Mycobacterium kyorinense sp. nov., a novel, slow-growing species, related to *Mycobacterium celatum*, isolated from human clinical specimens

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A novel, non-pigmented, slow-growing mycobacterium was identified on the basis of biochemical and nucleic acid analyses, as well as growth characteristics. Three isolates were cultured from clinical samples (two from sputum and one from pus in lymph nodes) obtained from three immunocompetent patients with infections. Bacterial growth occurred at 28–42 °C on Middlebrook 7H11-OADC agar. The isolates showed negative results for Tween hydrolysis, nitrate reductase, semiquantitative catalase, urease activity, 3 day arylsulfatase activity, pyrazinamidase, tellurite reduction and niacin accumulation tests, but positive results for 14 day arylsulfatase activity and heat-stable catalase tests. The isolates contained α -, keto-, and dicarboxymycolates in their cell walls. Sequence analysis revealed that all isolates had identical, unique 16S rRNA sequences. Phylogenetic analysis of the 16S rRNA, *rpoB*, *hsp65* and *sodA* gene sequences confirmed that these isolates are unique but closely related to *Mycobacterium celatum*. DNA–DNA hybridization of the isolates demonstrated less than 50% reassociation with *M. celatum* and *Mycobacterium branderi*. On the basis of these findings, a novel species designated *Mycobacterium kyorinense* sp. nov. is proposed. The type strain is KUM 060204^T (=JCM 15038^T=DSM 45166^T).

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *hsp65*, *proB* and *sodA* gene sequences of strains KUM 060204^T, NTH 512-121 and AHM 060905 are, respectively: AB370111, AB370169 and AB370170 (16S rRNA); AB370171, AB370176, and AB370177 (*hsp65*); AB370178, AB370182, and AB370183 (*rpoB*); AB370184, AB370188, and AB370189 (*sodA*).

INTRODUCTION

The advent of new molecular techniques has evoked great interest in the identification and classification of non-tuberculous mycobacteria that cause infectious diseases in mammals. Currently, there are more than 100 species of non-tuberculous mycobacteria, of which approximately 60 are considered to be potential pathogens (Brown-Elliott & Wallace, 2005). Among the non-tuberculous mycobacteria, *Mycobacterium celatum* was first described by Butler *et al.* (1993). It has unique species-specific 16S rRNA and superoxide dismutase sequences, resembling those of *Mycobacterium xenopi*, but is biochemically indistinguishable from the *Mycobacterium avium* complex (Butler *et al.*, 1993). Sequencing analysis of the 16S rRNA gene in additional *M. celatum* strains revealed the existence of a new subtype, which is distinct from but very similar to the two previously reported subtypes (types 1 and 2) (Butler *et al.*, 1993). Furthermore, *Mycobacterium branderi* was described as a novel species in 1995, and analysis of its 16S rRNA sequence has confirmed the close phylogenetic relationship of this organism to *M. celatum* (Koukila-Kahkola *et al.*, 1995).

In this study, slow-growing mycobacteria with a close phylogenetic relationship to *M. celatum* were isolated from clinical specimens from three Japanese patients with infections. Biochemical tests and genotypic analyses revealed that these isolates belong to the same novel species of the genus *Mycobacterium* for which the name *Mycobacterium kyorinense* is proposed.

METHODS

Bacterial strains. Strain KUM 060204^T was isolated from the sputum of a 62-year-old Japanese man with pneumonia at Kyorin University Hospital in Mitaka City, Tokyo, Japan. Three more isolates were obtained from the sputa of the same patient at different time points, but later analyses revealed that all four isolates were identical. Therefore, these four isolates were referred to as a single strain, KUM 060204^T. Strain NTH 512-121 was isolated from the sputum of a 70-year-old Japanese man with pneumonia at the National Hospital Organization Tokyo National Hospital in Kiyose City, Tokyo, Japan. Strain AHM 060905 was isolated from pus from cervical lymph nodes of a 64-year-old Japanese woman with non-tuberculous mycobacterial lymphadenitis at Aomori Prefectural Hospital in Aomori City, Aomori, Japan. None of these three patients suffered underlying immunocompromising disease. Since strains KUM 060204^T and AHM 060905 fulfil the criteria for infections of clinical significance (Medical Section of the American Lung Association, 1997), these two strains were considered to be clinically relevant in immunocompetent patients. *M. celatum* ATCC 51131^T, *M. branderi* ATCC 51789^T and *M. branderi* ATCC 51788 were purchased from ATCC and used as standard strains.

Phenotypic properties. Bacterial morphology and acid–alcohol-fastness were determined by Ziehl-Neelsen staining as described by Chapin (2007). Colony morphology, pigmentation, and the ability of the isolates to grow at various temperatures (25, 28, 30, 35 and 42 °C) were observed on Middlebrook 7H11-OADC agar (Nippon Becton Dickinson) and 1% Ogawa egg agar (Kyokuto Pharmaceutical Industrial Co. Ltd). The following biochemical tests were performed as described by Kent & Kubica (1985): Tween hydrolysis, nitrate reductase, pyrazinamidase, tellurite reduction, urease activity, niacin

accumulation, arylsulfatase activity (3- and 14-day), semiquantitative catalase and heat-stable catalase tests (68 °C).

Antimicrobial susceptibility testing. MICs for amikacin, clarithromycin, ethambutol, isoniazid, kanamycin, levofloxacin, rifampicin and streptomycin were determined based on the broth microdilution method with Broth MIC NTM (Kyokuto Pharmaceutical Industrial Co. Ltd) as described in monograph M24-A of the National Committee on Clinical Laboratory Standards, now CLSI (NCCLS, 2003).

Mycolic acid analysis by thin-layer chromatography (TLC). Mycolic acid analyses were performed using Silica gel TLC (Uniplate, 20 × 20 cm, 250 µm; Analtech) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) with an Ultraflex II (Bruker Daltonics) as described previously (Masaki *et al.*, 2006) with some modifications. *Mycobacterium tuberculosis* H37Rv ATCC 27294^T, *M. avium* ATCC 25291^T and *Mycobacterium intracellulare* ATCC 13950^T were used as controls.

Sequence determination and phylogenetic analysis. DNA was prepared after mechanical disruption of bacterial cells and subjected to sequence analysis of the 16S rRNA, *hsp65*, *rpoB* and *sodA* genes using methods as described by Kirschner *et al.* (1993), Telenti *et al.* (1993), Boor *et al.* (1995) and Domenech *et al.* (1997) with some modifications. Phylogenetic trees with bootstrap values were generated using the CLUSTAL W program (www.clustal.org) and displayed using TREEVIEW as described by Li *et al.* (2004). Phylogenetic analyses were also performed using the neighbour-joining method with Kimura's two-parameter distance correction model with 100 bootstrap replications in the MEGA version 2.1 software package. Homology searching with the 16S rRNA, *hsp65*, *rpoB* and *sodA* gene sequences was performed against sequences registered in GenBank/EMBL/DBJ using BLAST.

DNA–DNA hybridization. Quantitative microplate DNA–DNA hybridization for selected strains was carried out under optimal conditions as described by Ezaki *et al.* (1988, 1989).

RESULTS AND DISCUSSION

The three isolates examined were acid–alcohol-fast, non-motile, non-spore-forming bacilli. Mature colonies of all isolates developed in 4 weeks on Middlebrook 7H11-OADC agar. Growth was observed at temperatures in the range 28–42 °C, with optimal growth obtained at 30–35 °C. No growth was observed at 25 °C. Colonies were smooth and initially transparent, but became creamy white on prolonged culture.

All three isolates were negative for Tween hydrolysis, nitrate reductase, semiquantitative catalase, urease activity, 3-day arylsulfatase activity, pyrazinamidase, tellurite reduction and niacin accumulation, but positive for 14-day arylsulfatase activity and heat-stable catalase (Table 1). All isolates were distinguishable from *M. celatum* and *M. branderi* by a lack of growth at 25 °C and a negative pyrazinamidase test. They were also distinguishable from *M. celatum* by a negative result for 3-day arylsulfatase activity and the tellurite reduction test. The three isolates were also distinct from *Mycobacterium cookii* by their ability to grow at 45 °C and a lack of growth at 25 °C, absence of pigment production in the dark, and a negative semiquantitative catalase test. They were also distinct from

Table 1. Growth characteristics of strain KUM 060204^T in comparison with the closely related species *M. celatum* and *M. branderi*

+, Positive; –, negative; ND, not done.

Characteristic	KUM 060204 ^T	<i>M. celatum</i> *	<i>M. branderi</i> *
Growth temperature (°C)			
25	–	+	+
28	+	+	ND
30	+	+	ND
35	+	ND	ND
42	+	+	+
Heat-stable catalase (68 °C)	+	+	–
Arylsulfatase activity			
3 days	–	+	–
14 days	+	+	+
Pyrazinamidase	–	+	+
Tellurite reduction	–	+	ND

*Data taken from Butler *et al.* (1993), Koukila-Kahkola *et al.* (1995) and Vincent & Gutierrez (2007).

M. xenopi by the lack of pigment production in the dark (Koukila-Kahkola *et al.*, 1995).

Using antimicrobial susceptibility tests, all isolates were shown to be susceptible to clarithromycin and ethambutol, but resistant to isoniazid and rifampicin. Amikacin, kanamycin, levofloxacin and streptomycin showed low MIC values against all three isolates (Table 2).

Mycolic acid analyses of the three isolates by TLC produced identical multispot patterns composed of α - (C82-86)-, keto (C84-89)- and dicarboxy- (C64-69) mycolates. The length of the carbon chain of each mycolic acid methyl ester was assigned by the mass number and proposed structures as summarized in Table 3. The mycolic acids contained predominantly one or two cyclopropane rings. This pattern of mycolic acid subclasses is characteristic for

Table 2. Susceptibility of the three isolates to various antibiotics

Antibiotic	MIC ($\mu\text{g ml}^{-1}$)		
	KUM 060204 ^T	NTH 512-121	AHM 060905
Amikacin	<0.5	<0.5	<0.5
Clarithromycin	<0.03	<0.03	<0.03
Ethambutol	4	2	4
Isoniazid	16	32	16
Kanamycin	0.5	0.25	1
Levofloxacin	0.125	<0.03	0.125
Rifampicin	32	>32	>32
Streptomycin	0.25	0.25	0.25

the *Mycobacterium avium* complex, and is also seen in *M. celatum* and *M. branderi*, suggesting that the new isolates are closely related to these *Mycobacterium* species (Fig. 1) (Butler *et al.*, 1993; Brander *et al.*, 1992).

The sequence of the 16S rRNA gene was identical in all strains, but was different from all other available 16S rRNA sequences. The sequences of the *hsp65*, *rpoB* and *sodA* genes were also identical in the three isolates. It was assumed that these three isolates belong to the same species, and strain KUM 060204^T was used as a representative strain for genotypic analyses thereafter.

Sequence analysis revealed that the 16S rRNA gene sequence of the newly identified mycobacterium was closest to that of *M. celatum* ATCC 51130 (type 2) with 18 base mismatches out of 1469 bp (98.8 % identity). It was also highly similar to that of *M. celatum* NCTC 12882 (type 3) with 25 base mismatches out of 1476 bp (98.3 %), *M. celatum* ATCC 51131^T (type 1) with 27 base mismatches out of 1450 bp (98.1 %), *M. branderi* ATCC 51789^T with 32 base mismatches out of 1476 bp (97.8 %) and *M. cookii* ATCC 49103^T with 41 base mismatches out of 1477 bp (97.2 %). A phylogenetic tree was created based on the 16S rRNA gene sequences, incorporating all previously described species of slow-growing mycobacteria. Phylogenetic analysis revealed that strain KUM 060204^T is adjacent to the type strains of *M. celatum* (ATCC 51131^T), *M. branderi* (ATCC 51789^T) and *M. cookii* (ATCC 49103^T) (Fig. 2a).

The *hsp65* gene sequence of strain KUM 060204^T was identical to that of *M. celatum* ATCC 51130 (392/392, 100 %) and highly similar to those of *M. branderi* ATCC 51789^T and *M. celatum* ATCC 51131^T (98.8 and 98.1 %, respectively). A phylogenetic tree based on the *hsp65* sequences is shown in Fig. 2(b). Phylogenetic analysis of the *hsp65* gene gave consistent results, i.e. strain KUM 060204^T is located adjacent to the type strains of *M. branderi* (ATCC 51789^T) and *M. celatum* (ATCC 51131^T).

The sequence of the *rpoB* gene of strain KUM 060204^T was identical to that of *M. celatum* ATCC 51130 (306/306, 100 %) and highly similar to that of *M. branderi* ATCC 51789^T (96.7 %). It showed further differences from those of *M. celatum* ATCC 51131^T and *M. cookii* ATCC 49103^T (94.1 and 92.7 %, respectively). Strain KUM 060204^T clustered adjacent to *M. branderi* ATCC 51789^T and *M. celatum* ATCC 51131^T (Fig. 2c) in a phylogenetic tree based on *rpoB* sequences.

The *sodA* gene of strain KUM 060204^T was almost identical to that of *M. celatum* ATCC 51130 (411/413, 99.5 %) and highly similar to that of *M. celatum* ATCC 51131^T (96.6 %). It showed further differences from those of *M. branderi* ATCC 51789^T and *M. xenopi* ATCC 19250^T (90.1 and 86.4 %, respectively) (Fig. 2d).

Given the close relationship of strain KUM 060204^T to *M. celatum* and *M. branderi*, a DNA–DNA hybridization study was performed for these strains under optimal conditions (Table 4). Strain KUM 060204^T exhibited DNA similarity values below the suggested species threshold (70 %) to its

Table 3. The relationship between molecular species and mass numbers in each mycolic acid subclass

Underlining indicates a major component.

Strain	α -MA	Keto-MA	Dicarboxy-MA
KUM 060204 ^T Mass number [M + Na] ⁺	1230, 1258, 1286	1274, 1302, 1316, 1344	<u>1024</u> , 1038, <u>1052</u> , 1066, 1080, 1094
Molecular species (carbon-chain length)	82:2, <u>84:2</u> , 86:2	84:1, 86:1, <u>87:1</u> , 89:1	<u>64:1</u> , 65:1, <u>66:1</u> , 67:1, 68:1, 69:1
NTH 512-121 Mass number [M + Na] ⁺	1230, 1258, 1286	1302, 1316, 1344	<u>1024</u> , 1038, <u>1052</u> , <u>1066</u> , 1080, 1094
Molecular species (carbon-chain length)	82:2, <u>84:2</u> , 86:2	86:1, <u>87:1</u> , <u>89:1</u>	<u>64:1</u> , 65:1, <u>66:1</u> , <u>67:1</u> , 68:1, 69:1
AHM 060905 Mass number [M + Na] ⁺	1230, 1258, 1286	1302, 1316, 1344	1024, 1038, <u>1052</u> , <u>1066</u> , 1080, <u>1094</u>
Molecular species (carbon-chain length)	82:2, <u>84:2</u> , 86:2	86:1, <u>87:1</u> , <u>89:1</u>	64:1, 65:1, <u>66:1</u> , <u>67:1</u> , 68:1, <u>69:1</u>

phylogenetic neighbours *M. celatum* ATCC 51131^T, *M. branderi* ATCC 51789^T and *M. branderi* ATCC 51788 (similarity 49.9, 44.5 and 41.5%, respectively). These results provide further evidence for the genetic diversity between this strain and closely related species, indicating that this isolate comprises a new mycobacterial species.

Based on the genotypic and phenotypic data described above, it was concluded that strain KUM 060204^T represents a novel *Mycobacterium* species for which we propose the name *Mycobacterium kyorinense* sp. nov. Two other strains, NTH 512-121 and AHM 060905, were assumed to belong to the same species as strain KUM 060204^T based on sequence identity. Considering that strains KUM 060204^T and AHM 060905 fulfil the criteria for clinical significance, this newly identified *Mycobacterium* is considered to be a potential pathogen for infection in humans.

Description of *Mycobacterium kyorinense* sp. nov.

Mycobacterium kyorinense (kyo.rin.en'se. N.L. neut. adj. *kyorinense* of Kyorin, referring to the Kyorin University Hospital where the first strain was isolated).

Long, rod-shaped cells (approx. $3 \times 0.3 \mu\text{m}$); acid–alcohol-fast. Growth requires >4 weeks at 28–42 °C. No growth occurs at 25 °C. Colony diameters are 1–2 mm on Middlebrook 7H11-OADC agar. Colonies on 1% Ogawa egg agar are smooth and raised with round or lobate regular margins, and non-chromogenic. Negative for Tween hydrolysis, nitrate reductase, semiquantitative catalase, urease activity, 3-day arylsulfatase activity, pyrazinamidase, tellurite reduction and niacin accumulation; positive for 14-day arylsulfatase activity and heat-stable catalase tests. Susceptible to clarithromycin and ethambu-

Fig. 1. TLC patterns of mycolic acid methyl esters. (a) Plate developed three times with hexane/diethylether. (b) Plate developed three times with benzene. Lanes: 1, *M. tuberculosis* H37Rv ATCC 27294^T; 2, *M. avium* ATCC 25291^T; 3, *M. intracellulare* ATCC 13950^T; 4, strain KUM 060204^T; 5, strain NTH 512-121; 6, strain AHM 060905. MA, Mycolic acid.

Fig. 2. Phylogenetic trees for isolate KUM 060204^T and closely related type strains of other mycobacterial species based upon comparisons of (a) 16S rRNA, (b) *hsp65*, (c) *rpoB* and (d) *sodA* gene sequences using the neighbour-joining method. The trees were rooted using the sequences of *Nocardia asteroides* (16S rRNA and *hsp65*), *Rhodococcus equi* (*rpoB*) and *Nocardia abscessus* (*sodA*) as the outgroups. The scale bars represent a 1% sequence difference.

Table 4. Levels of DNA–DNA relatedness between strain KUM 060204^T and related *Mycobacterium* species

Strain	Similarity value (%) with labelled DNA from:			
	KUM 060204 ^T	<i>M. celatum</i> ATCC 51131 ^T	<i>M. branderi</i> ATCC 51789 ^T	<i>M. branderi</i> ATCC 51788
KUM 060204 ^T	100	49.1	46.0	47.6
<i>M. celatum</i> ATCC 51131 ^T	49.9	100	62.4	59.8
<i>M. branderi</i> ATCC 51789 ^T	44.5	51.3	100	100
<i>M. branderi</i> ATCC 51788	41.5	46.2	92.5	100

tol, but resistant to isoniazid and rifampicin. Sequence analysis of the 16S rRNA, *hsp65*, *rpoB* and *sodA* genes indicated that *M. kyorinense* is a mycobacterial species most closely related to *M. celatum* and *M. branderi*. DNA–DNA hybridization revealed that *M. kyorinense* exhibits DNA similarity values below the suggested threshold with its phylogenetic neighbours *M. celatum* ATCC 51131^T, *M. branderi* ATCC 51789^T and *M. branderi* ATCC 51788 (similarity 49.9, 44.5 and 41.5 %, respectively), thus defining it as a distinct species. *M. kyorinense* was placed in the slow-growing mycobacteria group.

The type strain is KUM 060204^T (=JCM 15038^T=DSM 45166^T), isolated from sputum in a patient with pneumonia.

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