

***Mycobacterium immunogenum* sp. nov., a novel species related to *Mycobacterium abscessus* and associated with clinical disease, pseudo-outbreaks and contaminated metalworking fluids: an international cooperative study on mycobacterial taxonomy**

^{1,2} Center for Pulmonary and Infectious Disease Control¹ and Department of Microbiology², The University of Texas Health Center, 11937 US Hwy 271, Tyler, TX 75708, USA

³ Institut für Medizinische Mikrobiologie, Medizinische Hochschule Hannover, 30623 Hannover, Germany

⁴ Institut Pasteur, Centre National de Référence des Mycobactéries, Paris, France

⁵ Texas Department of Health, Austin, TX, USA

⁶ Departamento de Medicina Preventiva, Facultad de Medicina, Universidad Autónoma de Madrid, Spain

⁷ BioSan Laboratories, Warren, MI, USA

Rebecca W. Wilson,^{1,2} Vincent A. Steingrube,² Erik C. Böttger,³ Burkhard Springer,³ Barbara A. Brown-Elliott,² Véronique Vincent,⁴ Kenneth C. Jost, Jr,⁵ Yansheng Zhang,² Maria J. Garcia,⁶ Sher H. Chiu,⁵ Grace O. Onyi,² Harold Rossmore,⁷ Donald R. Nash¹ and Richard J. Wallace, Jr^{1,2}

Author for correspondence: Rebecca W. Wilson. Tel: +1 903 877 7680. Fax: +1 903 877 7652. e-mail: becky.wilson@uthct.edu

PCR–restriction enzyme pattern analysis of a 439 bp *hsp65* gene segment identified 113 unique isolates among non-pigmented rapidly growing mycobacteria (RGM) from clinical and environmental sources that failed to match currently recognized species patterns. This group represented 40% of isolates recovered from bronchoscope contamination pseudo-outbreaks, 0% of disease-associated nosocomial outbreaks and 4% of routine clinical isolates of the *Mycobacterium abscessus*/*Mycobacterium chelonae* group submitted to the Mycobacteria/Nocardia laboratory for identification. It is grouped within the *Mycobacterium fortuitum* complex, with growth in less than 7 d, absence of pigmentation, positive 3-d arylsulfatase reaction and growth on MacConkey agar without crystal violet. It exhibited overlapping biochemical, antimicrobial susceptibility and HPLC characteristics of *M. abscessus* and *M. chelonae*. By 16S rRNA gene sequencing, these isolates comprised a homogeneous group with a unique hypervariable region A sequence and differed by 8 and 10 bp, respectively, from *M. abscessus* and *M. chelonae*. Surprisingly, this taxon contained two copies of the ribosomal operon, compared with single copies in the two related species. By DNA–DNA hybridization, this new group exhibited <30% homology with recognized RGM species. The name *Mycobacterium immunogenum* sp. nov. is proposed for this new taxon.

Keywords: *Mycobacterium immunogenum*, rapidly growing mycobacterium, mycobacterium taxonomy, metalworking fluids

INTRODUCTION

Mycobacterium abscessus, *Mycobacterium chelonae* and *Mycobacterium mucogenicum* have been the most

Abbreviations: BAL, bronchoalveolar lavage; HP, hypersensitivity pneumonitis; MWF, metalworking fluid; PRA, PCR–restriction enzyme pattern analysis; RGM, rapidly growing mycobacteria.

The EMBL accession number for the 16S rDNA sequence of strain ATCC 700506 is AJ011771.

commonly recovered mycobacteria involved in water-borne nosocomial outbreaks and pseudo-outbreaks (Band *et al.*, 1982; Fraser *et al.*, 1992; Maloney *et al.*, 1994; Petersen *et al.*, 1994; Wallace *et al.*, 1993a, b) and metalworking fluid (MWF)-associated hypersensitivity pneumonitis (HP) (Kreiss & Cox-Ganser, 1997; Muilenberg *et al.*, 1993). These rapidly growing mycobacterial (RGM) species are more resistant to free chlorine than are coliform bacteria (Carson *et al.*, 1988a; Collins *et al.*, 1984). They are found, along

with other environmental species of mycobacteria (Picardeau *et al.*, 1997), in 83–90% of samples from domestic water-purification systems (Carson *et al.*, 1988a; Schulze-Röbbecke *et al.*, 1992). They are prevalent in biofilms (Schulze-Röbbecke *et al.*, 1992), are relatively resistant to disinfecting agents such as 2% alkaline glutaraldehyde and up to 8% formaldehyde (Carson *et al.*, 1978) and are able to grow in distilled-water supplies (Carson *et al.*, 1978). These characteristics enhance the capacity of these organisms to survive and proliferate in hospital water systems, which are the source of most nosocomial non-tuberculous mycobacterial outbreaks (Band *et al.*, 1982; Bernstein *et al.*, 1995; Hoffman *et al.*, 1981; Kuritsky *et al.*, 1993; Szabo & Sarkozi, 1980; Villaneuva *et al.*, 1997).

HP has been characterized as a granulomatous lung disease similar to sarcoidosis and beryllium disease (Kreiss & Cox-Ganser, 1997). Since the first reported case in 1991, a total of 98 cases of HP have been associated with exposure of industrial workers to aerosolized water-based synthetic, semi-synthetic or soluble oil MWF (Kreiss & Cox-Ganser, 1997). While the HP generally resolved upon removal of patients from the MWF environment, a small number of cases were reported to have chronic interstitial lung disease and some had biopsy evidence of fibrosis. The complex microbial flora of used MWFs, acid-fast bacteria in particular, have been implicated as the aetiological agent of HP (Kreiss & Cox-Ganser, 1997).

The taxonomy and epidemiology of RGM and their involvement in nosocomial outbreaks and pseudo-outbreaks have been a major focus of the Mycobacteria/Nocardia Laboratory at the University of Texas Health Center at Tyler (UTHCT). Biochemical, antimicrobial susceptibility and PCR–restriction enzyme analysis (PRA) testing of mycobacterial isolates recovered from several outbreaks, some involving MWFs, have resulted in the identification of a group of isolates that produced a hybrid pattern of phenotypic and genotypic characteristics that were common to both *M. abscessus* and *M. chelonae*. The potential of these environmental and nosocomial pseudo-outbreak isolates to cause human disease (Kreiss & Cox-Ganser, 1997) prompted further examination, in order to determine their relationship to the established mycobacterial pathogens *M. abscessus* and *M. chelonae*.

METHODS

Organisms. The first of these unusual isolates were recognized during a study of the use of PRA for taxonomic identification of clinical mycobacteria (Wilson *et al.*, 1998). These isolates exhibited PRA band patterns from a 439 bp segment of the *hsp65* gene (Telenti *et al.*, 1993) that appeared to be a hybrid between those of *M. chelonae* and *M. abscessus*. The PRA pattern obtained with *Bst*EII was characteristic for *M. chelonae*, while the *Hae*III pattern was characteristic for *M. abscessus*. Environmental isolates with the same hybrid PRA pattern were subsequently identified

as part of an environmental study of contaminated MWF, a portion of which has been published (Moore *et al.*, 2000) (see Table 1). Most isolates had high minimum inhibitory concentrations (MICs) for tobramycin and cefoxitin, so isolates within our culture collection with the same susceptibility pattern were screened by PRA. Based on DNA fingerprinting results of 10 previous nosocomial outbreaks of disease and 10 pseudo-breaks involving *M. abscessus* and/or *M. chelonae* (Wallace *et al.*, 1993a; Zhang *et al.*, 1997), one isolate representative of each outbreak pattern was selected and screened by PRA.

In 10 selected previous mycobacterial nosocomial outbreaks associated with disease (Szabo & Sarkozi, 1980; Hoffman *et al.*, 1981; Lowry *et al.*, 1988; Kuritsky *et al.*, 1993; Wallace *et al.*, 1993a; Bernstein *et al.*, 1995; Villaneuva *et al.*, 1997), based on PRA species identification, *M. abscessus* was the predominant species, accounting for 9 of 10 (90%) outbreaks previously identified by biochemical testing as being due to *M. abscessus* or *M. chelonae*. *M. chelonae* was responsible for 1 of 10 (10%) of the outbreaks. The new taxon was not identified in any of these nosocomial outbreaks. Conversely, among the 10 pseudo-outbreaks studied, based on PRA species identification, strains of the new taxon were found in 5 of 10 (50%) water-related pseudo-outbreaks (Moore *et al.*, 2000; Fraser *et al.*, 1992; Wallace *et al.*, 1993a), compared with 2 of 10 (20%) that involved *M. abscessus* (Kuritsky *et al.*, 1993; Wallace *et al.*, 1998). One additional PRA pattern was identified in 3 of 10 (30%) water-related pseudo-outbreaks (referred to as new taxon II in Fig. 3) (Maloney *et al.*, 1994; Petersen *et al.*, 1994) and was not studied further. *M. chelonae* was not identified in any of the pseudo-outbreaks.

Clinical isolates belonging to the *M. chelonae*/*M. abscessus* group submitted for identification were also screened for the new taxon pattern by PRA. Among 165 clinical isolates identified as members of the *M. abscessus*/*M. chelonae* group, 67% were *M. abscessus*, 30% were *M. chelonae* and 4% belonged to the new taxon.

A total of 112 isolates were identified that exhibited the *M. chelonae*/*M. abscessus* hybrid PRA pattern. This included 98 environmental isolates from MWF, two isolates from five separate nosocomial pseudo-outbreaks involving contaminated bronchoscopes in Missouri (MC 779) (Maloney *et al.*, 1994) and Maryland (MC 926) (Wallace *et al.*, 1993a), one environmental isolate from the Midwest and 11 clinical isolates isolated from skin, cornea, urine, joint fluid, bronchoalveolar lavage (BAL) fluid, Groshong exit site, pacemaker pocket, Broviac site and blood (catheter site) submitted to the UTHCT Mycobacteria/Nocardia laboratory for susceptibility testing (Table 1).

Control isolates included randomly chosen clinical isolates submitted to the UTHCT laboratory for identification by PRA (Wilson *et al.*, 1997) and susceptibility testing, as well as reference isolates from the ATCC (Manassas, VA, USA). These comprised 11 *M. abscessus* isolates, including the type strain ATCC 19977^T, and 13 *M. chelonae* isolates, including ATCC 35749, ATCC 35751 and the type strain, ATCC 35752^T.

Phenotypic characteristics. Isolates were tested for their ability to utilize citrate, D-glucitol (D-sorbitol), *i*-myo-inositol and D-mannitol as sole carbon sources according to the methods of Tsukamura (1981, 1984). Tests for 3-d arylsulfatase activity, iron uptake, nitrate reductase activity and growth on MacConkey agar without crystal violet were performed according to standard methods (Kent & Kubica,

Table 1. Clinical characteristics of isolates of the new taxon of RGM

Isolates MC 779^T, MC 926 and MC 1991 have been reported previously, by Fraser *et al.* (1992), Wallace *et al.* (1993a) and Moore *et al.* (2000), respectively.

Isolate	Source	Disease	Location	Comments
Environmental isolates				
MC 779 ^T (= ATCC 700505 ^T)	BAL fluid	None	Missouri	Pseudo-outbreak. Isolates highly resistant to cefoxitin
MC 926	BAL fluid	None	Maryland	Pseudo-outbreak
MC 1903	Hospital environment	Unknown	Midwest USA	
MC 1991 (= ATCC 700506)	MWF		Wisconsin	
MC 1993	MWF		—	
MC 1995	MWF		—	
MC 1996	MWF		—	
MC 2233	MWF		—	
MC 2234	Environment		—	
MC 2236	MWF		—	
MC 2240	MWF		—	
MC 2241	Environment		—	Formaldehyde resistant
MC 2268	Environment		—	
Clinical isolates				
MC 835	Skin	Disseminated cutaneous infection	Texas	SCID
MC 1878	Cornea	Suspected keratitis	Texas	No clinical data available
MC 1911	Urine	Unknown	Florida	No clinical data available
MC 1988	Intravenous catheter site (Groshong)	Catheter-related infection	Texas	Host immune status unknown
MC 2110	Hand-aspirated joint fluid	Septic joint	Texas	Normal host
MC 2230	BAL fluid	Chronic pneumonia	Texas	SCID
MC 2181	Intravenous catheter site, blood	Catheter-related sepsis	Iowa	Bone-marrow transplant
MC 2462	Pacemaker pocket, blood	Pacemaker-related sepsis	Michigan	Normal host
MC 2540	Broviac site	Catheter-related infection	Massachusetts	Acute leukaemia
MC 2570	Skin	Disseminated cutaneous infection	Louisiana	Liver transplant
MC 2605	Blood	Unknown	N. Carolina	No clinical data available

SCID, Severe combined immunodeficiency syndrome.

1985). Growth on Löwenstein–Jensen medium containing 5% NaCl (Kent & Kubica, 1985) was tested at both 30 and 35 °C.

MIC values were determined for amikacin, cefoxitin, cefmetazole, ciprofloxacin, clarithromycin, doxycycline, imipenem, sulfamethoxazole and tobramycin by a broth microdilution method in cation-supplemented Müller–Hinton broth (Swenson *et al.*, 1985; Wallace *et al.*, 1993b). Disc-diffusion zone sizes for polymyxin b (10 µg), amikacin (30 µg) and kanamycin (30 µg) were determined using Müller–Hinton agar swabbed with oleic acid, albumin and dextrose and incubation for 3 d at 30 °C.

Genomic restriction endonuclease digestion and pulsed field gel electrophoresis (PFGE) analysis. All isolates of the new taxon were examined for genetic strain relatedness of their large restriction fragment (LRF) patterns by PFGE

(Tenover *et al.*, 1995). Genomic DNA was prepared and LRF patterns were obtained by restriction endonuclease digestions with *Dra*I and *Xba*I, with separation by PFGE according to methods described previously (Wallace *et al.*, 1993a). Strain relatedness was defined according to the methods of Tenover *et al.* (1995).

Fluorescence detection (FL)-HPLC. Ten strains of the new taxon, strains 2649, 2642^T (= ATCC 700505^T), 2648, 2645, 2623, 2647, 2646 (= ATCC 700506), 2652, 2644 and 2651, were analysed by FL-HPLC. Ten reference strains of *M. abscessus*, strains 2612, 2603, 0203^T (= ATCC 19977^T), 2605, 2600, 2611, 2608, 2607, 2606 and 2602, and 10 reference strains of *M. chelonae*, strains 0187^T (= ATCC 35752^T), 0189 (= ATCC 19236), 0188, 0183 (= ATCC 35749), 0195, 0186, 0194, 0185, 0191 and 0190, were used as comparative strains. For HPLC analysis, isolates were obtained from the ATCC or from the HPLC Users Group

Steering Committee (Butler *et al.*, 1996a, b). Mycobacterial isolates were analysed by FL-HPLC according to the method of Jost & Dunbar (1992) and Jost *et al.* (1995) with the modifications described by Brown *et al.* (1999). The method of Butler & Kilburn (1990) was used to number chromatographic peaks and to calculate peak-height ratios.

PCR amplification for PRA. DNA from cells was prepared for PCR amplification as described previously (Steingrube *et al.*, 1995a; Telenti *et al.*, 1993; Wilson *et al.*, 1998). A 439 bp segment of the *hsp65* gene was amplified from mycobacterial ground-cell supernatants, together with the appropriate positive and negative controls, according to a modification of the method of Telenti *et al.* (1993) (Steingrube *et al.*, 1995a; Wilson *et al.*, 1998).

PRA. Six restriction endonucleases, *Bst*EII, *Hae*III, *Hha*I, *Msp*I, *Hinf*I and *Bsa*HI (New England Biolabs and Promega), were used to produce PRA band patterns using methods described previously (Steingrube *et al.*, 1995a, b, c, 1997; Wilson *et al.*, 1997). Fragments of ≤ 60 bp were disregarded and restriction fragment sizes were rounded to the nearest 5 bp, as recommended by Telenti *et al.* (1993).

Sequence determination of 16S rRNA gene. The 16S rRNA gene regions chosen for analysis were based on published sequence data available in the EMBL/GenBank database (Kazda *et al.*, 1992; Kirschner *et al.*, 1992a, b, 1993a, b; Pitulle *et al.*, 1992; Rogall *et al.*, 1990; Stahl & Urbance, 1990). GenBank accession numbers for selected 16S rRNA gene sequences used for comparison were as follows: *Mycobacterium fortuitum* ATCC 6841^T, X52933; *M. abscessus* ATCC 19977^T, X82235; and *M. chelonae* ATCC 35752^T, X82236.

The methods used for DNA extraction, amplification of 16S rRNA gene fragments and sequence determination have been described previously (Hultman *et al.*, 1989; Kirschner *et al.*, 1993a, b). The sequences obtained were aligned with selected 16S rRNA gene sequences as described previously (Rogall *et al.*, 1990).

For phylogenetic analyses, only 16S rRNA gene regions that were available for the rapidly growing species were included (corresponding to *Escherichia coli* positions 126–554, 894–1084 and 1101–1332). Pairwise distances (Hamming distances) were calculated by weighting nucleotide differences and insertions/deletions equally. A phylogenetic tree was constructed using the neighbourliness method as described previously (Rogall *et al.*, 1990).

Sequence determination of *hsp65* gene. The *hsp65* gene region selected for analysis was described by Telenti *et al.* (1993). The methods used for DNA extraction, amplification, sequence determination and construction of the phylogenetic tree were described previously (Ringuelet *et al.*, 1999).

DNA–DNA hybridization. DNA–DNA homology experiments were performed as described previously (Domenech *et al.*, 1997). Genomic DNA (0.5 µg) was labelled *in vitro* using a nick-translation labelling kit (Boehringer Mannheim) and 30 µCi [α -³²P]dCTP (Amersham).

Portions (1 µg) of each unlabelled DNA were bound to nylon membrane filters (Amersham) by alkaline denaturation and UV fixation. Hybridizations were carried out under stringent conditions for 40 h. Filters were washed and counted with a liquid scintillation counter (Beckman). The relative binding ratios for each strain were calculated from the counts of homologous DNA bound and were expressed as percentages.

RFLP analysis of the 16S rRNA gene. RFLP analysis of the 16S rRNA gene was performed using genomic DNA digested with *Bam*HI and *Pst*I. The digests were separated by electrophoresis on horizontal gels containing 0.75% (w/v) agarose and then transferred to nylon membrane filters (Amersham).

An 804 bp fragment of the 16S rRNA gene of the *M. fortuitum* type strain, ATCC 6841^T, was obtained by PCR and used as a probe. For DNA amplification, the *Rc*I and *Rc*2 oligonucleotide sequences from the *Mycobacterium bovis* BCG 16S rRNA gene sequence were used in 50 µl amplification mixtures as described previously (Domenech *et al.*, 1997). The cycling profile consisted of 30 cycles of 1 min at 94 °C, 30 s at 58 °C and 1 min at 72 °C, followed by a final 5 min extension at 72 °C. The ribosomal probe was labelled using the prime- α -gene system (Promega) and 20 µCi [α -³²P]dCTP (Amersham).

RESULTS

Phenotypic characteristics

Isolates of the new taxon had the growth and biochemical characteristics of the *M. fortuitum* complex (Silcox *et al.*, 1981) (Table 2). This included typical morphology with acid-fast staining, the absence of pigmentation, growth on tryptic soy and Middlebrook 7H10 agar in less than 7 d incubation at 30 and 35 °C, no growth at 45 °C, growth on MacConkey agar without crystal violet and a positive 3-d arylsulfatase reaction (Silcox *et al.*, 1981). Most isolates grew better at 30 than at 35 °C.

These isolates appeared to be related to the *M. abscessus*/*M. chelonae* group, based on negative iron uptake and nitrate reductase reactions, properties common to the latter two species (Silcox *et al.*, 1981). They exhibited some reactions typical of *M. abscessus*, including the inability to utilize citrate, D-glucitol (D-sorbitol), *i*-myo-inositol and D-mannitol as sole carbon sources (Silcox *et al.*, 1981) (Table 2) and resistance to tobramycin (MIC ≥ 16 µg ml⁻¹) (Swenson *et al.*, 1985) (Table 3). However, they were unable to grow on Löwenstein–Jensen medium containing 5% NaCl at 35 °C (Table 2) and most isolates exhibited high levels of resistance to cefoxitin (MIC 256 µg ml⁻¹), phenotypic characteristics typical of *M. chelonae* (Silcox *et al.*, 1981; Swenson *et al.*, 1985; Wallace *et al.*, 1991b) (Table 3). Interestingly, isolates of both the new taxon and *M. chelonae* exhibited 25–50% growth in the presence of 5% NaCl at 30 °C compared with control growth on Löwenstein–Jensen medium.

In terms of drug susceptibilities, the new taxon differed from both *M. chelonae* and *M. abscessus*. The ratio of kanamycin to amikacin disc-zone sizes for the 10 control strains of *M. abscessus* ranged from 1.3 to 1.7, with a mean of 1.5, while for 16/18 isolates of the new taxon, the ratio was < 1.2 with a mean of 0.92. For the new taxon, 14/17 had cefoxitin MICs of ≥ 256 µg ml⁻¹ and 16/18 had tobramycin MICs of ≥ 16 µg ml⁻¹. All isolates of the new taxon had at least one of these features and 17/18 (95%) had two of the three. No isolate showed any (partial or complete) zone of

Table 2. Growth and biochemical properties of *M. abscessus*, *M. immunogenum* and *M. chelonae*

Data for clinical isolates were taken from Springer *et al.* (1995) and Wallace *et al.* (1993b). Characters are scored as: +, positive ($\geq 90\%$); –, negative ($\leq 10\%$).

Character	<i>M. abscessus</i>		<i>M. immunogenum</i>		<i>M. chelonae</i>	
	Clinical isolates	ATCC 19977 ^T	Ten isolates	ATCC 700505 ^T	Clinical isolates	ATCC 35752 ^T
Utilization of carbon sources:						
Citrate	–	–	–	–	+	+
D-Glucitol (D-sorbitol)	–	–	–	–	–	–
<i>i</i> -myo-Inositol	–	–	–	–	–	–
D-Mannitol	–	–	–	–	–	–
3-d Arylsulfatase activity	+	+	+	+	+	+
Growth in presence of 5% NaCl (35 °C)	+	+	–	–	–	–
Iron uptake	–	–	–	–	–	–
Nitrate reduction	–	–	–	–	–	–
Growth in < 7 d	+	+	+	+	+	+
Pigment production	–	–	–	–	–	–
Mycolic acids by HPLC	+	+	+	+	+	+

Table 3. Antimicrobial susceptibility results for *M. abscessus*, *M. immunogenum* and *M. chelonae*

Values are MICs ($\mu\text{g ml}^{-1}$), expressed as modal values for groups of isolates. NT, Not tested.

Agent	<i>M. abscessus</i>		<i>M. immunogenum</i>			<i>M. chelonae</i>			
	Ten clinical isolates	ATCC 19977 ^T	Ten isolates	ATCC 700505 ^T	ATCC 700506	Ten clinical isolates	ATCC 35749	ATCC 35751*	ATCC 35752 ^T
Amikacin	8	32	16	16	128	32	32	8	32
Cefoxitin	16	16	> 256	> 256	64	> 256	> 256	32	> 256
Cefmetazole	16	128	> 128	> 128	64	128	> 128	32	> 128
Ciprofloxacin	16	> 16	> 16	2	> 16	> 16	> 16	> 16	1
Clarithromycin	0.5	4	0.25	NT	0.125	0.25	0.25	0.25	0.25
Doxycycline	> 128	> 128	> 128	32	> 128	> 128	128	> 128	16
Imipenem	8	8	16	16	16	16	8	4	4
Sulfamethoxazole	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64
Tobramycin	16	16	> 16	> 16	> 16	2	4	16	4

* By PRA, this isolate is a strain of *M. abscessus*.

inhibition by polymyxin b. Susceptibility results for the isolates of the new taxon are compared with isolates of *M. chelonae* and *M. abscessus* in Table 3.

PFGE analysis

Fourteen different LRF patterns (genotypes) of the new taxon were identified by PFGE analysis. Details of some of the MWF results have already been presented (Moore *et al.*, 2000). The two isolates of the new taxon representative of the two nosocomial pseudo-outbreaks from contaminated bronchoscopes (MC 779 and MC 926, Table 1) were different genotypes. Nine of the 11 clinical isolates were unique genotypes, with the remaining two displaying the

predominant LRF pattern seen with the environmental isolates. As observed previously (Wallace *et al.*, 1993a), undigested genomic DNA satisfactory for PFGE analysis could be obtained from only 50% of the *M. abscessus* isolates, including those from nosocomial outbreaks, while 100% of the isolates of the new taxon in the current study produced satisfactory genomic DNA.

FL-HPLC analysis

Analysis of strains of the new taxon by FL-HPLC-generated mycolic acid patterns identified all strains as belonging to the *M. abscessus*/*M. chelonae* group. These strains exhibited mycolic acid elution patterns

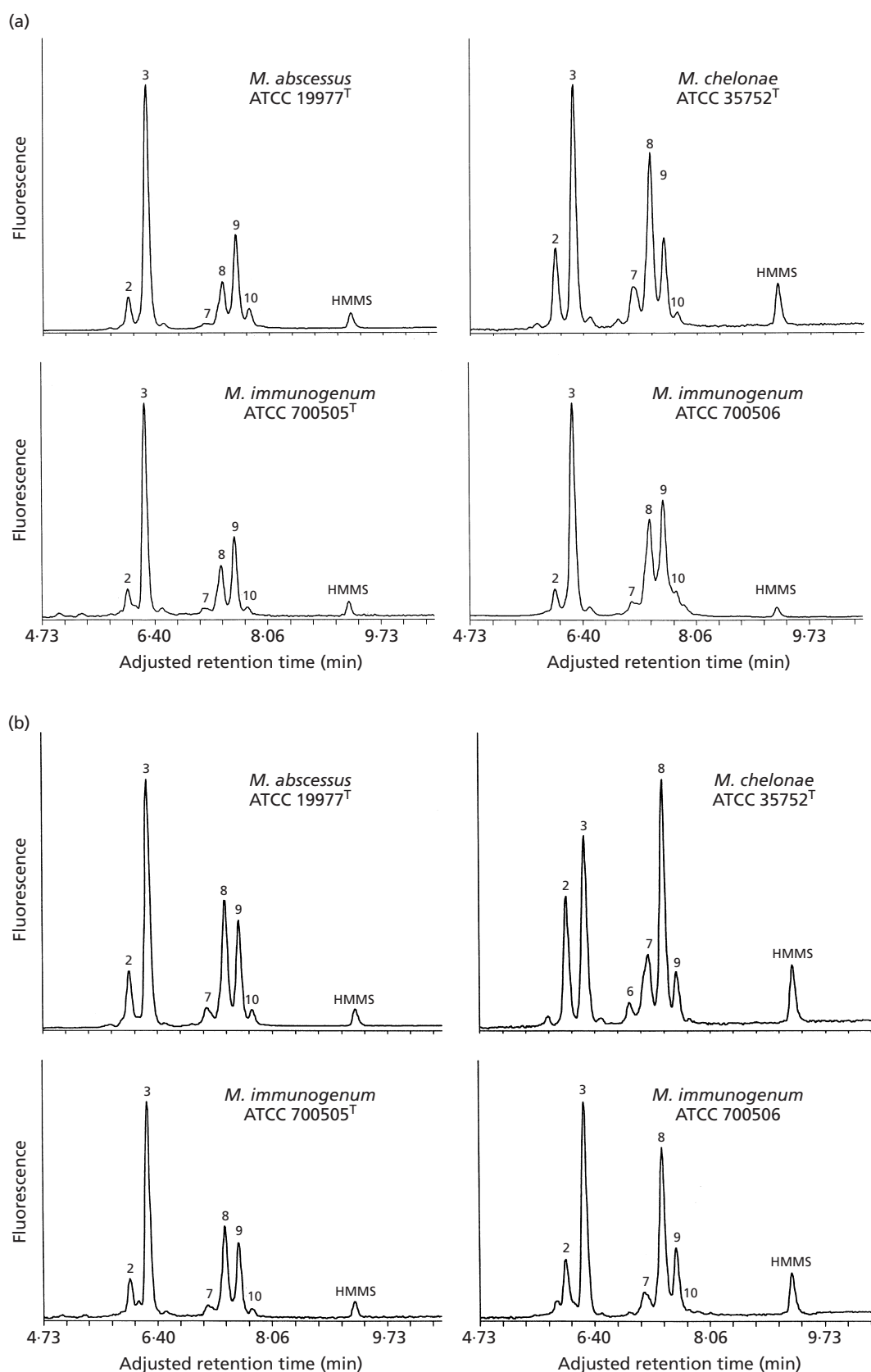


Fig. 1. Incubation-temperature-mediated variation of HPLC-generated mycolic acid 6,7-dimethoxy-4-coumarinylmethyl ester elution patterns for *M. abscessus*, *M. chelonae* and *M. immunogenum* strains grown on Löwenstein–Jensen medium at 30 (a) and 35 (b) °C. HMMS, High molecular mass internal standard. Peaks were named using the method of Butler & Kilburn (1990).

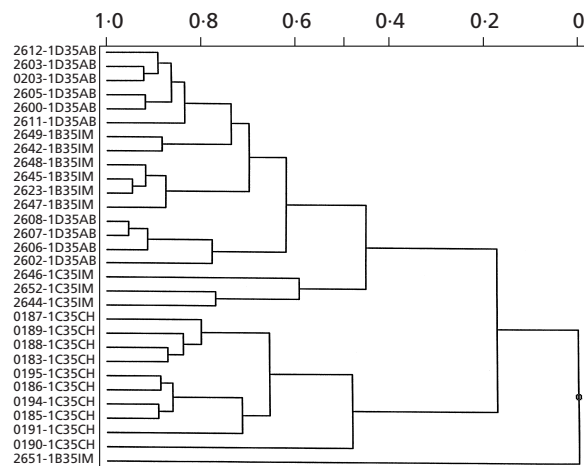


Fig. 2. Dendrogram illustrating phylogenetic relationships between *M. abscessus*, *M. chelonae* and *M. immunogenum* strains grown on Löwenstein–Jensen medium at 35 °C, based on mycolic acid composition analysis by HPLC. Suffixes AB, CH and IM respectively indicate strains of *M. abscessus*, *M. chelonae* and *M. immunogenum*. The scale indicates the similarity index.

that were characterized by two peak clusters, each cluster containing two to four peaks (Fig. 1a, b). Visually, most patterns from strains of the new taxon were indistinguishable from *M. abscessus* strains, but distinct from *M. chelonae* strains. *M. abscessus* and *M. chelonae* strains incubated at 35 °C produced peak 8/peak 9 height ratios that ranged from 0.34 to 0.54 and 1.06 to 2.66, respectively. The corresponding range for strains of the new taxon was 0.25–1.00. Butler & Kilburn (1990) observed peak 8/peak 9 height ratios of ≤ 0.86 for *M. abscessus* and ≥ 1.00 for *M. chelonae*.

Mean-centred hierarchical cluster analysis of aligned whole data obtained at 35 °C between the two internal standard markers revealed a single distinct cluster that contained all of the strains of *M. chelonae* (Fig. 2). At a similarity index of 0.70, the *M. abscessus* strains grouped into two clusters, one of six strains and a second of four strains. At a similarity index of 0.70, the strains of the new taxon were more polymorphic than either *M. abscessus* or *M. chelonae*. The strains of the new taxon grouped into two clusters, with one strain unclustered. The pattern of the unclustered strains

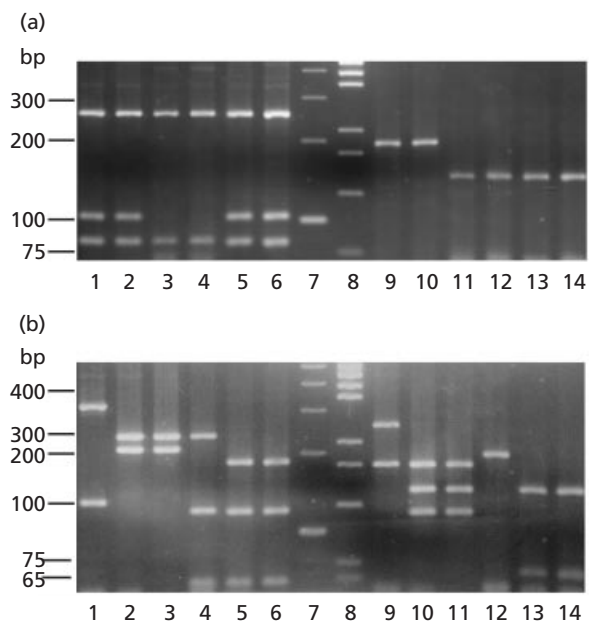


Fig. 4. Comparison of PRA patterns for *M. chelonae*, *M. abscessus* and the proposed new species, *M. immunogenum*. (a) Lanes 1–6 and 9–14: *Bsa*HI- (lanes 1–6) and *Hae*III- (lanes 9–14) derived patterns for *M. chelonae* ATCC 35749 (lanes 1 and 9), *M. chelonae* ATCC 35752 (2, 10), *M. immunogenum* ATCC 700506 (3, 11), *M. immunogenum* MC 1995 (4, 12), *M. abscessus* ATCC 19977^T (5, 13) and *M. abscessus* MC 1148 (6, 14). Lanes 7 and 8 contain size standards (100 bp and pGEM base pair ladders). (b) *Bst*EII- (lanes 1–3), *Hha*I- (4–6), *Msp*I- (9–11) and *Hae*III- (12–14) derived patterns for *M. chelonae* ATCC 35752^T (lanes 1, 4, 9 and 12), strain ATCC 35751 (2, 5, 10 and 13) and *M. abscessus* ATCC 19977^T (3, 6, 11 and 14). Lanes 7 and 8 contain size standards (100 bp and pGEM base pair ladders).

resembled that produced by *M. chelonae* at 30 °C and was distinct from all other patterns produced at 35 °C.

The incubation temperature exerted a pronounced effect on the HPLC patterns of *M. abscessus*, *M. chelonae* and the new taxon. Compared with patterns of strains incubated at 35 °C, patterns of the strains incubated at 30 °C exhibited a diminished front peak cluster height relative to rear peak cluster height. Within each peak cluster, a shift in peak height towards the earlier-eluting peaks was observed. Strains of *M. abscessus* and the new taxon incubated at 30 °C yielded patterns similar to those of *M. chelonae* strains incubated at 35 °C (Fig. 1).

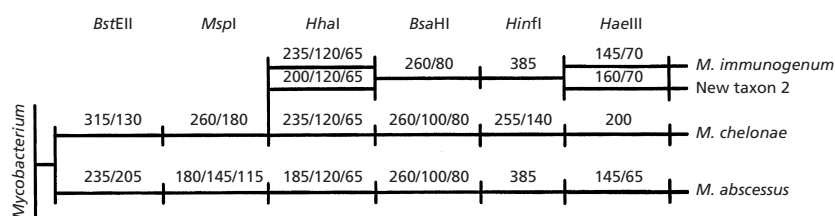


Fig. 3. Practical schematic illustrating the similarities and differences between *M. immunogenum*, new taxon 2, *M. abscessus* and *M. chelonae* by PRA of a 439 bp segment of the *hsp65* gene. PRA band sizes are expressed as the number of nucleotide bp rounded to the nearest 5 bp, as recommended by Telenti *et al.* (1993).

Table 4. Signature nucleotides within hypervariable region A of the 16S rRNA gene for the pathogenic RGM

The first nucleotide corresponds to *E. coli* position 175. *M. tuberculosis* is used as the reference species. Dots indicate nucleotides identical to the *M. tuberculosis* sequence. Dashes indicate the absence of insertions. Proposed or accepted type strains are indicated.

Taxon/species	Signature sequence
<i>M. tuberculosis</i> – <i>M. bovis</i>	CGGATAGG–ACCACGGGATGCATGTC–TTGTGGTG
<i>M. fortuitum</i> group	
<i>M. fortuitum</i> ATCC 6841 ^T	..A...T-.....C.C.T...GT-G.....
<i>Mycobacterium peregrinum</i> ATCC 14467 ^T	..A...T-...G..CAC.T.C..GT-G.....
<i>M. fortuitum</i> third biovariant complex (sorbitol-positive) ATCC 49403 ^T ; <i>M. senegalense</i>-.....C.C.T...GT-G.....
<i>M. fortuitum</i> third biovariant complex (sorbitol-negative) ATCC 49404 ^T	..A.....-...G..CTC.T...GG-G.....
<i>M. chelonae</i> / <i>M. abscessus</i> group	
<i>M. abscessus</i> ATCC 19977 ^T-.....ACAC.T...GT-GA.....
<i>M. chelonae</i> ATCC 35752 ^T-.....ACAC.T...GT-GA.....
<i>M. immunogenum</i> ATCC 700506-...G.ATGC.T...GT-G.....
<i>M. mucogenicum</i> ATCC 49650 ^T	..A.....-.....C.C.T...GT-G.....
<i>M. smegmatis</i> group	
<i>M. smegmatis sensu stricto</i> ATCC 14468	..A...CACC.TG.T..TC.....G.C.G..A.G.
<i>M. goodii</i> ATCC 700504 ^T	..A...TACC.TG.T..TC.....G.C.G....G.
<i>M. wolinskyi</i> ATCC 700010 ^T-.....TC.....GA-.....
<i>M. mageritense</i> ATCC 700351 ^T	..A.....-..TC...AC.....GT-C..G....

PRA

Amplicons were obtained from all the isolates tested. Patterns identical to those seen with *M. chelonae* were exhibited by 98% (110/112) of the isolates of the new taxon after *Bst*EII PRA and 100% of isolates after *Msp*I and *Hha*I PRA. Two isolates contained no *Bst*EII restriction site (Fig. 3). Isolates of the new taxon exhibited *Hinf*I and *Hae*III patterns that were identical to those seen with *M. abscessus* (Figs 3 and 4a, lanes 11–14) and differentiated them clearly from *M. chelonae* isolates (Figs 3 and 4a, lanes 9 and 10). The *Bsa*HI pattern of the new taxon lacked a 100 bp band (Figs 3 and 4a, lanes 3 and 4) that was present in the common *Bsa*HI pattern seen with *M. chelonae* and *M. abscessus* isolates (Figs 3 and 4a, lanes 1, 2, 5 and 6).

The three RGM isolates representing the three pseudo-outbreaks from contaminated bronchoscopes, which have been recognized but not characterized at this time, exhibited a unique PRA pattern, not seen previously, consisting of a 200 bp upper band with *Hha*I and a 160 bp upper band with *Hae*III, compared with 235 and 145 bp bands seen with the new taxon (Fig. 3).

Misidentification of ATCC 35751

In this laboratory, *M. chelonae* reference isolate ATCC 35751 was found to be negative for utilization of citrate, D-glucitol (D-sorbitol), *i*-myo-inositol and D-mannitol as sole carbon sources and found to grow in the presence of 5% NaCl (Silcox *et al.*, 1981) (Table 2).

Results of susceptibility testing indicated that this strain was moderately susceptible to cefoxitin and resistant to tobramycin (MIC 16 µg ml⁻¹), similar to values for isolates of *M. abscessus* (Swenson *et al.*, 1985; Wallace *et al.*, 1991b) (Table 3). In addition to phenotypic characteristics typical of *M. abscessus*, PRA of isolate ATCC 35751 with all six restriction endonucleases produced band patterns that matched those of *M. abscessus* isolates, including the *M. abscessus* type strain, ATCC 19977^T (Fig. 3). This strain appears to be misidentified and is a strain of *M. abscessus* rather than *M. chelonae*. A comparison of restriction fragment patterns for the *M. chelonae* type strain, ATCC 35752^T, ATCC isolate 35751 and the *M. abscessus* type strain, ATCC 19977^T, with *Bst*EII, *Hha*I, *Msp*I and *Hae*III is shown in Fig. 4(b).

Sequence determination of 16S rRNA gene

Five isolates of the new taxon were sequenced in hypervariable regions A and B, with identical results. The isolates of the new taxon had a hypervariable region B that matched *M. chelonae* and *M. abscessus*, but a hypervariable region A that differed from these two species as well as all other species of RGM (Table 4). Over the sequence of the entire 16S rRNA gene, the new taxon differed by 8 and 10 bp from *M. abscessus* and *M. chelonae*, respectively, while the latter two species are known to differ from one other by 4 bp (Kirschner *et al.*, 1993b; Springer *et al.*, 1995). The new taxon differs by more than 35 bp from all other species. A phylogenetic tree showed *M. chelonae*,

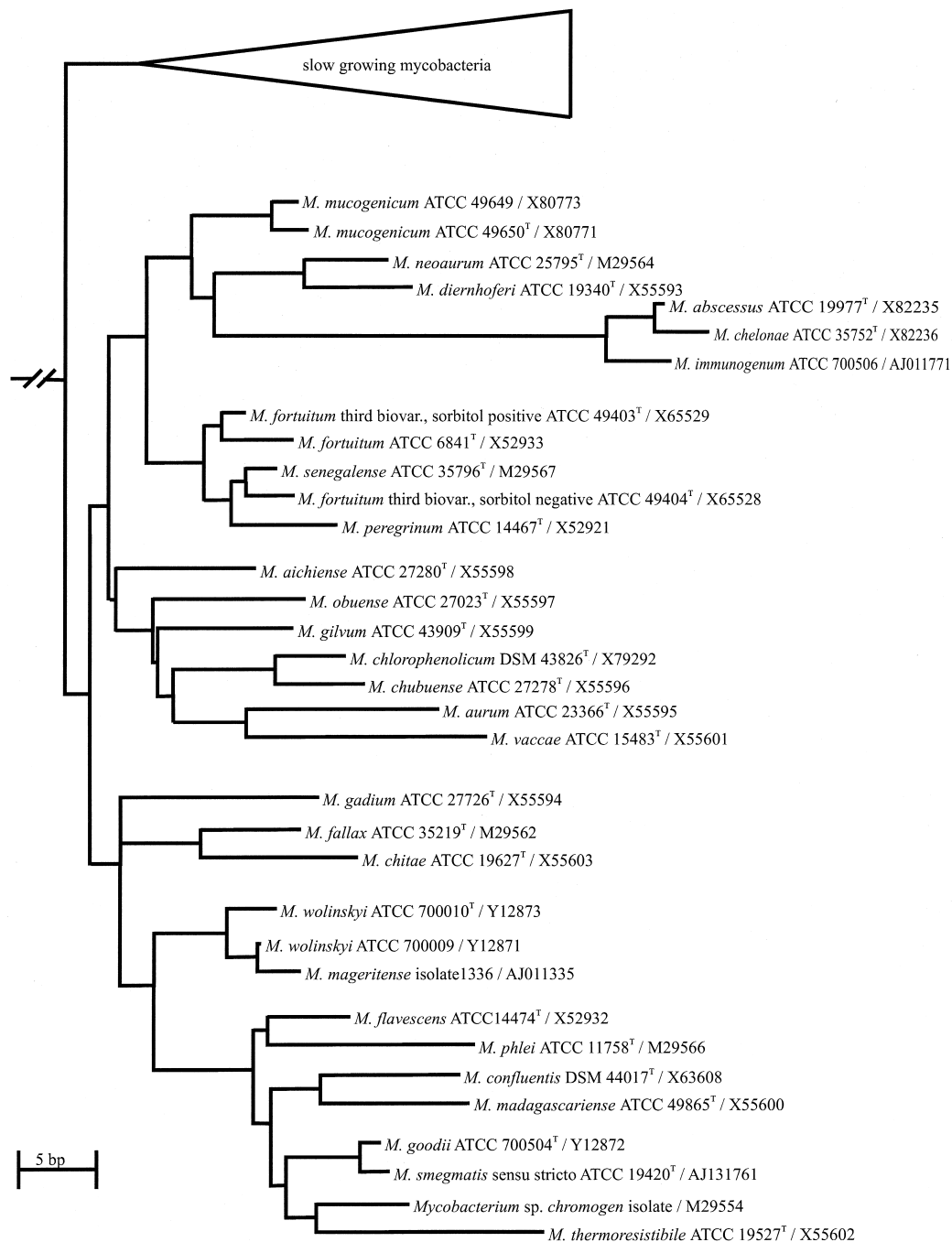


Fig. 5. Dendrogram illustrating the phylogenetic relationships on the basis of 16S rRNA gene sequences among 30 species and taxa of RGM including *M. immunogenum* ATCC 700506, *M. abscessus* ATCC 19977^T and *M. chelonae* ATCC 35752^T.

M. abscessus and the new taxon to occupy a separate branch of the tree, quite distant from other recognized species (Fig. 5).

Sequence determination of the *hsp65* gene

The 441 bp region described by Telenti *et al.* (1993), from nucleotide position 396 to 836 according to the published sequence from *Mycobacterium tuberculosis*

(Shinnick, 1987), was investigated. The *hsp65* sequences within this region differed by 21 and 33 bp, respectively, from *M. chelonae* and *M. abscessus*, while *M. chelonae* and *M. abscessus* differed from each other by 29 bp. As demonstrated previously, two hyper-variable regions of the *hsp65* gene were identified, between positions 624 and 664 and positions 683 and 725. A comparison of *M. chelonae*, *M. abscessus*, *M. mucogenicum* and the new taxon in the first hyper-

<i>M. tuberculosis</i>	AA	GGTCACCGAG	ACCCTGCTCA	AGGGCGCCAA	GGAGGTCGA
<i>M. abscessus</i> ATCC 19977 ^T	- -	- - - - -	- - G - - - G -	- - A - - - -	- - - - -
<i>M. chelonae</i> ATCC 35752 ^T	GC	C - - - -	- AGC	T - T - - - GG	- CTC - - - -
<i>M. immunogenum</i> ATCC 700505 ^T	- -	T - T - - - T	G - C - - - -	CT - - - - -	- - - - C - -
<i>M. mucogenicum</i> ATCC 49650 ^T	- -	- - - - -	- C	- GT - - - - -	- - - - - T - -

Fig. 6. Alignment of *hsp65* gene sequences of *M. tuberculosis* and four species of RGM in the hypervariable region (bp 624–664).

Table 5. DNA–DNA genomic pairing between *M. immunogenum* and phylogenetically related RGM

Test strain	Homology with ATCC 700505 ^T (%)
<i>M. immunogenum</i> ATCC 700505 ^T	100
<i>M. abscessus</i> ATCC 19977 ^T	15
<i>M. chelonae</i> ATCC 35752 ^T	18
<i>M. fortuitum</i> ATCC 6841 ^T	9.3
<i>M. mucogenicum</i> ATCC 49650 ^T	7
<i>M. senegalense</i> ATCC 35796 ^T	5.3

variable region is shown in Fig. 6. A phylogenetic tree based on this 441 bp *hsp65* sequence obtained with 10 species of RGM and the new taxon is shown in Fig. 7 (data for the 10 recognized species were published by Ringuet *et al.*, 1999).

DNA–DNA hybridization

DNA of the new taxon was hybridized with the following RGM reference isolates: *M. abscessus* ATCC 19977^T, *M. chelonae* ATCC 35752^T, *M. fortuitum* ATCC 6841^T, *M. mucogenicum* ATCC 49650^T and *Mycobacterium senegalense* ATCC 35796^T. The new taxon displayed < 25% homology with the five reference strains, with the highest degree of hybridization being shown with *M. chelonae* and *M. abscessus* (Table 5).

RFLP analysis of the 16S rRNA gene

*Bam*HI- and *Pst*I-generated chromosomal DNA restriction fragments from the new taxon and five other species of RGM were hybridized with an 804 bp, ³²P-labelled *M. fortuitum* 16S rRNA probe. Each of the six species of RGM produced a unique hybridization pattern with *Bam*HI- and *Pst*I-generated fragments. *M. abscessus* and *M. chelonae* had only a single copy of the ribosomal genes, while the new taxon and the other three species (*M. fortuitum*, *M. mucogenicum* and *M. senegalense*) had two copies.

DISCUSSION

This proposed newest member of the *M. fortuitum* complex resembled *M. abscessus* and *M. chelonae* so closely that molecular biological methods, including PRA and 16S rRNA gene sequencing, were required for its initial recognition. This almost certainly explains the failure until now to recognize this new taxon. Biochemically and by HPLC, it is related most closely

to *M. abscessus*, and it was identified as such in previous recoveries from nosocomial pseudo-outbreaks (Fraser *et al.*, 1992; Wallace *et al.*, 1993a). An unusual feature that differentiated isolates recovered from some pseudo-outbreaks from those of *M. abscessus* was the high level of resistance to the cephamycins cefmetazole and ceftioxin (Fraser *et al.*, 1992).

An unexpected finding was that, despite the similarity of new taxon to *M. chelonae* and *M. abscessus*, it has two copies of the rRNA operon, while the latter two species have only one. So far, *M. chelonae* and *M. abscessus* remain the only RGM species that contain just a single copy of the operon.

Isolates of the new taxon were recovered from 40% of nosocomial pseudo-outbreaks involving contaminated automated bronchoscope washers and were present in MWF used in the metal-grinding industry (Kreiss & Cox-Ganser, 1997). The new taxon was not identified among isolates that were recovered from 10 nosocomial outbreaks involving patient infections and were previously identified biochemically as *M. abscessus* and *M. chelonae*. The new taxon was responsible for a wide spectrum of clinical diseases (cutaneous infections, keratitis and catheter-related infections; Table 1), comparable to other species of RGM (Wallace *et al.*, 1991a, 1993b). In addition to the 11 clinical isolates of the new taxon identified in this study, two other clinical isolates that appear to belong to the new taxon have been reported by other investigators. One isolate (M.3666/3667) was identified earlier by 16S rRNA gene sequencing by one of the authors (B. Springer, unpublished), but was not available for further examination. The other isolate was designated as presumptive *M. chelonae* (Taylor *et al.*, 1997) and was referred to as *M. chelonae* II by Devallois *et al.* (1997). The latter isolate exhibited PRA patterns with *Bst*EII and *Hae*III that resembled very closely those of the new taxon in this study. These observations implicate this new mycobacterial taxon as a potential causative agent of human disease. Among clinical isolates of the *M. abscessus*/*M. chelonae* group submitted to the UTHCT laboratory for identification, 4% were identified by PRA as members of the new taxon. FL-HPLC did not resolve the new taxon from *M. abscessus*. However, *M. chelonae* could be distinguished from *M. abscessus* and the new taxon by either peak-height ratio or hierarchical cluster analysis of aligned whole chromatographic data.

By 16S rRNA gene sequencing, the new taxon isolates comprised a homogeneous group that exhibited a hypervariable region B sequence that was identical to

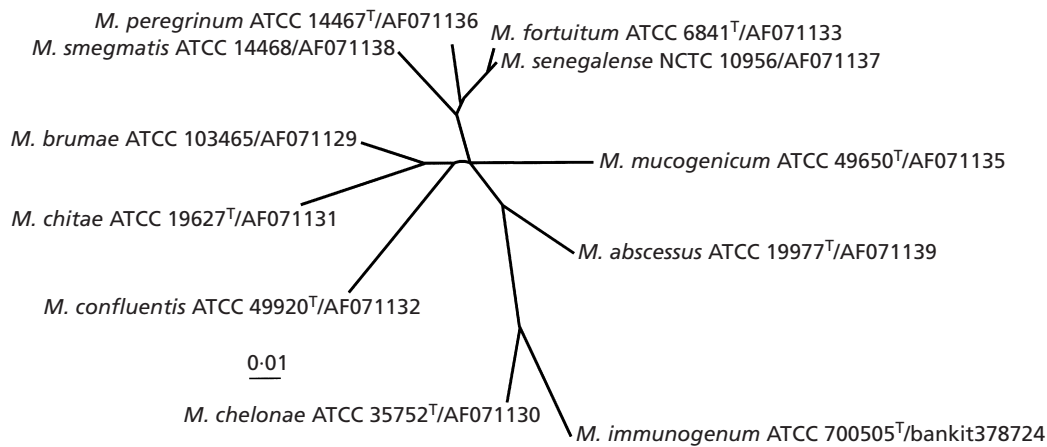


Fig. 7. Unrooted phylogenetic tree of species relatedness based on *hsp65* gene sequences. Bar, 1% estimated sequence divergence.

those of *M. chelonae* and *M. abscessus*. They exhibited a unique hypervariable region A sequence, however, and the entire sequence differed by 8 and 10 bp, respectively, from *M. abscessus* and *M. chelonae*.

Among non-ribosomal genes in the RGM, *hsp65* has been shown to be of special interest for taxonomic purposes (Ringuet *et al.*, 1999; Telenti *et al.*, 1993; Steingrube *et al.*, 1995a, b; Swanson *et al.*, 1996). As established previously, the *hsp65* gene displays more polymorphism than does the 16S rRNA gene. The *M. chelonae* and *M. abscessus* *hsp65* sequences differed by 29 bp in the 441 bp hypervariable region, whereas their 16S rRNA genes differed by only four nucleotides. Despite this, the phylogenetic tree based on *hsp65* sequences was consistent with the phylogeny based on 16S rRNA. Both trees showed that *M. chelonae* and *M. abscessus* are the more related species to the new taxon. However, due to the clear differentiation of *M. chelonae* and *M. abscessus* achieved by *hsp65* sequencing, the *hsp65* tree has an enlargement effect on the phylogenetic distances among the three groups and suggested that the new taxon is more related to *M. chelonae* than to *M. abscessus*. The use of phylogenetic trees of *hsp65* alleles from various mycobacterial species, both slowly and rapidly growing, has been reported previously (Swanson *et al.*, 1996; Kapur *et al.*, 1995; Ringuet *et al.*, 1999).

In a previous report on PRA identification of RGM (Steingrube *et al.*, 1995a, b), the only isolate of *M. chelonae* that failed to match the common PRA patterns for the species was the ATCC 35751 reference strain. More detailed examination of the original sample and a sample acquired recently from the ATCC revealed that it exhibited the biochemical, antimicrobial susceptibility and PRA pattern characteristics typical of *M. abscessus*. These data support the identification of reference strain ATCC 35751 as a strain of *M. abscessus* rather than *M. chelonae*.

Reference has been made to the difficulties involved in achieving objective, reproducible results with the biochemical tests available for the identification of aerobic actinomycetes (Devallois *et al.*, 1997; Silcox *et al.*, 1981; Steingrube *et al.*, 1995c, 1997), including the distinction between *M. abscessus* and *M. chelonae*. This difficulty was evident with evaluation of growth on Löwenstein–Jensen medium in the presence of 5% NaCl. Growth of *M. chelonae* and isolates of the new taxon was inhibited by 5% NaCl when incubated at 30 °C; however, 25–50% growth compared with positive controls was observed, and growth was not totally absent, as reported previously (Kent & Kubica, 1985; Springer *et al.*, 1995; Wallace *et al.*, 1993b). No growth was observed on Löwenstein–Jensen medium with 5% NaCl when *M. chelonae* and isolates of the new taxon were incubated at 35 °C. Growth of *M. abscessus* and strain ATCC 35751 in the presence of 5% NaCl was unaffected by temperature (30 or 35 °C), although the *M. abscessus* type strain, ATCC 19977^T, showed some degree of inhibition at both temperatures.

Previous disc-diffusion susceptibility studies of *M. abscessus* have shown that kanamycin disc zones of inhibition are larger than those for amikacin. A useful marker for the new taxon was that amikacin disc zones were generally equivalent to or larger than those for kanamycin and the isolates were resistant to cefoxitin and tobramycin. At least two of the three markers were present in 17/18 (95%) of isolates tested.

On the basis of the observed phenotypic (susceptibility) and genotypic characteristics that differentiate isolates of the new taxon from those of other species of *Mycobacterium*, we propose the name *Mycobacterium immunogenum* sp. nov. for these isolates. The specific epithet refers to the association of these mycobacterial isolates with cases of HP (Bolan *et al.*, 1985; Kreiss & Cox-Ganser, 1997; Muilenberg *et al.*, 1993).

Differentiation of *M. immunogenum* from related species by PRA

The PRA patterns of a 439 bp fragment of the *hsp65* gene of *M. immunogenum* are distinguishable from those of *M. abscessus* and *M. chelonae* and include: 315 and 130 bp bands with *Bst*EII; 140 and 70 bp bands with *Hae*III; 235, 120 and 65 bp bands with *Hha*I; 260 and 180 bp bands with *Msp*I; a 385 bp band with *Hinf*I; and 260 and 80 bp bands with *Bsa*HI. Differentiation of *M. immunogenum* from *M. abscessus* and *M. chelonae* can be accomplished with as few as two enzymes, e.g. *Bst*EII and *Hae*III, *Bst*EII and *Bsa*HI or *Msp*I and *Hinf*I.

Description of *Mycobacterium immunogenum* sp. nov.

Mycobacterium immunogenum (im.mu.no.gen'um. N. L. neut. adj. *immunogenum* eliciting an immune response).

A Gram-positive, acid- and alcohol-fast, curved bacillus that grows aerobically. It does not form spores or aerial hyphae. Visible growth appears in < 7 d. Colonies on Middlebrook 7H10 agar and trypticase soy agar are off-white in colour and usually rough, although smooth forms are observed. No pigment is produced. Growth occurs at 30 and 35 °C, but not at 45 °C. Isolates are susceptible *in vitro* to amikacin and clarithromycin and resistant to cefoxitin (MIC \geq 256 μ g ml⁻¹), cefmetazole, ciprofloxacin, doxycycline, imipenem, sulfamethoxazole and tobramycin (MIC \geq 16 μ g ml⁻¹). They are positive for 3-d arylsulfatase activity, negative for iron uptake and nitrate reductase, do not grow on Löwenstein-Jensen medium containing 5% NaCl at 35 °C and do not utilize citrate, D-glucitol (D-sorbitol), *i*-myo-inositol or D-mannitol as sole sources of carbon. Mycolic acids are present and produce HPLC chromatograms typical of the *M. abscessus*/*M. chelonae* group. Intact genomic DNA for PFGE can be obtained from all strains.

Two strains, ATCC 700505^T (formerly BH29^T, MC 779^T) and ATCC 700506 (formerly MN 3744, MC 1991), have been deposited with the ATCC (Manassas, VA, USA), with the type strain also deposited in the Mycobacterial Reference Collection of the Institute Pasteur as CIP 106684^T. The type strain, ATCC 700505^T, was recovered from a nosocomial pseudo-outbreak involving water-borne contamination of an automated bronchoscope washer in St Louis, MO, USA (Fraser *et al.*, 1992), and strain ATCC 700506 was isolated from contaminated industrial MWF (Moore *et al.*, 2000).

ACKNOWLEDGEMENTS

A portion of this work was presented as an abstract to the 98th General Meeting of the American Society for Microbiology, Atlanta, GA, USA, in May 1998. Contributions of authors: R.W.W., PRA technology and biochemical testing; V.A.S., computerized measurements, data evaluation and preparation of manuscript text and graphics;

E.C.B. and B.S., 16S rRNA sequencing; B.A.B., biochemical and susceptibility testing; V.V., *hsp65* gene sequencing and phylogenetic tree; S.H.C. and K.C.J., HPLC analyses; Y.Z., PFGE analyses; G.O., susceptibility testing; H.R., procurement of MWF isolates; M.J.G., DNA-DNA pairing and RFLP experiments; D.R.N., project coordinator; R.J.W., project director, traditional identification of isolates and editing manuscript text and graphics.

This work was supported by the Department of Microbiology and the Center for Pulmonary and Infectious Disease Control at UTHCT. We express our appreciation to Phyllis Pienta, former Collection Manager of Bacteriology, American Type Culture Collection, Manassas, VA, who kindly provided the reference strains used in this study; to Steven J. Moore, Department of Occupational and Environmental Medicine, UTHCT, for facilitating the acquisition of isolates from metalworking fluids; and to Professor Hans G. Trüper, Institute for Microbiology and Biotechnology University of Bonn, Bonn, Germany, for providing the correct Latin nomenclature. We would also like to thank Joanne Woodring for preparation of this manuscript.

This work is in memory of our co-author and friend Vincent Steingrube, a dedicated and meticulous researcher, who passed away prior to completion of this work.

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