**Mycobacterium paraintracellulare** sp. nov., for the genotype INT-1 of *Mycobacterium intracellulare*

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Three mycobacterial strains, isolated from independent Korean patients with pulmonary infections, belonging to the *Mycobacterium intracellulare* genotype 1 (INT-1) were characterized using a polyphasic approach. The sequences of the 16S rRNA gene and internal transcribed spacer 1 (ITS1) of the INT-1 strains were identical to those of *Mycobacterium intracellulare* ATCC 13950T. However, multilocus sequence typing (MLST) analysis targeting five housekeeping genes (hsp65, rpoB, argG, gnd and pgm) revealed the phylogenetic separation of these strains from *M. intracellulare* ATCC 13950T. DNA–DNA hybridization values of >70% confirmed that the three isolates belong to the same species, while the values of <70% between one of them and the type strains of *M. intracellulare* and *Mycobacterium chimaera* confirmed their belonging to a distinct species. In addition, phenotypic characteristics such as positive growth on MacConkey agar and in acidic broth culture, unique matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS profiles of lipids, and unique mycolic acids profiles further supported the taxonomic status of these strains as representatives of a novel species of the *Mycobacterium avium* complex named *Mycobacterium paraintracellulare*. The type strain is MOTT64T (=KCTC 29084T=JCM 30622T).

Non-tuberculous mycobacteria (NTM) are common in the environment and can be opportunistic pathogens. Recently, the application of molecular techniques to the taxonomy and identification of isolates from environmental sources and clinical specimens has led to an increased awareness of the diversity within NTM (Magee & Ward, 2012). Because combined molecular and conventional taxonomic methods have been applied to the isolates from Korean patients, we have successfully identified several novel NTM species to date (Kim et al., 2012d, 2013a, b; Lee et al., 2010a, b; Mun et al., 2008, 2007).

Recent advances in molecular taxonomy have fuelled the identification of novel species within the *Mycobacterium avium* complex (MAC) (Bang et al., 2008; Ben Salah et al., 2009; Murcia et al., 2006; Saini et al., 2009; Tortoli et al., 2004; van Ingen et al., 2009). *M. avium* and *Mycobacterium intracellulare* are well known as two representative species of the MAC. Additionally, another eight MAC species, including *Mycobacterium colombiense* (Murcia et al., 2006),

**Abbreviations**: DDH, DNA-DNA hybridization; ITS, intergenic transcribed spacer; MAC, *Mycobacterium avium* complex; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; OADC, oleic acid-albumin-dextrose-catalase; PNB, p-nitrobenzoic acid; TCH, thiopephene-2-carboxylic acid hydrazide.

The Genbank/EMBL/DDBJ accession numbers for the partial 16S rRNA gene, ITS1, hsp65, rpoB, gnd, argG and pgm of strain MOTT64T are KP670329, KP670345, KP670342, KP890661, KP670337 and KP670332 KP670349, respectively. The accession numbers for genes of other strains are listed in Table S1.

Four supplementary figures and seven supplementary tables are available with the online Supplementary material.
Mycobacterium vulneris (formerly sequevar MAC-Q) (van Ingen et al., 2009), Mycobacterium arosense (Bang et al., 2008), Mycobacterium boucheardhomonense, Mycobacterium marseillense, Mycobacterium timonense (Ben Salah et al., 2009) Mycobacterium chimaera (formerly sequevar MAC-A) (Tortoli et al., 2004) and Mycobacterium yongonense (Kim et al., 2013b), have recently been described.

Recently, via sequence analysis targeting three independent chromometer molecules, hsp65, the internal transcribed spacer 1 region (ITS1) and the 16S rRNA gene, we have reported that there exist five genetically distinct groups, INT-1 to INT-5, in M. intracellulare-related strains from Korean patients (Park et al., 2010). Our previous study demonstrated that INT-1 strains have an hsp65 sequence identical to that of M. chimaera (HG1 genotype) but an ITS1 sequence identical to that of M. intracellulare (MIN-A genotype). In this study, we sought to characterize the taxonomic status of the M. intracellulare INT-1 genotype, which is most prevalent among M. intracellulare-related groups in Korean patients as well as among Hanwoo, a native Korean cattle species (Bos taurus coreanae) (Kim et al., 2014), using a polyphasic taxonomic approach.

The three INT-1 strains (MOTT64, from a 60-year-old male patient and Asan 29591 from a 55-year-old male patient and Asan 37128 from a 71-year-old male patient) and two INT-2 strains (Asan 37016 from a 62-year-old female patient and Asan 38402 from an 89-year-old male patient) were also used for comparison with the three INT-1 strains including MOTT64. These strains were cultivated on Middlebrook 7H10 supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) and 0.5% glycerol at 37°C under 5% CO₂ for 2 weeks.

The phenotypic characteristics of M. intracellulare ATCC 13950 and M. chimaera JCM 14737 were also used for comparison with the three INT-1 strains including MOTT64. These strains were cultivated on Middlebrook 7H10 supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) and 0.5% glycerol at 37°C under 5% CO₂ for 2 weeks.

The optimal growth temperature of INT-1 strains was 37°C, and mature colonies were observed after 1 week on 7H10 agar plates. Colonies appeared smooth and white--yellow, unlike those of M. intracellulare ATCC 13950, showing a white and intermittently smooth-rough type of colonies. INT-1 strains showed positive responses for heat-stable catalase (68°C), pyrazinamidase and tellurite reduction, and negative results for arylsulfatase, nitrate reductase, urease activity and Tween 80 hydrolysis. In inhibition tests, INT-1 strains had resistance to 10 µg TCH ml⁻¹, 500 µg PNB ml⁻¹ and grew on MacConkey agar without crystal violet; however, no growth on media with ethambutol (8 µg ml⁻¹) or 5% NaCl was observed. In addition, these strains showed tolerance to acidic environments. The production of lipase (C14), valine arylamidase and cystine arylamidase was weakly positive, whereas the production of alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosamine, α-mannosidase and α-fucosidase] was also analysed with the API ZYM kit (bioMérieux) following the manufacturer's recommendations.

Although the drug susceptibility tests did not generally provide decisive information regarding mycobacteria taxonomy, it should be noted that INT-1 strains have generally higher MIC values than M. intracellulare ATCC 13950 for most of the antibiotics. Except for imipenem (>64 µg ml⁻¹) and tobramycin (8 µg ml⁻¹), MIC values of INT-1 strains were higher than those of M. intracellulare ATCC 13950 for seven other antibiotics, amikacin (16 or 32 µg ml⁻¹), cefoxitin (256 µg ml⁻¹), ciprofloxacin (16 µg ml⁻¹), clarithromycin (2 µg ml⁻¹), doxycycline (32 µg ml⁻¹), moxifloxacin (2 or 4 µg ml⁻¹) and rifampicin (4 µg ml⁻¹). Detailed information of phenotypic characteristics, biochemical activities and susceptibilities to antimicrobial agents for M. intracellulare ATCC 13950, M. chimaera JCM 14737 and the three INT-1 strains is shown in Table 1.

HPLC was used to analyse mycolic acids from the three type strains MOTT64, M. intracellulare ATCC 13950 and M. chimaera JCM 14737. For HPLC analysis, the strains were cultured on 7H10 agar plates for 1 week at 37°C. HPLC were processed as described previously using the standard method for sample preparation and UV spectrometric analysis (Butler & Guthertz, 2001).
Table 1. Comparison of cultural and biochemical characteristics and susceptibilities to antimicrobial agents among three INT-1 strains and the type strains *M. intracellulare* ATCC 13950T and *M. chimaera* JCM 14737T

Strains: 1, Asan 29591 (INT1); 2, Asan 37128 (INT1); 3, MOTT64T (INT1); 4, *M. intracellulare* ATCC 13950T (INT2); 5, *M. chimaera* JCM 14737T. ++, Good growth; +, positive/growth; –, negative/no growth; ±, variable. All species were positive for pyrazinamidase and tellurite reduction; negative for arylsulfatase, nitrate reductase, urease and Tween hydrolysis; positive for growth at 25°C and on 7H10 agar with TCH (10 µg ml⁻¹) or PNBl (500 µg ml⁻¹), and negative for growth on 7H10 agar with ethambutol (8 µg ml⁻¹) or 5% NaCl; weakly positive for lipase (C14), valine arylamidase and cystine arylamidase; and clearly positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and β-glucosidase.

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*S, smooth; Y, yellow; W, white; I, intermediate.
†AK, amikacin; CFX, cefoxitin; CIP, ciprofloxacin; CLA, clarithromycin; DOX, doxycycline; IPM, imipenem; MXF, moxifloxacin; RIF, rifampin; TOB, tobramycin.

The mycolic acid chromatograms from these mycobacteria evinced a different pattern (Fig. S1a, available in the online Supplementary Material). MOTT64T showed two representative peaks ranging from 3.2–3.82 min and from 6.1–6.4 min, respectively. The major peaks were generally detected in all three strains; however, the intensity of absorption units (AU) differed among strains. The AU of the first major peak from MOTT64T was about 0.20–0.50 (Fig. S1a) and those of *M. intracellulare* ATCC 13950T (Fig. S1b) and *M. chimaera* JCM 14737T (Fig. S1c) were 0.15–0.40 and 0.15–0.60, respectively. In addition, the AU of the second major peak from MOTT64T was 0.25–0.30; however, those of *M. intracellulare* ATCC 13950T and *M. chimaera* JCM 14737T were 0.45–0.5 and 0.3–0.5, respectively. The HPLC results also showed a characteristic peak ranging from 5.1 to 6.0 min in MOTT64T and *M. intracellulare* ATCC 13950T that was not found in *M. chimaera* JCM 14737T. The intensity of AU was also different between the two species, with those of MOTT64T and *M. intracellulare* ATCC 13950T ranging from 0.05 to 0.10 and 0.15 to 0.30, respectively.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used to compare total mass of the cell-wall lipid extracted with CHCl₃/CH₃OH (2:1, v/v) by a Voyager-DETM STR mass spectrometer (Applied Biosystems) with a pulse laser emitting at 337 nm (Rhoades et al., 2003). The MALDI-TOF MS profiles from Asan 29591 (Fig. 1a), Asan 37128 (Fig. 1b) and MOTT64T (Fig. 1c) were very similar to each other, but were different from those of *M. intracellulare* ATCC 13950T (Fig. 1d) and *M. chimaera* JCM 14737T (Fig. 1e). INT-1 strains exhibited two clusters, the first major peaks ranging from ~m/z 1199.9 to ~m/z 1476.1 and the second peaks from ~m/z 1869.3 to ~m/z 1967.4. The first major peaks were also detected in *M. intracellulare* ATCC 13950T and *M. chimaera* JCM 14737T; however, the second peaks were distinct only in INT-1 strains. These peaks were similar to serotype glycopeptidolipid (GPL) 7 ranging from ~m/z 1869.0 to ~m/z 1964.0 (Fujiiwara et al., 2007; Nakata et al., 2008).

Using the whole genome sequences of strain MOTT64T (Kim et al., 2012a) and two strains belonging to the INT-2 group, *M. intracellulare* ATCC 13950T (Kim et al., 2012b)
Fig. 1. MALDI-TOF MS analysis of extracted cell-wall lipids from strains Asan 29591 (a), Asan 37128 (b), MOTT64T (c), M. intracellulare ATCC 13950T (d) and M. chimaera JCM 14737T (e). The arrows indicate a unique cluster of peaks in the MALDI-TOF MS profiles of INT-1 strains.
and MOTT-02 (Kim et al., 2012c), a phylogenetic tree was inferred by the Mauve alignment algorithm and visualized by using the TreeViewX program.

The reconstructed tree revealed that strain MOTT64\(^T\) was clustered with the MAC complex strains, whereas in the \textit{M. intracellularare} ATCC 13950\(^T\)-related cluster, strain MOTT64\(^T\) was phylogenetically separated from a cluster of two INT-2 strains, \textit{M. intracellularare} ATCC 13950\(^T\) and MOTT-02, as an independent branch (Fig. S2). This result suggests that strain MOTT64\(^T\) may be a representative of an independent species that is distinct from \textit{M. intracellularare} ATCC 13950\(^T\).

For the phylogenetic analysis, partial 16S rRNA, ITS-1, \textit{hsp65}, \textit{rpoB}, \textit{gnd}, \textit{argG} and \textit{pgm} genes were amplified by PCR from chromosomal DNA extracted by the bead-beater phenol method (Jeon et al., 2014; Kim et al., 2005; Macheras et al., 2011); the primers for the target genes are shown Table S2. The PCR amplicons were compared with sequences in the GenBank database using the \texttt{BLAST} program (NCBI) and aligned using the multiple alignment algorithm in the MegAlign software package as previously described (Kim et al., 2005). Evolutionary distance matrices were generated according to the Jukes & Cantor model (Jukes & Cantor, 1969). Phylogenetic trees were inferred from the seven target gene sequences by the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods in the \texttt{MEGA} version 4.0 software (Kumar et al., 2008).

The \texttt{BLAST} analysis results of the 16S rRNA gene sequence (1305 bp) and ITS1 (280 bp) sequence showed that the sequences of INT-1 strains had 100\% sequence similarity with those of \textit{M. intracellularare} strain ATCC 13950\(^T\).
(GenBank accession nos GQ153276.1 and JQ411532.1, respectively) with a high bootstrap value (100) (Figs. 2a and S3a).

In addition, PCR amplicons of five housekeeping genes for hsp65, rpoB, argG, gnd and pgm gene sequences were further analysed for phylogenetic analysis. The partial hsp65 gene sequence (603 bp) of the three INT-1 strains showed a complete match (100 %) to that of M. chimaera DSM 44623^T (GenBank accession no. JF795578.1) but a 4 bp difference (those corresponding to the 192nd, 249th, 279th and 285th nucleotides) with the hsp65 gene sequence of M. intracellulare ATCC 13950^T (GenBank accession no. JF795578.1), resulting in 99.2 % similarity (Fig. 2b). The three INT-1 strains had identical rpoB (711 bp) sequences but showed a 5 bp difference corresponding to 2490th, 2526th, 2541st, 2544th and 2865th nucleotides of the rpoB gene sequence from M. intracellulare ATCC 13950^T (GenBank accession no. of GQ153307.1) and a 6 bp substitution (those corresponding to the 192nd, 249th, 279th and 285th nucleotides) with the hsp65 gene sequence of M. intracellulare ATCC 13950^T (GenBank accession no. JF795578.1).
corresponding to 2445th, 2526th, 2544th, 2865th and 3048th nucleotides) compared with the rpoB gene sequence from M. chimaera DSM 44623T (GenBank accession no. EF521908.1), resulting in 99.3 % and 99.2 % similarity, respectively (Fig. 2c, Table S3). The result of partial gnd gene analysis was generally similar to the phylogenetic tree based on rpoB. The gnd gene sequences (633 bp) from the three INT-1 strains were perfectly matched but showed a 6 bp difference (99.1 % similarity) with the sequence from M. intracellulare ATCC 13950T and an 11 bp substitution (98.3 % similarity) with the sequence from M. chimaera JCM 14737T (Fig. S3b, Table S4). The phylogenetic tree based on argG sequences (513 bp) demonstrated that strains MOTT64T and Asan 29591 had the same argG sequence, and differed by only 1 bp from another clinical isolate (Asan 37128) with 99.8 % similarity. The argG gene from MOTT64T showed sequence similarities of 99.2 % (6 bp difference) with M. intracellulare ATCC 13950T and 98.8 % (4 bp difference) with M. chimaera JCM 14737T (Fig. S3c, Table S5). The results of pgm analysis (633 bp) of these mycobacteria were similar to those obtained using rpoB. The partial gene from MOTT64T and Asan 29591 had the same sequence, which differed by 1 bp from Asan 37128 (99.8 % similarity). In comparison with the sequence of MOTT64T, there were differences of 5 bp with the pgm sequence of M. intracellulare ATCC 13950T and 19 bp with the pgm sequence of M. chimaera JCM 14737T, resulting in similarities of 99.2 % and 97.0 %, respectively (Fig. S3d, Table S6).

Finally, a phylogenetic tree based on five concatenated housekeeping genes [hsp65 (603 bp) + rpoB (711 bp) + gnd (633 bp) + argG (513 bp) + pgm (633 bp)] was reconstructed. The sequence similarity of the concatenated genes (3093 bp) among the three INT-1 strains showed that there were no nucleotide differences between MOTT64T and Asan 29591 and only a 2 bp difference with Asan 37128 (99.9 % similarity). The analysis based on concatenated sequences also revealed that the three INT-1 strains were more closely related to M. intracellulare ATCC 13950T, showing a similarity value of 99.2 % (26 bp differences), than to M. chimaera JCM 14737T, which had a similarity value of 98.6 % (40 bp differences) (Fig. 2d, Table S7).

DNA–DNA hybridization (DDH) was performed to verify the DNA relatedness among the three type strains (MOTT64T, M. intracellulare ATCC 13950T and M. chimaera JCM 14737T) and the other INT-1 and INT-2 strains (Table 2), as previously described (Sambrook & Russell, 2001; Park et al., 2007; Lee et al., 2011, 2012; Choi et al., 2013; Lo et al., 2014). Briefly, the genomic DNA extracted was blotted onto Hybond-N+ nylon membranes (Amerham Pharmacia Biotech) in triplicate after denaturation using NaOH solution and heating at 80 °C. Each DNA sample (4 µg) was digested by HaeIII restriction enzyme to label DNA probe for cross-hybridization, and the hybrids were detected on nylon membrane by using the DIG High Prime DNA Labeling kit (Roche Applied Science) according to the manufacturer’s instructions. The hybridization signals were detected by a scanner (HP Scanjet 3770) and analysed using Adobe Photoshop (version 7.0). The signal produced by the hybridization of the probe to the homologous target DNA was taken to be 100 %, and the signal intensities arising from the self-hybridization of the series of dilutions were used to calculate the levels of DNA relatedness.

When MOTT64T DNA was used as a probe, the DDH values between MOTT64T and M. intracellulare ATCC 13950T and between MOTT64T and M. chimaera JCM 14737T were 57.9±4.0 % and 49.1±4.9 %, respectively. When the type strains of the two recognized species were used as probes for cross check, the DDH values between MOTT64T and M. intracellulare ATCC 13950T and between MOTT64T and M. chimaera JCM 14737T were 53.1±0.2 and 47.2±1.6, respectively, which were below the 70 % that is generally accepted as a standard for species delineation (Wayne et al., 1987). The DDH values between MOTT64T and Asan 29591 and between MOTT64T and Asan 37128 were 85.9±7.9 % and 72.3±2.8 %, respectively. In addition, to check the fidelity of the DDH assay, it was further applied for the analysis of DDH values between the INT-2 strains and M. intracellulare ATCC 13950T. The DDH values between M. intracellulare ATCC 13950T and the two INT-2 strains, Asan 37016 and Asan 38402, were 86.6±6.23 % and 81.9±2.48 %, respectively, suggesting that they could be members of the same species. The DDH results are shown in Table 2 and the membrane images of DDH are shown in Fig. S4.

The genomic distances between MOTT-64T and M. intracellulare ATCC 13590T were determined from fully sequenced genomes using the average nucleotide identity (ANI; CLgenomics version 1.52; http://www.chunlab.com/) (Goris et al., 2007; Konstantinidis & Tiedje, 2005) and the web-based Genome-to-Genome Distance Calculator (GGDC version 2.1; http://ggdc.dsmz.de/).

The ANI values calculated between M. intracellulare ATCC 13950T and MOTT-64T were 98.56 %. Also, according to the Genome Blast Distance Phylogeny (GBDP) DDH prediction, the predicted DDH values between M. intracellulare ATCC 13950T and MOTT-64 were 89 %.

Taken together, the distinct sequence characteristics of the five independent genes (hsp65, rpoB, gnd, argG and pgm) together with biochemical characteristics, unique HPLC profiles of mycolic acids, MALDI-TOF profiles of lipids and DDH values of >70 % with the type strains of M. intracellulare and M. chimaera strongly support the conclusion that the INT-1 strains belong to a distinct mycobacterial species within the M. avium complex for which the name Mycobacterium paraintracellulare sp. nov. is proposed.

Description of Mycobacterium paraintracellulare sp. nov.

Mycobacterium paraintracellulare [pa.ra.in.tra.cell.lu.la’re. Gr. prep. para next to, resembling; N.L. neut. adj.
intracellular a bacterial epithet (intracellular); N.L. neut. adj., paraintracellular next to (Mycobacterium) intracellular.

A rod-shaped and acid-fast bacteria without spores or filaments under the microscope. The optimum temperature for growth is 37 °C on Middlebrook 7H110 agar medium, which results in smooth and white-yellow colonies after more than 7 days. Growth is observed on not only MacConkey agar without crystal violet but also in acidic environments (pH 5.5). Positive for heat-stable catalase (68 °C). Strongly resistant to seven antibiotics (aminicin, cefoxitin, ciprofloxacin, clarithromycin, doxycycline, moxifloxacin and rifampin). The lipid profile by MALDI-TOF MS and mycolic acid analysis by HPLC are distinctive and different from those of M. intracellular and M. chimaera. Unique sequences for five other housekeeping genes (hsp65, rpoB, gnd, argG and pgm) differentiate the species from M. intracellular.

The type strain, MOTT64T (=KCTC 29084T=JCM 30622T), was isolated from human sputum in Seoul, Korea.

Acknowledgements
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References

Table 2. DNA–DNA hybridization values among three INT-1 strains, M. intracellular ATCC 13950T, two INT-2 strains and M. chimaera JCM 14737T.

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<td>Mchi*</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>49.1±4.9</td>
<td>–</td>
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</tbody>
</table>

*Mint, M. intracellular ATCC 13950T; Mchi, M. chimaera JCM 14737T.


