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Virulence of *Mycobacterium avium* complex strains isolated from immunocompetent patients

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ABSTRACT

Mycobacterium avium complex (MAC) disease has been increasing worldwide not only in immunocompromised but also in immunocompetent humans. However, the relationship between mycobacterial strain virulence and disease progression in immunocompetent humans is unclear. In this study, we isolated 6 strains from patients with pulmonary MAC disease. To explore the virulence, we examined the growth in human THP-1 macrophages and pathogenicity in C57BL/6 mice. We found that one strain, designated 198, which was isolated from a patient showing the most progressive disease, persisted in THP-1 cells. In addition, strain 198 grew to a high bacterial load with strong inflammation in mouse lungs and spleens 16 weeks after infection. To our knowledge, strain 198 is the first isolated MAC strain that exhibits hypervirulence consistently for the human patient, human macrophages *in vitro*, and even for immunocompetent mice. Other strains showed limited survival and weak virulence both in macrophages and in mice, uncorrelated to disease progression in human patients. We demonstrated that there is a hypervirulent clinical MAC strain whose experimental virulence corresponds to the serious disease progression in the patients. The existence of such strain suggests the involvement of bacterial virulence in the pathogenesis of pulmonary MAC disease in immunocompetent status.

1. Introduction

Mycobacterium avium complex (MAC) is the most common cause of human infection due to nontuberculous mycobacteria. Initially MAC was regarded as only an opportunistic pathogen, primarily in acquired immunodeficiency syndrome (AIDS) patients [1]; however, it has now been shown to cause progressive pulmonary disease even in immunocompetent humans [2]. The American Thoracic Society indicates a wide range of clinical manifestation in patients with non-AIDS MAC disease; some patients keep a stable condition for years, whereas others progress their illness rapidly [3]. Furthermore, MAC infection can be more difficult to treat than *M. tuberculosis* due to even fewer available anti-microbial agents [3].

The pathogenesis of MAC infection has been recently investigated with respect to the host immune response. Interferon-gamma (IFN-γ) activates macrophages to produce proteolytic enzymes and other metabolites, which exhibit mycobactericidal effects. Tumor necrosis factor-alpha (TNF-α), of which production is also stimulated by IFN-γ, augments the bactericidal capacity of macrophages and plays a key role in the induction of the acquired immune response against mycobacteria [4]. A defective IFN-γ response has been shown recently to cause disseminated MAC disease in IFN-γ knock out mice and in humans with genetic mutations of IFN-γ receptor [5,6] or autoantibodies to IFN-γ in some young non-AIDS patients [7,8]. In addition to that, the activity of interleukin-10 (IL-10), which is known to inhibit cytokine synthesis by IFN-γ-producing type1 helper T cells (Th1 cells), has been shown to increase susceptibility to MAC infection in immunocompetent mice [9].

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Besides genetic factors of the host, bacterial virulence should play an important role for the development of MAC disease. While isolates of *M. tuberculosis* are genetically homogeneous at the nucleotide level [10], MAC has high genetic diversity, including the presence of multiple plasmids [11], and thus likely to have a large corresponding diversity in virulence. In the most complete study examining virulence, forty-one MAC isolates from the environment as well as infected humans and animals were compared for virulence in C57BL/6 mice by intravenous injection [12]. Monitoring of the virulence by CFU counts in lungs, livers, and spleens over 4 months revealed three virulence phenotypes; high (logarithmically increasing load), intermediate (chronic infection at a constant load), and low (initial load increase followed by a decrease until clearance). In addition, clinical studies have suggested severe disease outcome in patients infected with some specific strain type of MAC. For example, MAC serovars 1, 4, and 8 *Mycobacterium avium* are associated with disease severity in AIDS patients [13], and a serovar 4 *M. avium* isolate from an AIDS patient was more invasive and proliferative in blood mononuclear cell-derived human macrophages than a serovar 2 strain from chickens [14]. In non-AIDS MAC disease, *Mycobacterium intracellulare* is associated with greater disease progression [15], and moreover, our previous prospective study on 68 non-AIDS patients suggests that serovar 4 *M. avium* is linked to greater disease progression with a pulmonary MAC infection [16]. Taking these previous data into consideration, we hypothesize that relatively hypervirulent MAC strains exist and may be associated with serious disease progression in immunocompetent patients. In order to elucidate the involvement of mycobacterial virulence in the pathogenesis of human pulmonary MAC disease, in this study we examined the difference of mycobacterial virulence of clinical isolates from patients with different disease types using human macrophages and immunocompetent mice.

2. Results

2.1. Characteristics of mycobacterial strains

Six clinical isolates of MAC were isolated from sputum of non-AIDS patients with pulmonary MAC disease, and designated 27, 33, 36, 198, 288, and 347 (Table 1). Strains 33, 198 and 288 were derived from patients with progressive disease against combination chemotherapy recommended by the American Thoracic Society guideline (progressive type) [3]. The patients with progressive disease exhibited higher levels of erythrocyte sedimentation rate (ESR), diffuse and severe pulmonary lesions in chest X-ray findings, and numerous bacteria in the sputum. The patient infected with strain 198 exhibited the most serious disease outcome among study patients in that a right pneumonectomy was needed to prevent disease progression. Strains 27, 36, and 347 were derived from patients with little progression of disease without chemotherapy (silent type). They exhibited lower levels of ESR, segmental pulmonary lesions in chest X-ray findings, and fewer bacteria in the sputum. The isolates belonging to the progressive type consisted of *M. intracellulare* unclassified serovar similar to serovar 12 (strain 198) and *M. avium* apolar type (strains 33 and 288). The isolates belonging to the silent type consisted of *M. intracellulare* serovar 1 (strain 27) and *M. avium* apolar type (strains 36 and 347). For comparison, we employed 2 veterinary strains of *M. avium* ATCC 25291 (serovar 2) as a highly virulent strain in mice [12] and ATCC 35767 (serovar 4) as a low virulent strain. Four clinical isolates other than strains 33 and 347, and ATCC 25291 formed the transparent colony morphology. Strain 33 produced both transparent and rough colony morphologies. Strain 347 and ATCC 35767 displayed smooth opaque colony morphology.

2.2. Growth of clinical isolates in 7H9 broth

All strains showed logarithmic growth from 3 days after culture in 7H9 broth (Table 2). At day 5, two isolates from progressive type (strains 198 and 288) and one isolate from silent type (strain 36) grew significantly slower than ATCC 25291 ($P < 0.005$), and all clinical strains grew significantly slower than ATCC 35767 ($P < 0.0001$). The growth of strain 198 at day 5 was significantly slower than that of strain 27 ($P = 0.001$), and was not significantly different from that of other clinical isolates.

2.3. Virulence of clinical isolates in THP-1 monocyte-derived macrophages

We next studied intracellular survival of the isolates. THP-1 cells, a human monocytic cell line, were differentiated into macrophages by treatment with phorbol 12-myristate 13-acetate (PMA) and infected with MAC strains. Strain 198 grew in THP-1 cells significantly higher than any other strains during 7 days of infection ($P < 0.0001$) (Table 3). Strain 198 grew to approximately 20-fold during 2 days of infection ($P = 0.005$), and even at day 7, it kept the same level of bacterial load as day 0. Strain 36 also grew to approximately 2-fold during 2 days of infection ($P = 0.008$); however, it was rapidly eliminated at day 7, similar to the other strains except for strain 198. There was no significant difference in

Table 1
Characteristics of isolated strains and clinical findings.

Isolates	Species and serovar	Age	Sex	Duration of illness (years)	Erythrocyte sedimentation rate (mm/h)	Chest X-ray findings[a]	Sputum[b]	
							Smear	Culture
Progressive type								
33	*M. avium* apolar type	58	M	17	62	Advanced	2+	3+
198	*M. intracellulare* unclassified serovar[c]	62	F	3	108	Advanced	2+	2+
288	*M. avium* apolar type	56	F	12	78	Advanced	2+	2+
Silent type								
27	*M. intracellulare* serovar 1	67	F	17	50	Moderate	-	1+
36	*M. avium* apolar type	54	F	9	29	Moderate	-	1+
347	*M. avium* apolar type	79	F	14	50	Moderate	1+	1+

Data and sputum samples were collected at the enrollment of the study in 2003.

[a] Advanced chest X-ray findings were defined as bilateral cavities, giant cavities, or bilateral bronchiectasis, and moderate findings were defined as focal inflammation, small or fewer cavities, or mild bronchiectasis.

[b] Smear findings of sputum were defined as follows in high performance fields of microscopy; -: no bacteria in all fields, 1+: less than one bacteria in several fields, 2+: approximately 1–12 bacteria in one field. Culture findings were defined as follows using Ogawa egg agar; 1+: colonies less than 200, 2+: colonies more than 200 and less than 500, 3+: colonies more than 500 and less than 2000.

[c] The serovar of strain 198 was identified as a new type similar to serovar 12 determined by the liquid chromatography/mass spectrometry.

Table 2
Growth rate of MAC in 7H9 broth.

Strain	Ratio of CFUs at[a]		
	Day 1	Day 3	Day 5
33	0.91 ± 0.28	38 ± 0.86	63 ± 10
198	0.93 ± 0.17	12 ± 1.9	18 ± 4.0
288	0.85 ± 0.20	4.3 ± 1.9	16 ± 0.95
27	1.1 ± 0.25	7.3 ± 2.2	120 ± 21
36	0.83 ± 0.093	5.4 ± 0.21	11.0 ± 1.7
347	0.96 ± 0.16	2.7 ± 1.1	44 ± 19
25291	1.6 ± 0.25	4.8 ± 0.24	110 ± 16*
35767	0.97 ± 0.12	11 ± 3.0	370 ± 43**

* Significantly different ($P < 0.005$) from values for strain 198, 288, and 36 as calculated by Scheffé's test.
** Significantly different ($P < 0.0001$) from values for all clinical strains as calculated by Scheffé's test.
[a] Means ± standard deviations of the ratio of CFUs to those at day 0.

the growth rate among these strains except strain 198 during infection.

On light microscopic observation, THP-1 cell morphologies were not different between infected and uninfected cells (data not shown). We then assessed cytotoxicity by the levels of lactate dehydrogenase (LDH) released into the culture supernatants at day 7. The LDH release was detectable in strain 33 and the laboratory strains (strain 33; 5.8 ± 1.5%, ATCC 25291; 11 ± 1.0%, ATCC 35767; 12 ± 1.9%, without significant difference among these strains); however, it was not detectable in other clinical isolates.

2.4. Pathogenesis of clinical isolates in mice

Female C57BL/6 mice were infected by intratracheal instillation with each strain. Bacterial load in lungs, livers, and spleens were evaluated, and histological inflammation was visually analyzed in 5-mice per strain at defined time points during 16 weeks of infection. There was no significant difference in lung CFUs among strains tested 1 day after the inoculation.

Strain 198 showed high bacterial load, and tended to increase gradually both in lungs and spleens during 16 weeks of infection ($P = 0.08$ between day 1 and 16 weeks) (Fig. 1). Strain 198 was loaded in lungs significantly higher than strain 27 ($P = 0.04$) and 33 ($P = 0.0006$) at 8 weeks of infection, and than strain 33 ($P = 0.0009$), 288 ($P = 0.001$), 36 ($P = 0.0003$), and 347 ($P = 0.004$) at 16 weeks. Histologically, strain 198 induced strong inflammation in lungs, which was paralleled with bacterial loads (Fig. 2). ATCC 25291, known as highly a virulent strain in mice [12], showed initial reduction of bacterial load in lungs at 4 weeks of infection ($P = 0.01$ between day 1 and 4 weeks) and rapid increase in bacterial load in lungs after 4 weeks of infection. ATCC 25291 was comparatively virulent to strain 198 with respect to the high bacterial load in lungs and spleens, and severe pulmonary inflammation at 16 weeks of infection. By contrast, other clinical isolates did not increase profoundly in lung CFUs; however, these strains were never eliminated from lungs. ATCC 35767 was rapidly decreased and undetectable in lungs, spleens and livers within 16 weeks of infection. Overall, the clinical isolates other than strain 198 exhibited limited histological lesions with transient inflammatory changes in lungs 4 weeks after the inoculation, and thereafter the inflammation subsided at 16 weeks.

Table 3
Growth rate of MAC in THP-1 cells.

strain	Ratio of CFUs at[a]	
	Day 2	Day 7
33	0.29 ± 0.12	0.25 ± 0.16
198	18 ± 12*	1.30 ± 0.68*
288	0.47 ± 0.26	0.097 ± 0.055
27	0.54 ± 0.37	0.28 ± 0.11
36	2.4 ± 1.2	0.31 ± 0.11
347	1.1 ± 0.49	0.36 ± 0.23
25291	0.16 ± 0.048	0.11 ± 0.038
35767	0.059 ± 0.029	0.0070 ± 0.0048

* Significantly different ($P < 0.0001$) from values for any other strains studied as calculated by Scheffé's test.
[a] Means ± standard deviations of the ratio of CFUs to those at day 0.

3. Discussion

Virulence is defined as the quantitative ability of an agent to cause disease. The virulence of mycobacteria can be evaluated by the infection to macrophages and animals [17]. This is the first study that examined the virulence of MAC isolates from immunocompetent patients with different types of disease outcome. We found that strain 198, which derived from a patient with most serious disease, revealed high bacterial load both in THP-1 cells and in C57BL/6 mice among isolates studied. Strain-specific virulence of MAC has been implicated by some previous studies of the serovar 4 *M. avium* isolated from patients. In AIDS-related MAC disease, a serovar 4 *M. avium* isolate has shown to be one of the frequently isolated type [13], and a previous analysis of a serovar 4 isolate and ATCC strain has shown the superior virulence of serovar 4 *M. avium* in human macrophages [14]. In non-AIDS pulmonary MAC disease, our recent prospective study indicates that patients infected with serovar 4 *M. avium* has poorer prognosis than those infected with MAC of other serovars [16]; however, to our best knowledge, no study has shown the direct data of mycobacterial virulence of clinical isolates and clinical disease outcome. Strain 198 is the first MAC isolate whose experimental virulence corresponds to the serious disease outcome in humans. Thus, strain 198 has strain-specific strong virulence for immunocompetent humans and mice. We consider that strain 198 is worth further genetic investigation of virulence factors.

MAC strains hypervirulent for mice has been isolated previously by Pedrosa et al. including ATCC 25291 and MAC 101, which proliferate profoundly in mouse macrophages and in mice *in vivo* [12]. In this study, strain 198 proliferated in human macrophages, in correspondence with rapid clinical disease progression and additionally in mouse lungs. The consistency between experimental virulence in human cells and clinical disease outcome suggests that the capability of inducing such strong pathogenesis may be attributed mostly to the characteristics of the pathogen, i.e. virulence factor(s) for mammalian cells unique to strain 198. Previously Birkness et al. has shown the strong cytotoxic effect and growth of serovar 4 *M. avium* isolated from an AIDS patient in blood mononuclear cell-derived human macrophages compared with a serovar 2 strain from chickens (ATCC 35713). Therefore, we evaluated cytotoxicity of MAC strains by the microscopic morphology and by the LDH release from infected THP-1 cells; however, contrary to the expectation, strain 198 was not cytotoxic to THP-1. In addition, the release of LDH was lower in strain 33, ATCC 25291, and ATCC 35767 than in the previous experiment of *M. tuberculosis* infection to THP-1 cells (cytotoxicity in cases of *M. tuberculosis* H37Rv and H37Ra; approximately 30%) [18]. We assume that cytotoxic effect may not play a major role in displaying the virulence of MAC during infection, suggested by the similar result by Huttunen et al. showing the lack of cytotoxic effect of MAC in human 28SC macrophage and A549 lung epithelial cell lines evaluated by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) assay [19]. We speculate the virulence of strain 198 depends on the ability to survive or proliferate in macrophages rather than cytotoxic effect, which, may be causative for severe pulmonary MAC disease with rapid disease progression within a few years.

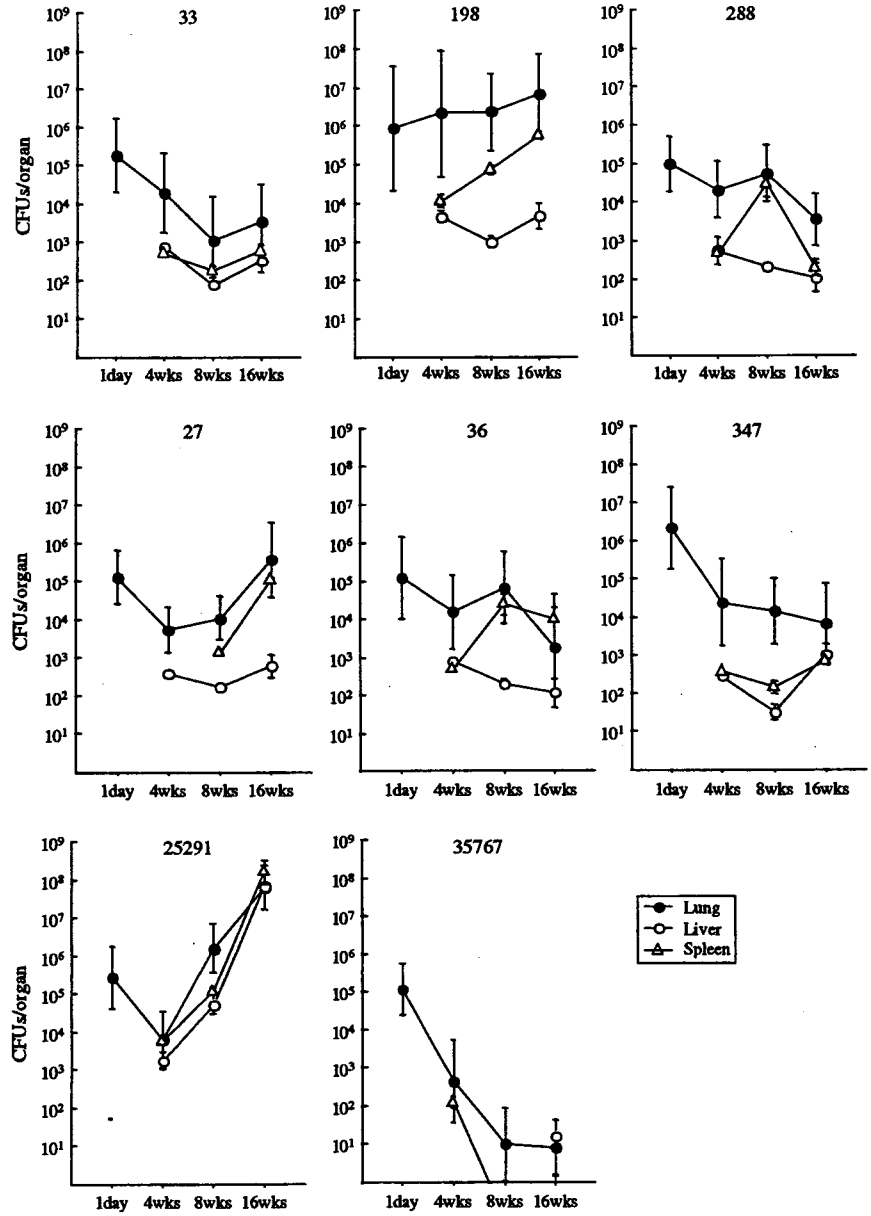

Fig. 1. Time course of mycobacterial growth in lungs, spleens and livers of C57BL/6 mice. Bacterial suspensions containing 1×10^5 CFUs were inoculated intratracheally to female C57BL/6 mice at the age of 7 weeks ($n = 20$ per strain). The lungs, livers and spleens of 5 mice per strain were sectioned at day 1 (only lungs), 4, 8, and 16 weeks later from challenge Data were presented as means ± standard deviations of CFUs/organ.

In this study, strain 198 showed strong virulence for mice at 16 weeks of infection similar to ATCC 25291; however, virulence for THP-1 cells was quite different, and pathogenic effects for mice within 4 weeks of infection was dissimilar between these two strains. These differences can be explained by the difference of immune response between host species and by the difference of immune phase. First, strain 198 could proliferate, but ATCC 25291 was rapidly eliminated in THP-1 cells (Table 3). In mouse macrophages mycobactericidal activity is attributed to nitric oxide produced by inducible nitric oxide synthase [20], whereas in human macrophages, it is attributed to Toll-like receptor signaling-dependent production of anti-microbial peptides [21,22]. Strain 198 is capable of proliferating under these two patterns of mycobactericidal activities, which suggests that strain 198 may have some virulence factors advantageous to survive both in human and mouse macrophages against mycobactericidal activity of the hosts, in contrast to ATCC 25291 which may lack virulence factors to survive in human macrophages. Second, strain 198 showed high bacterial load in lungs continuingly during 16 weeks of infection in mice, while ATCC 25291 proliferated after initial reduction in lungs at 4 weeks of infection (Fig. 1). The *in vitro* infection model using cell lines and the *in vivo* infection model using mice within 4 weeks reflects early stages of infection; on the other hand, the *in vivo* model after 8 weeks reflects chronic phase of infection [17,23]. The difference of pathogenic effects for mice within 4 weeks of infection suggests that strain 198 may resist both innate and acquired immunity, while ATCC 25291 may resist acquired immunity only. We assume that strain 198 and ATCC 25291 may possess different virulence mechanisms to persist in *in vivo* after development of acquired immunity.

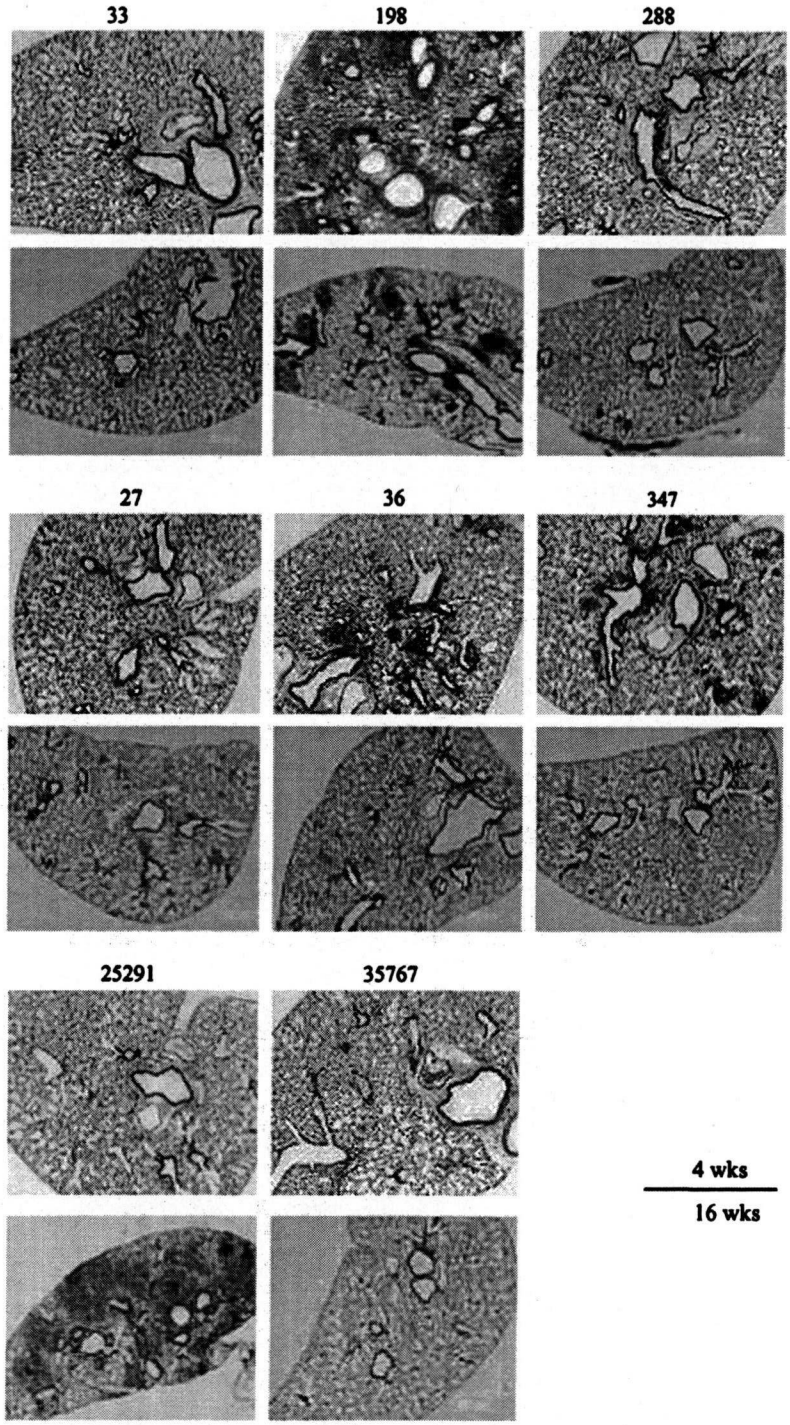

Fig. 2. Histological pictures of the lungs during 4 weeks or 16 weeks of infection in C57BL/6 mice by hematoxylin–eosin staining. Magnification, ×40.

In this study, clinical strains except for strain 198 did not show consistent virulence-associated phenotype among THP-1 cells, C57BL/6 mice, and clinical disease outcome. Similarly, Pedrosa et al. has also revealed that the growth of MAC in bone-marrow derived macrophages does not necessarily predict the virulence in mice by comparing the growth of 41 MAC isolates from various derives including humans, animals, and environment [12]. Although some clinical cases of pulmonary MAC disease may be caused by hypervirulent strains such as strain 198, these findings of clinical and natural isolates suggest that virulence may not the only determinant of the pathogenesis of pulmonary MAC disease in the majority of clinical cases. The development of pulmonary MAC disease depends on the balance between bacterial virulence and host defense. It is widely accepted that patients with pulmonary MAC disease have some characteristics of clinical background, such as males in their 40s and early 50s who have a history of cigarette smoking and excessive alcohol use, and such as postmenopausal, nonsmoking females [3], and these patient characteristics might

possibly indicate unknown predisposing conditions which enhance susceptibility for pulmonary MAC infection. The diverse phenotype of clinical MAC strains may be attributed to the disease susceptibility of the hosts. We propose that pathogenic mechanism of human pulmonary MAC disease include two patterns; one is that the strong virulence of MAC strains such as strain 198 induces rapid mycobacterial growth and serious disease outcome, and the other is that relatively weak to moderate virulence interacts with predisposing conditions of the host, leading the wide range of clinical outcome.

This study was preliminary in that we did not identify the mechanism of hypervirulence of strain 198. We observed the consistency between hypervirulence in human macrophages bedsides in immunocompetent mice and severe clinical outcome only in strain 198, not in any other isolates studied. From this finding, we speculate the existence of strain-specific virulence factors of strain 198. Recent exponential advances have enabled whole genome sequence of two *M. avium* strains, *M. avium* 104 and *M. avium* subsp. *paratuberculosis* K-10. Based on these exhaustive information, comparative genomics of MAC organisms has revealed the different genomic components regarding virulence factors, such as *ser2* encoding glycosylation enzyme of the lipopeptide core to generate the glycopeptidolipids, mammalian cell entry (*mce*) gene homologs, and PE/PPE genes (i.e., with Pro Glu and Pro Pro Glu motifs) [24]. In addition, there are large sequence polymorphisms among MAC organisms, suggesting a large corresponding diversity in virulence [11,24]. We speculate that the virulence of MAC strains including strain 198 may be determined by insertion or deletion of virulence genes encoding known [24] or unknown virulence factors.

In summary, we demonstrated that certain clinical strain derived from patients of the progressive pulmonary MAC disease exhibits strong virulence in human macrophages and in immunocompetent mice. Among clinical isolates, strain 198 is the first isolate hypervirulent to both human macrophages and mice. Our data suggest that strain-to-strain differences in virulence may play a significant role in disease progression in humans. Although Sarmento et al. showed that capability of TNF-α production from macrophages inversely correlates with the virulence of MAC strains [25], we could not find such relationship among the isolates (data not shown). In future studies, we will identify the virulence/pathogenicity-associated factor(s) of strain 198 and survey the frequency of strain variation in immunocompetent patients with pulmonary MAC disease.

4. Materials and methods

4.1. Bacterial strains

We used six clinical isolates from non-AIDS patients with pulmonary MAC disease and two laboratory strains, *M. avium* ATCC 25291 (serovar 2) and *M. avium* ATCC 35767 (serovar 4), in this study. Clinical isolates were obtained between September and November in 2003 at Toneyama National Hospital. Informed consent was obtained from all patients according to the guideline of Institutional Review Board of Toneyama National Hospital. Diagnosis of pulmonary MAC disease was made according to the American Thoracic Society guideline [3]. The samples were derived from two groups of patients; one group exhibited progressive disease in spite of the combination chemotherapy including clarithromycin, ethambutol and rifampin recommended by the American Thoracic Society guideline (progressive type) [3], the other displayed no exacerbation without anti-microbial chemotherapy for approximately ten years or more (silent type). These types were determined by the laboratory findings at the period of sputum sampling (including sputum smear and culture, chest X-ray findings, and erythrocyte sedimentation rate) and the rapidness of disease progression (Table 1). Sputum specimens were mixed with 2% sodium hydroxide, and *N*-acetyl-L-cysteine and then centrifuged for 15 min at 3000 *g*. The supernatants were discarded, and the sediment was mixed at 1:10 (vol/vol) with sterile water. The bacteria were cultivated in Middlebrook 7H9 broth supplemented with albumin–dextrose–catalase, 0.02% glycerin and 0.05% Tween 80, and then kept at −80 °C until following experiments. Identification of MAC was made by polymerase chain reaction using a commercially available kit (AMPLICOR Mycobacterium Tuberculosis Test, Roche, Basel, Switzerland). The serovars of clinical isolates were identified by the liquid chromatography/mass spectrometry as described previously [26]. Strains not containing serovar-specific oligosaccharides were defined as apolar type.

4.2. Growth in 7H9 broth

Bacterial suspension was adjusted to be 0.2 by optical density (OD) at 630 nm. The samples were cultured in 5 ml of 7H9 media in plastic tubes without agitation After vortexing to dissolve aggregates, cultivated bacterial suspensions were inoculated at days 1, 3, and 5 by serial 10-fold dilutions on Middlebrook 7H11 agar plates supplemented with oleic acid–albumin–dextrose–catalase, and 0.05% glycerol (7H11-OADC) agar plates in triplicate. The number of CFUs was counted after cultivating at 37 °C for 3 weeks.

4.3. Infection of THP-1 cells with MAC in vitro

THP-1 cells were purchased from Health Science Research Resources Bank (Tokyo, Japan). The cells were cultured in RPMI1640 containing 10% heat-inactivated fetal bovine serum (FBS; Equitech-bio, TX), and subcultured every 3–4 days. THP-1 cells were differentiated by 100 nM PMA (Sigma–Aldrich, St Louis, MO) for 48 h before infection. Before 48 h of infection, 1 ml of 2×10^5/ml cells was cultured in RPMI1640 containing 5% human serum (AB-blood group) in 24-well plates. Then, 1 ml of 2×10^4 CFUs/ml bacteria was exposed to the cultured cells for 24 h without opsonization (multiplicity of infection; 0.1 bacteria/cell). After that, the cells were treated with 20 μg/ml of gentamicin for 3 h to kill extracellular bacteria, followed by washing 4 times by RPMI1640. The infected cells were cultured in 2 ml of RPMI1640 containing 5% human serum. At days 0 and 7, uninfected bacteria were removed by washing with RPMI1640 4 times, and 500 μl of filter-sterilized phosphate buffered saline containing 0.5% Triton X-100 (Wako, Osaka, Japan) was treated per well to lyse cell membrane. The intracellular survival of bacteria was determined by counting CFUs by inoculating the cell lysate on 7H11-OADC agar plates. The experiment was performed in triplicate.

4.4. Assays for cytotoxicity

Cytotoxic effects were evaluated by the release of LDH from the cells. LDH activity of culture supernatants was determined by a commercially available kit (Roche, Basel, Switzerland). Supernatants were diluted to be 10^{-1} by distilled water for optimal reaction. The diluents were reacted with reaction mixture for 30 min, and then the OD was measured at 492 nm. Supernatants of completely lysed uninfected cells with filter-sterilized phosphate buffered saline containing 20% Triton X-100 and those of uninfected cells untreated with Triton X-100 were served as high and low controls, respectively. Cytotoxicity (%) was calculated as follows; $(OD_{sample} - OD_{low\ control}) \times 100/(OD_{high\ control} - OD_{low\ control})$. The measurement was performed in triplicate.

4.5. Animal studies

Female C57BL/6 mice aged at 6 weeks were purchased from CLEA Japan (Tokyo, Japan). All mice were kept under specific pathogen free conditions in animal facility of Osaka City University Graduate School of Medicine according to the institutional guidelines for the animal experiments. Twenty mice were used per group for infecting with each strain. One hundred microlitre of bacterial suspension containing 1×10^5 CFUs of MAC was inoculated into the trachea of the 7 weeks-aged mice anesthetized with pentobarbital sodium. Lungs, spleens and livers were removed on day 1 (only lungs) and 4, 8, 16 weeks after inoculation from 5-mice per strain. The organs were homogenized in 1 ml saline, and 0.1 ml of 10-fold dilutions of the homogenates was plated on 7H11-OADC agar followed by cultivating for 3 weeks. Bacterial burden was evaluated by CFUs per organ. Histological sections were made by standard methods including formalin fixation, dehydration, embedding in paraffin, and staining with hematoxylin and eosin.

4.6. Statistical analysis

Data were analyzed using the statistical analysis software package StatView 5.0 (SAS Institute, Cary, NC). The difference of mycobacterial growth in 7H9 broth, THP-1 cells, and mice was compared by a post hoc test of Scheffé among the strains tested. The difference of mycobacterial growth at defined time points during infection in THP-1 cells as well as in mice was compared by repeated measurement ANOVA with a post hoc test of Scheffé in the individual strains. Difference was considered statistically significant at $P < 0.05$.

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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/DDBJ 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	*M. celatum**	*M. branderi**
Growth temperature (°C)			
25	–	+	+
28	+	+	ND
30	+	+ (31 °C)	ND
35	+	ND	ND
42	+	+ (45 °C)	+ (45 °C)
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 & Gutierres (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 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).

Table 2. Susceptibility of the three isolates to various antibiotics

Antibiotic	MIC (μg 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 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	1024, 1038, 1052, 1066, 1080, 1094
	Molecular species (carbon-chain length)	82:2, 84:2, 86:2	84:1, 86:1, 87:1, 89:1	64:1, 65:1, 66:1, 67:1, 68:1, 69:1
NTH 512-121	Mass number [M+Na]+	1230, 1258, 1286	1302, 1316, 1344	1024, 1038, 1052, 1066, 1080, 1094
	Molecular species (carbon-chain length)	82:2, 84:2, 86:2	86:1, 87:1, 89:1	64:1, 65:1, 66:1, 67:1, 68:1, 69:1
AHM 060905	Mass number [M+Na]+	1230, 1258, 1286	1302, 1316, 1344	1024, 1038, 1052, 1066, 1080, 1094
	Molecular species (carbon-chain length)	82:2, 84:2, 86:2	86:1, 87:1, 89:1	64:1, 65:1, 66:1, 67:1, 68:1, 69:1

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 × 0.3 μm); 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-

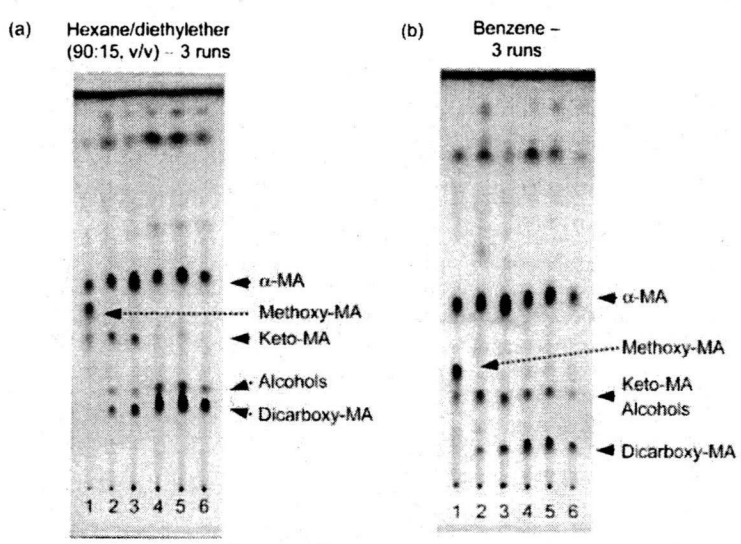

Fig. 1. TLC patterns of mycolic acid methyl esters. (a) Plate developed three times with hexane/diethylether. (b) Plate developed three times with benzene. Lanes: 1, *M. tuberculosis* H37Rv ATCC 27294^T; 2, *M. avium* ATCC 25291^T; 3, *M. intracellulare* ATCC 13950^T; 4, strain KUM 060204^T; 5, strain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

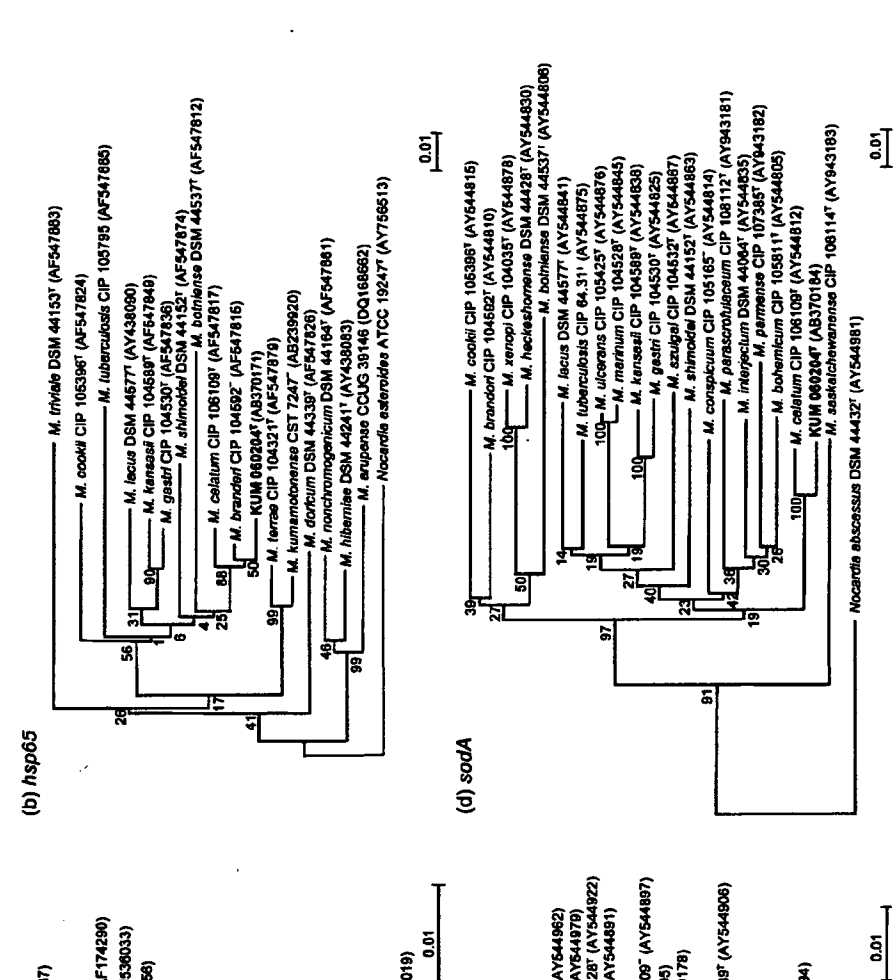

Fig. 2. Phylogenetic trees for isolate KUM 060204^T and closely related type strains of other mycobacterial species based upon comparisons of (a) 16S rRNA, (b) *hsp65*, (c) *rpoB* and (d) *sodA* gene sequences using the neighbour-joining method. The trees were rooted using the sequences of *Nocardia 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	*M. celatum* ATCC 51131^T	*M. branderi* ATCC 51789^T	*M. branderi* ATCC 51788
KUM 060204^T	100	49.1	46.0	47.6
M. celatum ATCC 51131^T	49.9	100	62.4	59.8
M. branderi ATCC 51789^T	44.5	51.3	100	100
M. branderi 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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