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 (Hockey 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 16:1ω7c (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: 15:0ω6c (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 %); a-15:0 (1:58–2:18 %); 15:0 (1:32–2:37 %); a-17:0 (1:30–1:38 %); 14:1ω5c (1:06–1:60 %); and 20:0 (0:1–2:55 %). 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. feelei, Legionella geotiana, Legionella moravica, Legionella quaterius, Legionella quinlivanii, Legionella sanitcruis, Legionella worsleiensis, Legionella sp. Montbéliard A1 and Legionella sp. Greoux 11D13 contain predominantly 16:1ω7c. However, all of these species, with the exception of Greoux 11D13, had a 16:1ω7c 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 and other species of Legionella ranged from 91.5 % to 95.8 %, the highest degree of relatedness being for Legionella birnhagenensis. Sequence similarities of rpoB and mip between K9951 and other Legionella species respectively ranged from 76.3 % (L. geotiana) to 85.0 % (Legionella hackelliae) and from 64.7 % (L. worsleiensis) to 76.5 % (L. feelei). 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, 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, 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).
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 16:1ω7c, 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 K9951T (KCTC 12084T = ATCC BAA-518T). It has a G+C content of 37.5 mol%.

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