***Mycobacterium wolinskyi* sp. nov. and *Mycobacterium goodii* sp. nov., two new rapidly growing species related to *Mycobacterium smegmatis* and associated with human wound infections: a cooperative study from the International Working Group on Mycobacterial Taxonomy**

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Previous investigations demonstrated three taxonomic groups among 22 clinical isolates of *Mycobacterium smegmatis*. These studies were expanded to 71 clinical isolates, of which 35 (49%) (group 1) were identical to five ATCC reference strains including the type strain ATCC 19420^T. Twenty-eight isolates (39%) were group 2, and eight isolates (11%) were group 3. Isolates of groups 2 and 3 were most often associated with post-traumatic or post-surgical wound infections including osteomyelitis, were susceptible to sulfamethoxazole, amikacin, imipenem and the tetracyclines, variably resistant to clarithromycin, and susceptible (group 1), intermediately resistant (group 2) or resistant (group 3) to tobramycin. The three groups were similar by routine biochemical and growth characteristics, but had different mycolic acid dimethoxy-4-coumarinylmethyl ester elution patterns by HPLC and different PCR-restriction enzyme patterns of a 439 bp fragment of the *hsp-65* gene. Group 3 isolates differed from group 1 by 18 bp by 16S rRNA sequencing and exhibited < 25% homology by DNA–DNA hybridization, being most closely related to *Mycobacterium mageritense*. The 16S rRNA of group 1 and group 2 isolates differed by only 3 bp, but by DNA–DNA hybridization they exhibited only 40% homology. The following names are proposed: *Mycobacterium goodii* sp. nov. for group 2 isolates (type strain ATCC 700504^T = MO69^T), *Mycobacterium wolinskyi* sp. nov. for group 3 isolates (type strain ATCC 700010^T = MO739^T) and *Mycobacterium smegmatis sensu stricto* for group 1 isolates.

Keywords: *Mycobacterium goodii*, *Mycobacterium wolinskyi*, *Mycobacterium smegmatis*, rapidly growing mycobacteria

INTRODUCTION

Mycobacterium smegmatis was first described in 1885 by Lustgarten as a cause of syphilitic penile ulcers

(Lustgarten, 1885). Although subsequently shown to have no relationship to syphilis, the organism was named for the genital secretions (smegma) from whence it was isolated (Lehmann & Neumann, 1931).

Abbreviations: ART, adjusted retention time; FL-HPLC, fluorescence detection HPLC; PRA, PCR-restriction enzyme pattern analysis.

The EMBL accession numbers for the 16S rRNA sequences in this paper are Y12873 (ATCC 700010^T), Y12871 (ATCC 700009) and Y12872 (ATCC 700504^T) (MO69^T).

The first modern day summary of isolates was in 1953 (Gordon & Smith, 1953) and described 56 strains, all from culture collections and none from human sources. Several studies (Tsukamura, 1984; Kasatiya *et al.*, 1974) have demonstrated the frequent presence of the organism in environmental sources.

M. smegmatis is currently recognized as an aetiologic agent of bovine mastitis (Richardson, 1970), feline and human post-traumatic wound infections (Plaus & Hermann, 1991; Richardson, 1970, 1971; Wallace *et al.*, 1988; Wilkinson *et al.*, 1982; Wolinsky & Rynearson, 1968) and human lung infections (Vonmoos *et al.*, 1986), although for its first 85 years it was regarded as a saprophytic organism of no clinical significance. The first well-described human case involved the lung and pleura in a patient with underlying lipoid pneumonia, and was reported less than 15 years ago (Vonmoos *et al.*, 1986). Since that date there have been a number of well-documented cases of human disease (Plaus & Hermann, 1991; Wallace *et al.*, 1988).

Although early taxonomic studies of *M. smegmatis* prior to the 1980s suggested the species was homogeneous, these analyses were based on limited phenotypic characteristics and/or involved only a few reference isolates (Kubica *et al.*, 1972; Tsukamura, 1984). Typical tests used to distinguish this species included; a negative 3 d arylsulfatase reaction, positive iron uptake and positive nitrate reductase reactions, growth at 45 °C and in the presence of 5 % NaCl, utilization of D-glucitol (D-sorbitol), i-myo-inositol and D-mannitol as sole carbon sources, and late (10–14 d incubation) yellow to orange pigmentation in 50 % of isolates (Gordon & Smith, 1953; Wayne & Kubica, 1986; Tsukamura, 1984; Wallace *et al.*, 1988). In subsequent studies in which more recently developed taxonomic techniques were used to evaluate clinical isolates, the heterogeneity among isolates within the species became readily apparent (Steingrube *et al.*, 1995b; Telenti *et al.*, 1993; Wallace *et al.*, 1988; Zhang *et al.*, 1992). This was first observed in a study of 22 clinical isolates of *M. smegmatis* in which three groups (1, 2 and 3) were identified based on differences in antibiotic susceptibilities, especially to tobramycin (Wallace *et al.*, 1988).

These interspecies differences were reinforced by genetic methodologies including PCR restriction analysis (PRA) of selected gene sequences. Two studies based on PRA of a 439 bp sequence of the *hsp-65* gene demonstrated the existence of genetic heterogeneity among isolates of *M. smegmatis*. In the first study, Telenti *et al.* (1993) reported two isolates of *M. smegmatis* (phenotypic or antibiogram group unknown) that differed from each other as well as from all other mycobacterial taxa with *Bst*EII and *Hae*III. Steingrube *et al.* (1995b) reported a second study that included nine isolates of *M. smegmatis* antibiogram groups 1 and 2 (Wallace *et al.*, 1988) among 129 clinical isolates and reference strains of rapidly growing mycobacteria in which PRA with *Bst*EII, *Hae*III,

*Acc*I and *Cfo*I (isoschizomer of *Hha*I) were compared. These studies found a close relationship between all nine isolates of *M. smegmatis* examined, although antibiogram group 1 and group 2 isolates could be separated with *Acc*I.

Heterogeneity of the β -lactamases extracted from *M. smegmatis* isolates was noted in a study by Zhang *et al.* (1992). They found two IEF patterns on polyacrylamide gels that were designated type 1 and type 2. The type 1 β -lactamase pattern was identified in 100 % of 41 isolates belonging to antibiogram group 1 (Wallace *et al.*, 1988), while the type 2 β -lactamase pattern was exhibited by 100 % of 13 isolates of groups 2 and 3 (Wallace *et al.*, 1988).

For the past 19 years, susceptibility testing and/or identification of isolates of rapidly growing mycobacteria has been a primary function of the Mycobacteria/Nocardia laboratory at the University of Texas Health Center at Tyler (UTHCT). Seventy-one isolates of *M. smegmatis* were identified during this period. Susceptibility testing produced three different patterns (antibiograms) among these isolates that included varying levels of susceptibility and resistance to tobramycin compared to the findings of Wallace *et al.* (1988). These groups were designated as *M. smegmatis* group 1, group 2 and group 3.

These observations prompted a cooperative study of these three groups as well as the newly described species *Mycobacterium mageritense* (Domenech *et al.*, 1997) by the International Working Group on Mycobacterial Taxonomy (IWGMT). This was a polyphasic analysis, and included standard biochemical analysis, antimicrobial susceptibility testing, GLC, HPLC, PCR restriction analysis of the 65 kDa heat-shock protein gene, 16S rRNA sequencing, 16S RFLP analysis and DNA–DNA hybridization assays following recently presented guidelines for recognition of new mycobacterial species (Levy-Frbault & Portaels, 1992).

METHODS

Organisms. Clinical isolates of *M. smegmatis* submitted to the UTHCT Mycobacteria/Nocardia laboratory from 1978 to 1998 were studied. Routine clinical information including culture source was obtained from the referring laboratory at the time each clinical isolate was received. Organisms were stored at –70 °C in trypticase soy broth with 15 % glycerol following initial identification and susceptibility testing. Five reference strains of *M. smegmatis* were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA): the type strain ATCC 19420^T, ATCC 35797, ATCC 35798, ATCC 14468 and ATCC 607. These five reference strains and 22 of the clinical isolates were partially characterized by Wallace *et al.* (1988). Five recently characterized strains of *M. mageritense* (1336, 1470, 1635, 1636 and the type strain 938^T = ATCC 700351^T) (Domenech *et al.*, 1997) were included for comparison with isolates of the *M. smegmatis* groups.

For HPLC analysis, reference strains of *Mycobacterium fortuitum* ATCC 6841^T, ATCC 35754 and I70715, *Myco-*

bacterium peregrinum ATCC 14467^T, ATCC 35755; and I70660; and *M. fortuitum* third biovariant complex I70868, I70876 and I71013 were used as comparative strains from the *M. fortuitum* complex. These isolates came from the ATCC or from the HPLC Users Group Steering Committee.

For the RFLP analysis of the 16S rRNA genes, control strains included *M. fortuitum* ATCC 6841^T, *M. fortuitum* third biovariant ATCC 49403^T, *M. fortuitum* third biovariant ATCC 49404^T, *Mycobacterium parafortuitum* strain CNMIVS, *M. peregrinum* ATCC 14467^T, *Mycobacterium porcinum* ATCC 27406^T, *Mycobacterium senegalense* NCTC 10956^T, *Mycobacterium chelonae* ATCC 35752^T and *Nocardia asteroides* strain CMNIVS.

For the DNA–DNA pairing studies, control strains included *M. mageritense* ATCC 700351^T, *M. smegmatis* ATCC 19420^T, *M. fortuitum* ATCC 6841^T and *Mycobacterium phlei* IMRU 500.

Cultural and biochemical characterization. All clinical and reference isolates received prior to 1988 were identified as *M. smegmatis* using standard growth and biochemical methods (Gordon & Smith, 1953; Kubica *et al.*, 1972; Silcox *et al.*, 1981; Wayne & Kubica, 1986; Swenson *et al.*, 1985; Tsukamura, 1984). This included typical colony morphology and growth within 7 d on trypticase soy agar and Middlebrook 7H10 agar, the absence of early pigmentation (within 7 d), positive nitrate reductase and positive iron-uptake reactions, a negative 3 d arylsulfatase reaction, growth at 45 °C, utilization of *i*-myo-inositol and *D*-mannitol as sole carbon sources (Silcox *et al.*, 1981; Wallace *et al.*, 1988), and drug susceptibility patterns that included susceptibility to amikacin, ciprofloxacin, doxycycline and sulfamethoxazole, and resistance to cefmetazole and cefoxitin (Wallace *et al.*, 1988). Isolates seen after 1988 were identified on the basis of typical growth and susceptibility patterns, the absence of early pigmentation, a negative 3 d arylsulfatase reaction, growth at 45 °C, utilization of *i*-myo-inositol and *D*-mannitol as sole carbon sources, and/or by PRA (Steingrube *et al.*, 1995b; Telenti *et al.*, 1993; Wilson *et al.*, 1998). Additional biochemical features including late pigmentation on 7H10 agar at 10–14 d, growth on MacConkey agar without crystal violet, degradation of *para*-aminosalicylate (PAS) to catechol, and utilization of citrate, *L*-arabinose, *D*-galactose, *D*-glucitol (*D*-sorbitol), *L*-rhamnose, *D*-trehalose and *D*-xylose, as sole carbon sources were determined for selected isolates (Silcox *et al.*, 1981; Tsukamura, 1984). Because all isolates were not tested simultaneously, some biochemical tests were not performed on all isolates.

Susceptibility testing. Susceptibility to nine antimicrobial agents was tested by a broth microdilution method in cation-supplemented Mueller–Hinton broth as previously described (Brown *et al.*, 1992; Swenson *et al.*, 1985). The antimicrobials tested included amikacin, cefmetazole, cefoxitin, ciprofloxacin, clarithromycin, doxycycline, imipenem, sulfamethoxazole and tobramycin. Because all isolates were not tested simultaneously, some isolates were not tested against all of the antimicrobials. Breakpoints used for MICs were those suggested by the National Committee for Clinical Laboratory Standards (NCCLS) (1990) for organisms that grow aerobically, except for cefoxitin, for which an MIC > 32 µg ml⁻¹ rather than > 16 µg ml⁻¹ was used as the resistance breakpoint.

For taxonomic purposes, susceptibility to tobramycin was established as an MIC of ≤ 1 µg ml⁻¹, intermediate suscep-

tibility as an MIC of 2–8 µg ml⁻¹, and resistance as an MIC of ≥ 16 µg ml⁻¹. Isolates in the susceptible category were designated group 1, those in the intermediate category as group 2, and those that were resistant as group 3 (Wallace *et al.*, 1988).

Susceptibilities to ethambutol, rifampin and isoniazid were performed by the proportion method on Ogawa egg medium by Tsukamura (1984) and on Mueller–Hinton agar supplemented with OADC (oleate-albumin-dextrose-catalase). Agar disk diffusion (Wallace *et al.*, 1980) with 30 µg chloramphenicol per disk and 10 µg tobramycin per disk was performed on Mueller–Hinton agar (Becton Dickinson Microbiology Systems) for 40 and 71 isolates, respectively. Thirty-five isolates of *M. smegmatis* group 1, including the five ATCC reference strains, and 28 isolates of *M. smegmatis* group 2 were tested against tobramycin, while 17 *M. smegmatis* group 1 and 15 *M. smegmatis* group 2 isolates were tested against chloramphenicol. All eight isolates of *M. smegmatis* group 3 were tested against both tobramycin and chloramphenicol. Zone sizes were determined after 3 d incubation at 30 °C. Isolates with zone sizes > 30 mm for tobramycin were considered to be susceptible; those with 11–30 mm zone sizes were considered to be of intermediate susceptibility, and those with zones ≤ 10 mm were considered to be resistant.

HPLC

Sample processing for fluorescence detection HPLC (FL-HPLC). A culture for FL-HPLC was inoculated onto Löwenstein–Jensen slants and incubated at 35 °C for 5 d. A sterile wooden applicator stick was used to transfer a small amount of cell mass to a test tube containing 2.0 ml 25% potassium hydroxide in methanol/water (1:1, v/v). The tube was capped and autoclaved for 60 min at 121 °C. After the tube was cooled to room temperature, 1.5 ml chloroform and 1.8 ml concentrated hydrochloric acid/water (1:1, v/v) were added. The tube was vortexed for 60 s and the layers were allowed to separate. The chloroform layer was transferred to a 13 × 100 mm screw cap test tube and evaporated to dryness at 85 °C at 30% full vortex speed in a Rapid-Vap (Labconco). One hundred microlitres of 2% potassium bicarbonate in water/methanol (1:1, v/v) were added to the tube and evaporated to dryness at 85 °C. The tube was cooled to room temperature and 100 µl chloroform containing 125 µg 4-bromomethyl-6-7 dimethoxycoumarin and 15 µg 18-crown-6 ether (both from Sigma) were added. The tube was sealed with a Teflon-lined cap and heated at 90 °C for 20 min. After the tube was cooled to room temperature, 1.5 ml chloroform and 1 ml concentrated hydrochloric acid/water/methanol (1:1:2, by vol.) were added. The tube was vortexed briefly and the layers were allowed to separate. The chloroform layer was transferred to a tube and evaporated. The sample was dissolved in 300 µl chloroform that contained 50 ng ml⁻¹ and 200 ng ml⁻¹, respectively, of Low and High Molecular Mass Internal Standards (LMWS and HMWS) for fluorescence-HPLC (Ribi Immunochem Research); 100 µl of this solution was transferred to an autosampler vial that contained 400 µl chloroform.

FL-HPLC. HPLC was performed with a Hewlett Packard 1050 series pump and autosampler. The pump gradient has been described previously (Butler *et al.*, 1996a). Sample injection volume was 5 µl. Mycolates were separated with a Beckman 4.6 × 70 mm, 3 µm C-18 particle cartridge column (Beckman) maintained at 35 °C. Mycolates were detected with a Jasco FP-920 fluorescence detector (Jasco) equipped

with a high-sensitivity photomultiplier (gain = 1000) and a 16 µl flowcell. Excitation and emission were set at 351 and 430 nm, respectively. Emission bandwidth was 18 nm. The digital filter was used (S = 3) and the signal was collected at 2 Hz. *M. smegmatis* groups 1, 2 and 3 strains were processed and analysed by a standardized UV-absorbance HPLC (UV-HPLC) method (Butler *et al.*, 1996a).

Chromatographic data analysis. Raw FL-HPLC chromatograms were imported into Pirouette Software, version 2.03 (Infometrix). Adjusted retention times (ART) for each chromatogram were derived by alignment of the two internal standards, as has been done previously with mycolic acid *p*-bromophenacyl esters detected by UV absorption (Butler *et al.*, 1996a, b). Hierarchical cluster analysis was performed on raw normalized chromatographic data from the region between the two internal standards using a mean-centred data preprocess.

GLC. Whole-cell fatty acid analyses were performed by GLC with the Microbial Identification System (MIS; MIDI). The MIS included an HP model 6890A gas chromatograph equipped with a 25 m × 0.2 mm fused silica capillary column, a flame-ionization detector, an automatic sampler, an integrator and a microcomputer (Hewlett Packard). Peaks were automatically identified and quantified by the system. Organism identification was done by comparison with the MIDI mycobacterial library database (version 3.9). The manufacturer's protocol was followed for all stages of cultivation, extraction, saponification, methylation and chromatography procedures.

Measurement of DNA G + C composition. Mycobacteria were cultured on Löwenstein-Jensen slants. DNA extraction, purification, degradation and G + C content determinations by HPLC were performed as previously described by Mesbah *et al.* (1989), except that a Waters 625 LC System, with a Waters 486 Tunable Absorbance Detector and a Waters 746 Data Module (Millipore/Waters), was used.

PRA of a 439 bp segment of the *hsp-65* gene. All isolates of *M. smegmatis* group 3 and *M. mageritense*, and selected isolates of *M. smegmatis* group 1 and *M. smegmatis* group 2 were subjected to PCR amplification of a 439 bp segment of the *hsp-65* gene as described by Telenti *et al.* (1993). Cells were grown on tryptic soy agar and ground cell supernatants were used as DNA templates as previously described (Steingrube *et al.*, 1995b; Wilson *et al.*, 1998) using the appropriate positive and negative controls (Telenti *et al.*, 1993).

Screening of multiple commercial restriction endonucleases in previous studies (Steingrube *et al.*, 1995a, b, 1997) resulted in the selection of seven restriction endonucleases (*Bst*II, *Hae*III, *Hha*I, *Msp*I, *Hinf*I, *Bsa*HI and *Acc*I) (New England Biolabs and Promega) for further characterization of isolates. Enzymes were used according to the manufacturer's recommendation, except for *Bsa*HI. To achieve complete digestion with *Bsa*HI, acetylated BSA was substituted for BSA and the digestions mixture was incubated at 60 °C for 1 h.

Restriction fragments were electrophoresed on 3% Meta-phor agarose (4 bp resolution; FMC Bioproducts) containing ethidium bromide (0.625 µg ml⁻¹) in a Mini-SubCell electrophoresis system (Bio-Rad) at 95 V for 1.5–2.0 h. Fragment sizes (in bp) were estimated on a computerized Bio Image system (Millipore). Fragments of 60 bp were disregarded as PCR primer artifacts as recommended by Telenti *et al.* (1993).

16S rRNA sequencing. The 16S rRNA regions chosen for analysis were based on previously published sequence data available in the EMBL/GenBank database (Domenech *et al.*, 1997; 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 sequences used for comparison studies were as follows: *M. fortuitum* ATCC 6841^T, X52933; *M. fortuitum* third biovariant complex sorbitol-positive ATCC 49403^T, X65528; *M. fortuitum* third biovariant complex sorbitol-negative ATCC 49404^T, X65529; *Mycobacterium abscessus* ATCC 19977^T, X82235; *M. chelonae* ATCC 35752^T, X82236; *Mycobacterium mucogenicum*, X80771; *M. smegmatis* ATCC 14468, X52922; *Mycobacterium mageritense* ATCC 700351^T, X99938; and *Mycobacterium peregrinum*, X52921.

The methods used for DNA extraction, gene fragment amplification of 16S rRNA, and sequence determination have been described previously (Rogall *et al.*, 1982; Böttger, 1989; Böddinghaus *et al.*, 1990; Edwards *et al.*, 1989; Kirschner *et al.*, 1993a, b; Rogall *et al.*, 1990). Briefly, to extract nucleic acids, a colony from a Löwenstein-Jensen slant was suspended in 500 µl of 10 mM Tris-1 mM EDTA (TE). The bacterial pellet was harvested by centrifugation in a microcentrifuge and then dispersed in 100 µl of TE containing acid-washed glass beads (diameter, 100 µm; Sigma). A tissue disintegrator (H. Mickle) was used to disrupt the cells (2 min at the maximum speed). After centrifugation for 5 min, 5 µl of the supernatant was used for PCR.

After amplification of the 5' part of the 16S rRNA gene using primers AGAGTTTGATCCTGGCTCAG (positions 8–28) in combination with TGCACACAGGCCACAAG-GGA (positions 1046–1026), the nucleic acid sequences of hypervariable regions A and B (Kirschner *et al.*, 1993b) were determined. Based on the grouping determined by this comparison, representative strains were chosen for nearly complete 16S rRNA analysis. The 3' part of the 16S rRNA gene was amplified using primers GTGTGGGTTTCCT-TTCCTTGG (positions 830–847) and AAGGAGGTGATC-CAGCCGCA (positions 1542–1522). Sequence information on nearly the complete 16S rRNA gene was obtained by PCR-mediated *Taq*-cycle sequencing using an ABI 373 sequencer (Hultman *et al.*, 1989).

The sequences which we obtained were aligned with selected 16S rRNA sequences as described previously (Rogall *et al.*, 1990). For phylogenetic analyses, only those 16S rRNA regions corresponding to *Escherichia coli* positions 126–554, 894–1082 and 1101–1332 that were available for rapidly growing mycobacterial species were included. Pairwise (Hamming) distances were calculated by weighting nucleotide differences and insertions-deletions equally. A phylogenetic tree was constructed by using the neighbourliness method. (Rogall *et al.*, 1990; Sourdís & Nei, 1998).

DNA-DNA hybridization experiments. 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 using alkaline denaturation and UV fixation. Hybridizations were carried out under stringent conditions for 40 h. The filters were then 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. Control strains included *M. fortuitum* ATCC 6841^T and *M. phlei* IMRU 500.

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

An 804 bp fragment of the 16S rRNA gene of *M. fortuitum* type strain ATCC 6841^T was obtained by PCR and used as a probe. For DNA amplification the R1 and Rc2 oligonucleotide sequences from the *Mycobacterium bovis* BCG 16S rRNA sequence were used in 50 µl amplification mixtures as previously described (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 incubation at 72 °C for 5 min. The ribosomal probe was labelled using the Prime-α-Gene system (Promega) and 20 µCi [α -³²P]dCTP.

RESULTS

Organisms

A total of 71 clinical isolates of *M. smegmatis*, identified during the 19-year study period from 1978 to the present, and five ATCC reference strains were divided into three groups on the basis of susceptibility to tobramycin and PRA (Wallace *et al.*, 1988). Thirty-five (49 %) of the clinical isolates and all five ATCC reference strains (including the type strain ATCC 19420^T) were highly susceptible to tobramycin with MICs $\leq 1 \mu\text{g ml}^{-1}$ and disk zones $> 30 \text{ mm}$, had a single PRA pattern, and were designated *M. smegmatis* group 1. Twenty-four (38 %) of the isolates were intermediately susceptible to tobramycin with MICs of $2\text{--}8 \mu\text{g ml}^{-1}$ and disk zones of $11\text{--}30 \text{ mm}$, exhibited a second unique PRA pattern, and were designated as a new species *M. smegmatis* group 2. Four additional isolates were highly susceptible to tobramycin, but

exhibited the second PRA pattern and were included in the latter group. The remaining eight (11 %) isolates were resistant to tobramycin with MICs $\geq 16 \mu\text{g ml}^{-1}$ and no zones of inhibition by disk diffusion, exhibited a third PRA pattern, and were designated as *M. smegmatis* group 3.

Eight of eight (100 %) isolates of *M. smegmatis* group 3, 22 of 28 (79 %) isolates of *M. smegmatis* group 2, and 34 of 37 (92 %) isolates of *M. smegmatis* group 1 were recovered from non-pulmonary sources including post-traumatic or post-surgical infections of skin, soft tissue and/or bone. Clinical histories were available for seven of eight *M. smegmatis* group 3 isolates (Table 1) and 24 of 28 isolates of *M. smegmatis* group 2 (Table 2). Six of eight (75 %) *M. smegmatis* group 3 infections occurred in Texas, while infections with *M. smegmatis* group 2 were more ubiquitous and only 11 of 28 (39 %) occurred in Texas. Of the *M. smegmatis* group 3 isolates, three were recovered from surgical wound infections while the remaining four came from traumatic wound infections. Multiple positive cultures were recovered from most patients, and 50 % of the wound infections involving *M. smegmatis* group 3 and 67 % of the wound infections involving *M. smegmatis* group 2 were associated with osteomyelitis (Table 1). Community-acquired soft tissue infections due to *M. smegmatis* group 2 were usually osteomyelitis following a motor vehicle accident, often with metal hardware insertion. Pulmonary disease was strongly associated with the histologic finding of lipoid pneumonia, including disease such as achalasia with chronic aspiration (Table 2).

Morphological and biochemical characterization

Ninety-three per cent of the *M. smegmatis* group 1 isolates and 78 % of the *M. smegmatis* group 2 isolates produced late (10–14 d incubation period) yellow to

Table 1. Clinical information for the eight patients infected with *M. smegmatis* group 3

Isolate no.	Patient age (yrs)	Sex	Geographic location	Source	Disease (reference)*
ATCC 700009	69	F	Texas	Sternum	Surgical wound infection, osteomyelitis (cardiac surgery) (Wallace <i>et al.</i> , 1988)
ATCC 700010 ^T	35	F	Switzerland	Face	Surgical wound infection (plastic surgery) (Pennekamp <i>et al.</i> , 1997)
MO 178	9	M	Texas	Foot	Cellulitis, osteomyelitis following stepping on a nail (Wallace <i>et al.</i> , 1988)
MO 472	29	F	California	Thigh	Cellulitis, localized abscess following MVA
MO 527	NA	M	Texas	Axilla	NA
MO 817	55	M	Texas	Arterial/venous ports	Infected arterial–venous shunt (on hemodialysis)
MO 116#2	55	F	Texas	Elbow	Cellulitis, osteomyelitis following open fracture (MVA) (Wallace <i>et al.</i> , 1988)
MF1425	40	F	Texas	Calf	Cellulitis following local trauma

NA, Not available.

* MVA, motor vehicle accident.

Table 2. Clinical information for 28 patients infected with *M. smegmatis* group 2

Isolate no.	Patient age (yrs)	Sex	Geographic location	Source	Diagnosis (reference)*
Community-acquired wound/bone infections					
ATCC 700504 ^T (MO 69)	22	M	Florida	Calcaneus heel	Osteomyelitis from penetrating trauma (Wallace <i>et al.</i> , 1988)
MO 82	20	F	Texas	Femur	Osteomyelitis from open fracture (MVA) (Wallace <i>et al.</i> , 1988)
MO 239	73	M	Alabama	Right third toe	Osteomyelitis from stepping on a nail
MO 471	21	M	California	Tibia	Cellulitis/osteomyelitis from puncture wound (MVA) (Newton <i>et al.</i> , 1993)
MO 116#1	55	F	Texas	Elbow	Cellulitis/osteomyelitis from open fracture (MVA) (Wallace <i>et al.</i> , 1988)
MO 648	18	M	Florida	Leg	Infected wound (?osteomyelitis)
MO 382	30	F	Texas	Tibia	Osteomyelitis following fracture
MO 776	NA	F	Minnesota	Femur	Osteomyelitis post-surgical (hardware) replacement
MO 881	26	F	California	Left thigh, femur	Cellulitis/osteomyelitis from an open avulsion injury (MVA) (Newton <i>et al.</i> , 1993)
MO 65	12	M	Texas	Cheek	Cellulitis (MVA) (Wallace <i>et al.</i> , 1988)
MO 947	16	M	Russia	Femur	Osteomyelitis from open fracture (MVA) hardware insertion
MO 541	13	M	Utah	Thigh	Cellulitis/chronic draining sinus from puncture wound with a nail
Nosocomial disease					
MO 80	64	M	Texas	Blood	Intravenous catheter sepsis (Wallace <i>et al.</i> , 1988)
MO 143	62	M	Texas	Sternum	Cardiac bypass infection (osteomyelitis)
MO 793	26	F	Oklahoma	Right breast	Infection following breast reduction surgery
MO 730	20	F	Missouri	Pacemaker	Infected pacemaker site
MO 850	91	F	California	Pacemaker	Infected pacemaker site
MO 57	60	M	California	Sternum	Wound infection (?osteomyelitis) (Wallace <i>et al.</i> , 1988)
Respiratory disease					
MO 123	58	M	Australia	Pleural fluid, lung biopsy	Lipoid pneumonia (Wallace <i>et al.</i> , 1988)
MO 584	76	M	Oklahoma	Open biopsy of lung mass	Chronic granulomatous disease
MO 815	56	M	Texas	Bronchial wash	Necrotizing granulomatous pneumonia
MO 375	34	F	Texas	Open lung biopsy	Bilateral lipoid pneumonia with prior gastrectomy
MO 386	18	M	Indiana	Bilateral open lung biopsy	Exogenous lipoid pneumonia (mineral oil)
MO 659	53	M	Ohio	Sputum	Achalasia with pulmonary infiltrates
Diagnosis unknown					
MO 288	90	M	Texas	Cerebrospinal fluid	NA
MO 302	40	M	Texas	Right thigh tissue	NA
MO 709	69	M	Massachusetts	Knee aspirate	NA
MO 547	18	F	Texas	Arm	NA

NA, Not available.

* MVA, motor vehicle accident.

orange pigmentation in contrast to isolates of *M. smegmatis* group 3 and *M. mageritense* that produced no pigment (Table 3).

The clinical and reference isolates of *M. smegmatis* group 1, the clinical isolates of *M. smegmatis* group 2, and the clinical isolates of *M. smegmatis* group 3 all

Table 3. Growth characteristics, biochemical features, β -lactamase patterns, and susceptibility features of *M. smegmatis* groups 1, 2 and 3

Some data are from a previous study (Wallace *et al.*, 1988).

Feature	<i>M. smegmatis</i> group 1				<i>M. smegmatis</i> group 2		<i>M. smegmatis</i> group 3		
	All isolates	ATCC 19420 [†]	ATCC 700011	ATCC 700012	All isolates	ATCC 700504 [†]	All isolates	ATCC 700009	ATCC 700010 [†]
Smooth colony	76% (25/33)	—	+	+	93% (26/28)	+	100% (8/8)	+	+
Growth at 45 °C at 3 d	100% (29/29)	+	+	+	100% (23/23)	+	100% (8/8)	+	+
Late pigmentation (> 10 d) on 7H10 agar	95% (18/19)	+	+	+	78% (18/23)	+	0% (0/8)	—	—
3 d arylsulphatase	5% (1/21)	—	—	—	5% (1/21)	—	0% (0/8)	—	—
Degradation of <i>p</i> -aminosalicylate	0% (0/13)	—	—	—	100% (8/8)	+	100% (3/3)	+	ND
Growth on MacConkey agar (no crystal violet)	100% (15/15)	+	+	+	100% (13/13)	+	100% (8/8)	+	ND
Nitrate reduction	92% (12/13)	+	—	+	100% (16/16)	+	100% (8/8)	+	+
Iron uptake	67% (10/15)	+	+	+	100% (17/17)	+	100% (8/8)	+	+
Growth on 5% NaCl	100% (15/15)	+	+	+	100% (18/18)	+	88% (7/8)	+	+
Semiquantitative catalase									
< 45 mm	87% (13/15)	+	+	+	89% (14/16)	+	50% (4/8)	+	+
< 60 mm	100% (11/11)	+	+	+	100% (11/11)	+	100% (8/8)	+	+
68 °C catalase	0% (0/15)	ND	ND	—	7% (1/14)	—	100% (8/8)	+	+
Utilization of carbon sources:									
L-Arabinose	100% (20/20)	+	+	+	100% (18/18)	+	63% (5/8)	+	+
Citrate	20% (4/20)	+	—	—	26% (5/19)	—	50% (4/8)	—	+
D-Galactose	80% (8/10)	+	+	—	42% (5/12)	—	88% (7/8)	+	+
D-Glucitol (D-sorbitol)	100% (17/17)	+	+	+	100% (18/18)	+	100% (8/8)	+	+
D-Mannitol	100% (23/23)	+	+	+	100% (21/21)	+	100% (8/8)	+	+
<i>i</i> -myo-Inositol	100% (23/23)	+	+	+	100% (21/21)	+	100% (8/8)	+	+
L-Rhamnose	100% (14/14)	+	+	+	100% (16/16)	+	100% (8/8)	+	+
D-Trehalose	60% (6/10)	+	+	—	50% (8/16)	—	88% (7/8)	+	+
D-Xylose	100% (20/20)	+	+	+	100% (18/18)	+	63% (5/8)	+	+
β-Lactamase pattern*									
pI 4.4	100% (40/40)	+	+	+	0% (0/6)	—	0% (0/3)	—	ND
pI 4.8, 4.6	0% (0/40)	—	—	—	100% (6/6)	+	100% (3/3)	+	ND
Disk diffusion susceptibility									
Tobramycin (10 µg disk)									
No zone (6 mm)	0% (0/35)	—	—	—	0% (0/28)	—	100% (8/8)	+	+
11–30 mm	3% (1/35)	—	—	—	86% (24/28)	+	0% (0/8)	—	—
> 30 mm	97% (34/35)	+	+	+	14% (4/28)	—	0% (0/8)	—	—
Chloramphenicol (30 µg disk)									
≥ 10 mm	100% (17/17)	+	+	+	100% (15/15)	+	50% (4/8)	—	+
MIC determinations									
Tobramycin									
MICs ≤ 1 µg ml ^{−1}	100% (28/28)	+	+	+	19% (5/27)	—	0% (0/8)	—	—
MICs 2–8 µg ml ^{−1}	0% (0/28)	—	—	—	81% (22/27)	—	100% (8/8)	—	—
Antituberculous drug susceptibilities (Ogawa media)									
Ethambutol (5 µg ml ^{−1})	100% (11/11)	+	+	+	100% (8/8)	—	100% (3/3)	ND	ND
Rifampin (25 µg ml ^{−1})	0% (0/11)	—	—	—	0% (0/8)	—	0% (0/3)	ND	ND
Isoniazid (10 µg ml ^{−1})	0% (0/11)	—	—	—	0% (0/8)	—	0% (0/3)	ND	ND

ND, Not determined.

* Data from Zhang *et al.* (1992).

exhibited the classical growth and biochemical profile of *M. smegmatis* (Wayne & Kubica, 1986) (Table 3). Thus, conventional biochemical testing could not distinguish between these three taxa.

Eight of eight (100%) isolates of *M. smegmatis* group 2 and three of three (100%) isolates of *M. smegmatis* group 3 tested degraded PAS to catechol, whereas none of the 13 (0%) isolates of the *M. smegmatis* group 1 were positive for PAS degradation. As noted above, isolates of all three taxa tested exhibited typical *M. smegmatis* biochemical profiles. This was also true with regard to carbohydrate utilization as sole carbon sources (Table 3). All isolates of *M. smegmatis* groups

1, 2 and 3 tested utilized D-glucitol (D-sorbitol), *i*-myo-inositol, D-mannitol and L-rhamnose. Likewise, only 11–50% of all isolates tested were capable of utilizing citrate as a sole carbon source. Utilization of L-arabinose, D-galactose, D-trehalose and D-xylose differed somewhat between the three taxonomic groups. Twenty of 20 (100%) isolates of *M. smegmatis* group 1 utilized L-arabinose and D-xylose, while 19 of 20 (95%) and 20 of 20 (100%) isolates of *M. smegmatis* group 2 and five of eight (63%) isolates of *M. smegmatis* group 3 were able to utilize these two substrates. Ten of 13 (77%) and seven of eight (88%) isolates of *M. smegmatis* group 1 and *M. smegmatis* group 3, respectively, and four of ten (40%) and three

Table 4. MICs by broth microdilution including MIC₅₀ and MIC₉₀ of *M. smegmatis* groups 1, 2 and 3

Drug	Group 1				Group 2				Group 3			
	No. tested	MIC range	MIC ₅₀	MIC ₉₀	No. tested	MIC range	MIC ₅₀	MIC ₉₀	No. tested	MIC range	MIC ₅₀	MIC ₉₀
Amikacin	25	≤ 0.25–1	≤ 0.5	≤ 1	28	≤ 0.25–4	0.5	≤ 1	8	2–16	2	16
Tobramycin	34	≤ 0.25–2	≤ 0.5	2	28	≤ 0.5–8	2	8	8	> 16–> 32	> 32	> 32
Doxycycline	23	≤ 0.25–1	≤ 0.25	0.5	28	≤ 0.25–1	≤ 0.25	0.5	8	0.5–4	1	4
Clarithromycin	17	≤ 0.063–> 128	4	> 128	17	≤ 0.25–> 128	32	> 128	8	8–> 64	> 16	64
Imipenem	24	≤ 0.5–4	≤ 0.5	2	28	≤ 0.5–8	2	8	8	2–8	4	8
Cefoxitin	22	≤ 2–128	8	64	28	≤ 8–> 256	64	> 256	8	16–128	32	64
Cefmetazole	12	≤ 1–32	16	32	22	≤ 2–> 128	32	> 128	8	16–32	16	32
Ciprofloxacin	25	≤ 0.063–1	0.25	1	28	≤ 0.063–1	0.25	0.5	8	0.25–2	1	2
Sulfamethoxazole	23	≤ 1–4	≤ 1	4	28	≤ 1–8	≤ 1	8	8	≤ 1–4	≤ 1	4

MIC₅₀, MIC which inhibits 50% of strains tested.

MIC₉₀, MIC which inhibits 90% of strains tested.

Fig. 1. Comparison of HPLC-generated mycolic acid 6,7-dimethoxy-4-coumarinylmethyl ester elution patterns for *M. smegmatis* group 1, *M. mageritense* and the proposed new species *M. smegmatis* groups 2 and 3. Abbreviations: LMWS, low molecular weight standard; and HMWS, high molecular weight standard.

of ten (30%) isolates of *M. smegmatis* group 2, utilized D-galactose and D-trehalose, respectively, as sole carbon sources.

Susceptibility testing

Isolates of *M. smegmatis* group 1, *M. smegmatis* group 2 and *M. smegmatis* group 3 were susceptible to amikacin and sulfamethoxazole, intermediately sus-

ceptible to ciprofloxacin and doxycycline, and variably susceptible to cefmetazole, cefoxitin and clarithromycin. Isolates of *M. smegmatis* group 3 exhibited higher MICs to amikacin, doxycycline and tobramycin, and lower MICs to cefoxitin than the other two species (Table 4). Isolates of all three taxa were susceptible to 5 µg ethambutol ml⁻¹ and resistant to 25 µg rifampin ml⁻¹ and 10 µg isoniazid ml⁻¹ (Table 3). By comparison, *M. mageritense* was resistant to

Fig. 2. Dendrogram illustrating phylogenetic relationships between *M. smegmatis* group 1, *M. mageritense* and the proposed new species *M. smegmatis* groups 2 and 3 based on mycolic acid composition analyses by HPLC. Abbreviations: Msm, *M. smegmatis* group 1; Mgo, *M. smegmatis* group 2; Mwo, *M. smegmatis* group 3; and Mmg, *M. mageritense*. Scale indicates the similarity index.

ethambutol as well as isoniazid (Domenech *et al.*, 1997).

Disk zone sizes were determined for 30 µg chloramphenicol and 10 µg tobramycin. Chloramphenicol zones were compared for 40 selected isolates of the three species. Twenty isolates of *M. smegmatis* group 1, including all five reference strains, had zones of inhibition to the chloramphenicol disk ≥ 10 mm with a range of 11–44 mm and a mean zone diameter of 17 mm. Twelve *M. smegmatis* group 2 isolates had zones of inhibition ≥ 10 mm with a range of 10–27 mm and a mean of 15 mm. Conversely, all eight isolates of *M. smegmatis* group 3 exhibited zones of 6–12 mm with a mean of 9 mm. Only four of the eight isolates had zones > 10 mm.

Tobramycin disk diffusion zone diameters were determined for 52 isolates and, as noted (Wallace *et al.*, 1988), were used for initial separation of the three taxonomic groups. Isolates of *M. smegmatis* group 1 were susceptible to tobramycin by disk diffusion, *M. smegmatis* group 1 isolates exhibited intermediate susceptibility to tobramycin, and *M. smegmatis* group 3 isolates were resistant to tobramycin (Table 3).

HPLC

FL-HPLC chromatograms aligned by using the two internal standards resulted in highly coincident peaks (Fig. 1). Strains analysed by both FL-HPLC and UV-HPLC yielded highly comparable chromatograms (data not shown). *M. smegmatis* groups 1, 2, 3 and *M. mageritense* produced two cluster mycolic acid patterns; each cluster contained four to five peaks. These same four taxa differed in the relative abundance of both the front and rear clusters and the peaks within a cluster.

All strains of *M. mageritense* clustered strongly and apart from the other three taxa (Fig. 2). *M. smegmatis* group 3 strains yielded moderately conserved patterns

that were intermediate between *M. mageritense* and some strains of *M. smegmatis* group 2. *M. mageritense* and *M. smegmatis* group 3 were characterized by low front cluster height; the height of the tallest front cluster peak ranged from 0.13 to 0.17 and 0.28 to 0.40 of the tallest rear cluster peak for *M. mageritense* and *M. smegmatis* group 3, respectively. *M. mageritense* and *M. smegmatis* group 3 strains produced a rear cluster with a single prominent peak at approximately 7.68 min ART; the second tallest rear cluster peak ranged from 0.27 to 0.37 and 0.46 to 0.69 of the tallest peak for *M. mageritense* and *M. smegmatis* group 3, respectively.

Compared to either *M. mageritense* or *M. smegmatis* group 3, *M. smegmatis* group 1 and *M. smegmatis* group 2 strains showed more diversity in their patterns and exhibited substantial front cluster peaks; the tallest front cluster peak was ≥ 0.50 of the tallest rear cluster peak, strains MO195, ATCC 14468 and ATCC 35798 excepted. *M. smegmatis* group 1 was differentiated from *M. smegmatis* group 3 and *M. mageritense* by the relative abundance, within the rear cluster, of a peak at approximately 7.48 min ART. Most *M. smegmatis* group 1 and 2 strains were differentiated by the relative height of a peak at approximately 6.14 min ART to the tallest front cluster peak; this value was generally ≥ 0.81 and ≤ 0.79 for *M. smegmatis* groups 1 and 2, respectively.

The HPLC patterns produced by *M. smegmatis* groups 1, 2, 3 and *M. mageritense* could be distinguished from the closely related patterns produced by the *M. fortuitum* group (*M. fortuitum*, *M. fortuitum* third biovariant and *M. peregrinum*) based on an ART difference of a prominent peak in the front cluster. For *M. smegmatis* groups 1, 2, 3 and *M. mageritense*, this peak ranged from 6.38 to 6.43 min ART. For the *M. fortuitum* group, the corresponding peak ranged from 6.33 to 6.34 min ART.

Examples of the typical HPLC patterns for these three

Fig. 3. Scheme for the differentiation of *M. smegmatis* group 1, *M. mageritense* and *M. smegmatis* groups 2 and 3 by PRA of a 439 bp segment of the *hsp-65* gene.

M. smegmatis groups as well as *M. mageritense* are shown in Fig. 1. A dendrogram of the relatedness among these same taxa based on mycolic acid composition is shown in Fig. 2.

GLC

GLC-derived fatty acid profiles for isolates *M. smegmatis* groups 1, 2, 3 and *M. mageritense* did not differ from one another. Comparison of these profiles to fatty acid profiles for *M. chelonae* and *M. fortuitum* revealed some quantitative differences but there were no qualitative differences. No 2-methyl branched-chain fatty acids were observed in the fatty acid profiles for any of the rapidly growing mycobacterial isolates analysed in this study.

G + C composition of DNA

The G + C content of DNA for isolates of *M. smegmatis* group 3 was 68 mol % for strain ATCC 700009 and 66 mol % for ATCC strain 700010^T, respectively, with a mean value of 68 ± 2 mol % for all eight isolates of *M. smegmatis* group 3. The type strain ATCC 700504^T of *M. smegmatis* group 2 had a G + C content of 66 mol %. The type strain ATCC 700351^T of *M. mageritense* had a G + C content of 66 mol %, and the mean value for all five isolates of *M. mageritense* was 67 ± 3 mol %.

PRA of the 439 bp *hsp-65* gene sequence

Sixty-one isolates were evaluated by PRA, including the five published strains of *M. mageritense* (Domenech *et al.*, 1997) (including ATCC 700351^T), 25 isolates of *M. smegmatis* group 1 (including ATCC 14468, ATCC 35798, ATCC 700011, ATCC 700012 and ATCC 19420^T), 23 *M. smegmatis* group 2 isolates (including ATCC 700504^T), and eight *M. smegmatis* group 3 isolates (including ATCC 70009 and ATCC 700010^T). BstEII digests of PCR amplification products produced similar PRA patterns from all 61 (100 %) isolates examined (Fig. 3). Digests with MspI readily differentiated isolates of *M. smegmatis* group 3 from those of *M. smegmatis* groups 1, 2 and *M. mageritense* as shown in Figs 3 and 4a; lanes 12–14, 1

Fig. 4. (a) *MspI*- and (b) *HhaI*-derived PRA band patterns for isolates of *M. smegmatis* group 1, *M. mageritense* and *M. smegmatis* groups 2 and 3. Lanes 1–3, *M. smegmatis* group 1 isolates ATCC 19420^T, ATCC 700011 and ATCC 700012; lanes 4–6, *M. smegmatis* group 2 isolates ATCC 700504^T, Mo 57 and Mo 65; lanes 7 and 8, sizes markers (100 bp and pGEM base pair ladders, respectively); lanes 9–11, *M. mageritense* isolates 1636, 1635 and 1336; and lanes 12–14, *M. smegmatis* group 3 isolates ATCC 700009, ATCC 700010^T and Mo 116.

to 3, 4–6 and 9–11, respectively. *HhaI*-derived PRA patterns separated *M. mageritense* and *M. smegmatis* group 3 from *M. smegmatis* groups 1 and 2 (Figs 3 and 4b; lanes 9–11, 12–14, 1–3 and 4–6, respectively). PRA patterns with *AciI* further differentiated all four species (Figs 3 and 5a). The combination of *MspI*-, *HhaI*-, *AciI*- and *BsaHI*-derived (Figs 3 and 5b) PRA patterns enabled complete differentiation of all four species.

Isolates of *M. smegmatis* groups 1 and 2 exhibited only a single PRA pattern with each enzyme. Three of the eight isolates of *M. smegmatis* group 3 exhibited a second PRA pattern with *HaeIII* and *AciI* that differed from the common pattern for the majority of isolates as shown in Fig. 3. Intraspecies polymorphism with *BsaHI* resulting in three different PRA patterns that differed from the common pattern for the majority of isolates was also observed for these isolates (Fig. 3).

Fig. 5. (a) *Acil*- and (b) *Bsa*HI-derived PRA band patterns for isolates of *M. smegmatis* group 1, *M. mageritense* and *M. smegmatis* groups 2 and 3. Lanes as for Fig. 4.

Four of 25 (16%) clinical and reference isolates with a tobramycin susceptibility pattern of *M. smegmatis* group 1 subjected to PRA had a *M. smegmatis* group 2 PRA pattern (with the latter considered to be the

species definitive test). All other isolates from the three tobramycin susceptibility groups matched the PRA grouping.

16S rRNA sequencing

M. smegmatis group 1 isolates ATCC 14468 and ATCC 19420^T, *M. smegmatis* group 2 isolate ATCC 700504^T, *M. mageritense* isolate ATCC 700351^T, and six *M. smegmatis* group 3 isolates (ATCC 700009, ATCC 700010^T, MO 178, MO 472, MO 527 and MO 116) were selected for 16S rRNA sequence analysis of the hypervariable regions A and B (Kirschner *et al.*, 1993b). By comparing the sequence of hypervariable region A, *M. smegmatis* groups 1, 2 and 3 were readily differentiated from one another as well as from *M. mageritense* and members of the *M. fortuitum* complex (Table 5). In addition to other base pair changes, the *M. smegmatis* groups 1 and 2 isolates had an extra cytosine inserted at two sites in the hypervariable region A that corresponded to positions 182–183 and 199–200 in the comparable region of *Escherichia coli*. With the exception of a small group of thermotolerant pigmented species, the former two species are the only rapidly growing mycobacterial species or taxa that have this extra cytosine. Similar insertions were not seen with any of the 6 isolates of *M. smegmatis* group 3 (Table 5).

Table 5. Signature nucleotides within the hypervariable region A of the 16S rRNA gene (the first nucleotide corresponds to *E. coli* position 175) for the pathogenic rapidly growing mycobacteria using *M. tuberculosis* as the reference species

Sequences obtained in part from references Domenech *et al.* (1997), Kirschner *et al.* (1992a, b), Springer *et al.* (1995). -, = no nucleotide insertion; N, uncertain base pair; ·, the base pair was identical to the *M. tuberculosis* base pair; ^T = proposed or accepted type strain.

Taxon/species	Signature sequence*											
<i>M. tuberculosis</i> – <i>M. bovis</i>	CGG	ATA	GG-	ACCA	CGG	GAT	GCA	TGT	C-TT	GTG	GTG	
<i>M. fortuitum</i> ATCC 6841 ^T	..A	...	T..		..C	..C.	T..	..G	TG..	...	...	
<i>M. peregrinum</i> ATCC 14467 ^T	..A	...	T..	...G	..C	AC.	T.C	..G	T-G.	...	...	
<i>M. fortuitum</i> third biovariant complex (sorbitol-positive) ATCC 49403 ^T , ATCC 49934 and ATCC 49936	...	...	...		..C	..C.	T..	..G	T-G.	...	...	
<i>M. fortuitum</i> third biovariant complex (sorbitol-negative) ATCC 49404 ^T , ATCC 49935, ATCC 49937, ATCC 49939	..A	...	...	...G	..C	TC.	T..	..G	G-G.	...	...	
<i>M. abscessus</i> ATCC 19977 ^T	...	...	...		..AC	AC.	T..	..G	TG-A	...	...	
<i>M. chelonae</i> ATCC 35752 ^T	...	...	...		..AC	AC.	T..	..G	TG-A	...	...	
<i>M. mucogenicum</i> ATCC 49649, ATCC 49650 ^T , ATCC 49651	..A	...	...		..C	..C.	T..	..G	TG..	...	...	
<i>M. smegmatis</i> group 1 ATCC 19420 ^T , ATCC 14468	..A	...	CAC	C.TG	..T.	..TC	...	..G	..CTG	..A	..G.	
<i>M. smegmatis</i> group 2 ATCC 700504 ^T	..A	...	TAC	C.TG	..T.	..TC	...	..G	..CTG	...	..G.	
<i>M. smegmatis</i> group 3 ATCC 700010 ^T , MO 178, MO 472, MO 527, MO 116#2	...	...	...		...	TC.	...	..G	A...	...	...	
ATCC 700009 (MF 238)	..A	...	...	..TC	...	NC.	...	..G	TN..	...	...	
<i>M. mageritense</i> ATCC 700351 ^T	..A	...	...	..TC	...	AC.	...	..G	TC..	..G.	...

Fig. 6. Dendrogram illustrating the phylogenetic relationships among 30 species and taxa of rapidly growing mycobacteria including *M. smegmatis* group 1 (*M. smegmatis sensu stricto*) (ATCC 19420^T), *M. mageritense* (938^T [Domenech et al., 1997]), *M. smegmatis* group 2 (*M. goodii*) (ATCC 700504^T) and *M. smegmatis* group 3 (*M. wolinskyi*) (ATCC 700009 and ATCC 700010^T).

Isolates were then selected from each of these three groups and these isolates were subjected to complete 16S rRNA sequencing, namely *M. smegmatis* group 1 (ATCC 19420^T), *M. smegmatis* group 2 (ATCC 700504^T), *M. mageritense* 1336 and *M. smegmatis* group 3 (ATCC 700009 and ATCC 700010^T). The sequence of *M. smegmatis* group 1 ATCC 19420^T was identical to the EMBL sequence for *M. smegmatis* group 1 ATCC 14468.

A dendrogram illustrating the 16S rRNA phylogenetic relationships among species of the rapidly growing mycobacteria including *M. smegmatis* groups 1, 2, 3 and *M. mageritense* is shown in Fig. 6, while a summary of the degree of relatedness based on Hamming distances is given in Table 6. *M. smegmatis* groups 1 and 2 showed a close phylogenetic relationship with a Hamming distance of 4. These two species share a common branch point in the 16S rRNA phylogenetic tree (Fig. 6) and share the characteristic insertion of two cytosine nucleotides in the hypervariable region A as noted above in Table 6. *M. smegmatis* groups 1 and 2 were clustered with other thermotolerant species (e.g. *M. phlei*, *Mycobacterium*

thermoresistibile, etc.) with this same cytosine insertion.

The two isolates of *M. smegmatis* group 3 (ATCC 700009 and ATCC 700010^T) exhibited sequences that differed from those of all previously described species of rapidly growing mycobacteria. They shared a common, but clearly separated, branch point in the 16S rRNA phylogenetic tree that was also shared with *M. mageritense* as illustrated in Fig. 6. Five of the six *M. smegmatis* group 3 isolates including the type strain ATCC 700010^T exhibited identical 16S rRNA sequences that differed from that of *M. smegmatis* group 1 by a Hamming distance of 18, from *M. smegmatis* group 2 by a distance of 16, from *M. mageritense* by a distance of 9, and from 28 other species and taxa of rapidly growing mycobacteria by a distance of 16 or greater (Table 6). The hypervariable region A rRNA sequence of the sixth *M. smegmatis* group 3 isolate (ATCC 700009) differed from those of the other five isolates by 4 bp. Unlike the hypervariable region A sequences that were different for each species, the hypervariable region B sequences for *M. mageritense*, *M. smegmatis* groups 2 and 3 were

Table 6. Hamming distances derived from 16S rRNA gene sequences for the proposed new species *M. goodii* and *M. wolinskyi*, *M. smegmatis* sensu stricto, 34 other species and taxa of mycobacteria, and *N. asteroides*

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	Taxon
0	55	51	55	54	59	61	47	44	51	57	63	53	50	54	59	65	61	60	68	54	59	48	45	62	59	64	66	46	47	46	49	62	58	54	53	53	1. <i>N. asteroides</i>	
0	4	6	11	18	41	36	39	30	41	47	29	34	35	36	30	36	38	35	41	33	39	34	31	36	30	52	56	27	29	30	36	40	34	29	31	29	2. <i>M. simiae</i>	
0	8	13	20	42	38	40	30	43	47	31	35	37	36	32	36	39	36	42	33	39	36	31	36	31	36	31	54	57	29	31	32	37	41	35	31	32	31	3. <i>M. lentiflavum</i>
0	14	21	43	39	39	33	42	48	32	38	38	39	33	39	41	39	44	37	43	37	43	37	34	39	34	56	57	30	32	33	38	44	38	32	33	32	4. <i>M. interjectum</i>	
0	28	41	36	37	31	43	49	28	38	34	35	32	36	43	39	46	36	39	36	31	40	35	51	54	29	31	32	38	43	37	30	31	30	31	30	5. <i>M. genavense</i>		
0	45	43	43	43	43	43	38	48	39	46	41	49	41	37	41	44	51	42	43	39	40	42	39	49	49	38	40	41	40	49	43	42	42	42	42	6. <i>M. intermedium</i>		
0	31	25	27	38	42	43	30	28	36	30	40	34	34	42	40	12	28	33	51	39	40	43	20	22	29	26	46	33	26	25	40	7. <i>M. diernhoferi</i>						
0	15	16	31	42	29	17	22	24	26	40	40	30	40	30	42	23	30	9	20	31	34	41	43	19	15	6	11	32	29	21	19	26	8. <i>M. fortuitum</i>					
0	22	27	34	35	22	26	32	27	40	40	37	43	31	29	11	27	43	37	39	42	22	18	15	9	39	35	29	26	32	9. <i>M. peregrinum</i>								
0	34	38	23	15	16	18	27	35	26	43	23	25	16	22	35	33	49	52	20	21	12	16	34	26	16	16	21	10. <i>M. atchiense</i>										
0	27	24	27	27	27	48	30	47	44	43	53	39	40	30	44	50	46	41	45	30	35	32	30	46	42	35	32	27	11. <i>M. aurum</i>									
0	35	30	34	50	41	27	32	30	42	29	38	39	50	38	38	59	62	37	42	39	39	35	32	41	37	31	12. <i>M. vaccae</i>											
0	21	17	31	30	38	39	36	51	32	40	26	31	37	37	54	57	25	30	23	27	37	35	24	25	10	13. <i>M. chubuense</i>												
0	18	30	28	39	35	28	44	25	31	17	27	38	35	49	52	17	22	13	18	31	28	22	19	18	14. <i>M. gilvum</i>													
0	29	28	34	34	32	49	28	26	16	30	39	39	47	50	22	16	18	33	32	21	22	23	15. <i>M. obuense</i>															
0	29	33	38	31	40	27	31	26	16	34	27	57	60	29	27	22	26	25	30	21	21	29	16. <i>M. chitae</i>															
0	40	36	30	26	28	32	24	24	39	27	48	51	25	23	20	26	28	29	22	21	33	17. <i>M. gadum</i>																
0	18	17	33	21	36	37	32	23	19	61	64	40	42	37	36	25	19	31	27	35	18. <i>M. confluentis</i>																	
0	16	33	25	33	36	35	30	22	63	66	40	42	36	36	29	16	27	25	37	19. <i>M. madagascariense</i>																		
0	22	9	30	31	30	22	14	60	63	34	36	27	31	16	3	18	15	33	20. <i>M. smegmatis</i>																			
0	25	41	42	37	36	20	55	58	41	40	39	41	26	22	36	33	48	21. <i>M. thermoresistibile</i>																				
0	34	25	28	18	14	55	58	31	30	21	26	13	10	24	21	29	22. <i>M. flavescens</i>																					
0	28	28	47	36	39	42	19	21	28	31	42	29	25	23	39	23. <i>M. neoaurum</i>																						
0	20	34	34	37	40	19	15	4	5	32	31	20	20	26	24. <i>M. senegalense</i>																							
0	32	25	45	48	25	23	16	23	30	28	21	21	30	25. <i>M. fallax</i>																								
0	20	58	61	38	37	30	37	24	25	32	31	36	26. <i>M. phlei</i>																									
0	57	60	32	31	30	34	17	14	27	24	34	27. <i>M. chromogenicum</i>																										
0	4	36	35	40	40	64	60	48	48	55	28. <i>M. abscessus</i>																											
0	39	38	43	43	67	63	51	51	58	29. <i>M. chelonae</i>																												
0	4	15	22	38	34	25	23	22	30. <i>M. mucogenicum</i> (ATCC 49649)																													
0	11	18	39	36	27	25	27	31. <i>M. mucogenicum</i> (ATCC 49650)																														
0	9	28	27	16	16	22	32. <i>M. fortuitum</i> (ATCC 49403)																															
0	34	30	21	20	24	33. <i>M. fortuitum</i> (ATCC 49404)																																
0	15	29	28	36	34. MICRO 10																																	
0	16	13	32	35. <i>M. goodii</i> (ATCC 700504 ³)																																		
0	4	20	36. <i>M. wolinskyi</i> (ATCC 70010 ³)																																			
0	22	37. <i>M. wolinskyi</i> (ATCC 700009)																																				
0	38. <i>M. chlorophenolicus</i>																																					

Table 7. Second region in the 16S rRNA gene (corresponding to *E. coli* positions 1001–1027) that differentiated the common species belonging to the *M. fortuitum* complex, and *M. smegmatis* groups 1 and 3

·, The base pair was identical to the *M. tuberculosis* base pair; ^T, proposed or accepted type strain. Strain numbers are the same as in Table 4.

Taxon	Sequence
<i>M. tuberculosis</i> – <i>M. bovis</i>	ACAGGACGCGTCTAGAGATAGGCGTT
<i>M. fortuitum</i>	AC.GC.....GT.GT..
<i>M. peregrinum</i>	CAG.....TTG...
<i>M. fortuitum</i> third biovariant complex (sorbitol-negative)	CAG.....TTG...
<i>M. abscessus</i>	TAC.....TA..
<i>M. chelonae</i>	G.....TA.....TA..
<i>M. fortuitum</i> third biovariant complex (sorbitol-positive)	CGGC.....GTCG...
<i>M. mucogenicum</i>	CGGC.....GTCG...
<i>M. mageritense</i> ATCC 700351 ^T	CGGC.....GTCG...
<i>M. smegmatis</i> group 1	CGGC.....GTCG...
<i>M. smegmatis</i> group 2 (ATCC 700504 ^T)	CGGC.....GTCG...
<i>M. smegmatis</i> group 3	
ATCC 700010 ^T (MO739), MO 178, MO 472, MO 527,	TCGGC.....GTCG...
MO 116#2	
ATCC 700009, (MF 238)	TCGGC.....GTCG...

Table 8. Levels of DNA–DNA homology (%) between *M. smegmatis* groups 2 and 3 and related taxa of rapidly growing mycobacteria

Species/strain	<i>M. mageritense</i> ATCC 700351 ^T	Group 1 ATCC 19420 ^T	Group 2 ATCC 700504 ^T	Group 3 ATCC 700009	Group 3 ATCC 700010 ^T
<i>M. mageritense</i> ATCC 700351 ^T	100	–	–	–	–
<i>M. smegmatis</i> group 1 ATCC 19420 ^T	4±3	100	–	–	–
<i>M. smegmatis</i> group 2 ATCC 700504 ^T	11±5	39±14	100	–	–
<i>M. smegmatis</i> group 3					
ATCC 700009	13±4	17±7	17.3±8	100	–
ATCC 700010 ^T	11±6	19±8	21±8	84±15	100
MF1425	12.2±1.5	14.3±7	19±5.4	99±7	91±15
MO116	11.2±5	11±4.5	19±5	> 100	98±20
MO178	7.7±1.5	15±7	16.4±5.6	> 100	> 100
MO472	11.2±3	26±10	23±9	–	104±20
MO527	9.7±2.7	20±8	19±8	–	80±15
MO817	5±0.5	9±3	18.2±5	–	65±7
<i>M. fortuitum</i>					
ATCC 6841 ^T	20.2±5	14±6	17.6±7	23.5±0.6	13±3
<i>M. phlei</i> IMRU 500	5.7±8	21±5	17±8	9±3	11±1.5

identical with *M. smegmatis* group 1. Separation of *M. smegmatis* group 3 from all other taxa could also be made by examining the 1001–1027 bp region (i.e. signature sequences designated by the *E. coli* numbering system) depicted in Table 7.

DNA–DNA hybridization

The mean results of four DNA–DNA hybridization analyses per sample are shown in Table 8. Most isolates of *M. smegmatis* group 3 showed 20% or less

Fig. 7. *Bam*HI- and *Pst*I-derived RFLP patterns of 16S rRNA genes from *M. smegmatis* groups 2 and 3 compared to *M. mageritense*, *M. smegmatis* and other species of rapidly growing mycobacteria, and *N. asteroides*. (a) *Bam*HI digests. Lane 3, *M. goodii* isolate ATCC 700504^T. Lanes 1, 2 and 4–9, *M. smegmatis* group 3 isolates ATCC 700009, MO 1425, MO 116, MO 178, MO 472, MO 527, ATCC 700010^T, MF 817, respectively. Lanes 10–20, *M. chelonae* ATCC 35752^T, *M. fortuitum* ATCC 6841^T, *M. fortuitum* third biovar strain ATCC 49403^T, *M. fortuitum* third biovar strain ATCC 49404^T, *M. mageritense* ATCC 700351^T, *M. parafortuitum* strain CNMIVS, *M. peregrinum* ATCC 14467^T, *M. porcinum* ATCC 27406^T, *M. senegalense* NCTC 10956^T, *Mycobacterium* sp., and *N. asteroides* strain CMNIVS. (b) *Pst*I digests. Lanes 1–19 are the same as for (a). Abbreviations: CIPT, Collection Institut Pasteur Unité de Tuberculose; NCTC, National Collection of Type Cultures, and CNMIVS, Centro Nacional de Microbiología, Immunología y Virología Sanitarias.

homology with the type strains of *M. mageritense* (ATCC 700351^T) (Domenech *et al.*, 1997), *M. smegmatis* group 1 (ATCC 19420^T) and *M. smegmatis* group 2 (ATCC 700504^T). Most isolates of *M. smegmatis* group 3 exhibited 90 % or greater homology within the group including the two reference strains ATCC 700009 and ATCC 700010^T. The *M. smegmatis* group 2 strain ATCC 700504^T exhibited only 39 %

homology with the *M. smegmatis* group 1 strain ATCC 19420^T.

RFLP analysis of 16S rRNA gene

Strain comparisons included *M. abscessus*, *M. chelonae*, *M. fortuitum*, *M. fortuitum* third biovariant sorbitol-positive, *M. fortuitum* third biovariant sorbitol-negative, *M. mageritense*, *M. parafortuitum*, *M. peregrinum*, *N. asteroides*, *Mycobacterium porcinum*, *Mycobacterium senegalense*, and *M. smegmatis* groups 1, 2 and 3.

The eight isolates of *M. smegmatis* group 3 gave identical RFLP hybridization patterns with two *Bam*HI-derived bands and four *Pst*I-derived bands. These patterns were unique from all control strains. Likewise, isolates of *M. smegmatis* group 2 including the type strain ATCC 700504^T gave a very different RFLP-hybridization pattern from that of the *M. smegmatis* group 3 isolates, and was unique from those of all other control strains (Fig. 7).

DISCUSSION

These three taxonomic groups exhibited typical characteristics of the genus mycobacterium. They grew well aerobically on Middlebrook 7H10 agar and Löwenstein–Jensen agar, were Gram-positive and appeared as acid-fast bacilli after staining with Ziehl–Neelsen stain, contained mycobacterial typical mycolic acids and whole-cell fatty acids on HPLC and GLC, respectively, and exhibited more than 95 % identity with other mycobacterial 16S rRNA sequences. Their mean of 67 mol % G + C content is within the range of 62–70 mol % typical of the genus mycobacterium (Wayne & Kubica, 1986).

The genetic studies clearly delineated differences between the three *M. smegmatis* groups, including by PRA of the 65 kDa heat-shock protein gene, 16S rRNA RFLP patterns, 16S rRNA sequences and DNA–DNA pairing experiments. PRA with *Bst*EII resulted in a single band pattern for *M. mageritense*, and *M. smegmatis* groups 1, 2 and 3, including the ATCC reference strains, suggesting a close relationship between the groups. As previously demonstrated (Steingrube *et al.*, 1995b; Telenti *et al.*, 1993) the use of both *Bst*EII and *Hae*III separated these four taxa from all other rapidly growing mycobacterial species and taxa studied. The use of other restriction enzymes separated all of the four groups. Isolates of *M. smegmatis* groups 1 and 2 were differentiated from one another by two of the five restriction enzymes (*Acc*II and *Bsa*HI) and from *M. smegmatis* group 3 by three enzymes (*Msp*I, *Hha*I and *Acc*II).

By 16S rRNA sequencing data, the common sequence seen in five of six *M. smegmatis* group 3 isolates differed from all other mycobacterial taxa, except *M. mageritense*, by a Hamming distance of at least 16. The Hamming distance of 9 between the common sequence for *M. smegmatis* group 3 (including ATCC 700010^T)

and *M. mageritense* suggested these two species were more closely related to one another than to *M. smegmatis* groups 1 and 2. Likewise, the latter two taxa differed by a Hamming distance of 3 and thus are among the most closely related of all the mycobacterial taxa studied by 16S rRNA sequencing. [A few mycobacterial species have fewer differences, e.g. *Mycobacterium ulcerans* and *Mycobacterium marinum* which differ by 2 bp (Tønjum *et al.*, 1998) and *Mycobacterium gastri* and *Mycobacterium kansasii* which have identical 16S rRNA sequences (Rogall *et al.*, 1990).]

M. smegmatis groups 2 and 3 exhibited <40% DNA–DNA homology with one another or with *M. mageritense* or *M. smegmatis* group 1. Despite differing by a small Hamming distance of 4 in their 16S rRNA sequences, *M. smegmatis* groups 1 and 2 exhibited different PRA patterns, phenotypic patterns, and <40% DNA–DNA homology. This degree of relatedness is well less than the approximately 70% DNA–DNA relatedness recommended by Wayne *et al.* (1987). Conversely, *M. smegmatis* group 3 strains ATCC 700009 and ATCC 70010^T exhibited a Hamming distance of 4, but were identical by PRA and phenotypic characteristics and exhibited 84% DNA–DNA homology.

FL-HPLC has been shown to be a sensitive and practical method for the identification of mycobacteria (Jost *et al.*, 1995). In the current study, the relationships between the four species as determined by HPLC mycolic acid patterns were similar to those observed by 16S rRNA sequencing. Previously, Butler & Kilburn (1990) reported that *M. smegmatis* could not be distinguished from the *M. fortuitum* group based on their HPLC patterns. However, in the current study a small but consistent difference in peak ART values clearly differentiated *M. smegmatis* groups 1, 2, 3 and *M. mageritense* from the *M. fortuitum* group.

Consistent with previous reports (Baba *et al.*, 1988; Yassin *et al.*, 1993), whole-cell fatty acid profiles were very homogeneous among the rapidly growing mycobacteria tested and did not distinguish between *M. smegmatis* groups 1, 2, 3, or *M. mageritense*. Apart from minor quantitative differences, fatty acid profiles also did not differentiate the latter species from *M. chelonae* or *M. fortuitum*. Valero-Guillén & Martin-Luengo (1983) reported that 2-methyl branched-chain fatty acids were detected only in isolates of *M. fortuitum* and *M. smegmatis*. These authors identified 2-methyl-2-octadecanoic acid in some but not all isolates of *M. fortuitum* and have identified three different whole-cell fatty acid patterns in this species by GLC (Valero-Guillén & Martin-Luengo, 1983). They have also reported 2,4-dimethyl-2-eicosenoic acid to be present in two of three isolates of *M. smegmatis*. Notably, the ATCC 607 strain of *M. smegmatis* lacked these acids. However, in the current study no 2-methyl branched-chain fatty acids were detected in any of the rapidly growing mycobacterial isolates tested, findings that parallel those of Yassin *et al.* (1993).

The phenotypic similarity of the three *M. smegmatis* groups is one of the major reasons *M. smegmatis* groups 2 and 3 went undetected until now. The three groups of *M. smegmatis* exhibited the same conventional growth and biochemical tests, including a negative 3 d arylsulfatase reaction, positive iron uptake and positive nitrate reductase reactions, growth at 45 °C, and utilization of D-glucitol (D-sorbitol), i-myo-inositol and D-mannitol as sole carbon sources. Another reason *M. smegmatis* group 3 isolates have gone unrecognized was an insufficient number of isolates available for study, as evidenced by their 11% proportion of the *M. smegmatis* isolates examined in this study. A comparison of the common biochemical markers for the three *M. smegmatis* groups and other closely related rapidly growing mycobacteria is presented in Table 8. Routine laboratory testing of rapidly growing mycobacteria does not include PAS degradation, 68 °C stable catalase assay, IEF of β -lactamases, PRA or DNA sequencing, methodologies that would have made earlier recognition of these proposed new species more likely.

The phenotypic data that provided the earliest and clearest indication of the existence of other taxa among isolates identified as *M. smegmatis* was susceptibility testing. Wallace *et al.* (1988) reported the presence of three different antimicrobial susceptibility patterns among clinical isolates. Based on the groupings in the 1988 study, these three groups of isolates, were designated herein as *M. smegmatis* groups 1, 2 and 3, respectively. All eight of the *M. smegmatis* group 3 isolates were resistant to tobramycin, the *M. smegmatis* group 2 isolates exhibited an intermediate level of resistance to tobramycin, while *M. smegmatis* group 1 isolates were susceptible.

Description of *Mycobacterium wolinskyi* sp. nov.

Mycobacterium wolinskyi (wo.lins'ky.i. M.L. gen. n. *wolinskyi* of Wolinsky, named after Emanuel Wolinsky in honour of, and in recognition for, significant contributions to the study of the non-tuberculous mycobacteria).

A Gram-positive, acid- and alcohol-fast bacillus that produces visible growth in 2–4 d as smooth to mucoid, off-white- to cream-coloured colonies on Middlebrook 7H10 and trypticase soy agar. No pigment is produced and growth occurs at 30, 35 and 45 °C. Isolates grow on MacConkey agar without crystal violet and in the presence of 5% NaCl, are negative for arylsulfatase activity at 3 d, and are positive for iron uptake and nitrate reductase. They produce low-level semi-quantitative catalase activity that is stable at 68 °C, pH 7.0. As sole carbon sources, 100% of isolates utilize D-glucitol (D-sorbitol), i-myo-inositol, D-mannitol and L-rhamnose, 88% utilize D-galactose and D-trehalose, and 50–63% utilize L-arabinose, citrate and D-xylose. The type strain is positive for galactose, arabinose, citrate, trehalose and xylose. Isolates are susceptible to amikacin and sulfamethoxazole, intermediately sus-

ceptible to doxycycline and ciprofloxacin, variably susceptible to cefmetazole, cefoxitin, chloramphenicol and clarithromycin, and resistant to isoniazid, rifampin and tobramycin. Mycolic acids and whole-cell fatty acids are typical of mycobacterial species. By PRA, isolates exhibit a unique, distinctive set of band patterns with *MspI* (135, 120, 110 and 75 bp bands), *HhaI* (185, 105 and 65 bp bands) and *AciI* (125 and 90 bp major band pattern, and 135, 125 and 85 bp minor band pattern). The proposed type strain, ATCC 700010^T (MO739), was recovered from a post-surgical facial abscess in Switzerland and was originally identified as *M. smegmatis* (Pennekamp *et al.*, 1997). An additional reference strain, ATCC 700009 (MO238), was isolated from a sternal wound infection following open heart surgery in Texas.

Description of *Mycobacterium goodii* sp. nov.

Mycobacterium goodii [good.i.i. M.L. gen. n. *goodii* of Good, named after Robert (Bob) Good, in honour of, and in recognition for, significant contributions to the study of mycobacteria].

A Gram-positive, acid- and alcohol-fast bacillus that produces visible growth in 2–4 d as smooth to mucoid, off-white- to cream-coloured colonies on Middlebrook 7H10 and trypticase soy agar. Yellow to orange pigment is produced by 78% of isolates after 10–14 d incubation and growth occurs at 30, 35 and 45 °C. Isolates grow on MacConkey agar without crystal violet and in the presence of 5% NaCl, are negative for arylsulfatase activity at 3 d, and are positive for iron uptake and nitrate reductase. They produce low-level semi-quantitative catalase activity that is stable at 68 °C, pH 7.0. As sole carbon sources, 95–100% of isolates utilize L-arabinose, D-glucitol (D-sorbitol), i-myo-inositol, D-mannitol, L-rhamnose and D-xylose, and only 11 to 33% utilize citrate, D-galactose and/or D-trehalose. Isolates are susceptible to amikacin, ethambutol, sulfamethoxazole, intermediately susceptible to ciprofloxacin, doxycycline and tobramycin, variably susceptible to cefmetazole, cefoxitin and clarithromycin, and resistant to isoniazid and rifampin. Mycolic acids and whole-cell fatty acids are typical of mycobacterial species. By PRA, isolates exhibit a distinctive set of band patterns with *MspI* (180, 130 and 115 bp bands), *HhaI* (235 and 165 bp bands), *AciI* (130, 110 and 60 bp bands) and *BsaHI* (260, 100 and 80 bp bands). The proposed type strain, ATCC 700504^T (=MO69^T), was recovered from a patient with a post-traumatic osteomyelitis of the calcaneus (heel).

The new species *M. goodii* and *M. wolinskyi* are clearly clinically significant, and occur in the same settings as *M. smegmatis* (*sensu stricto*) and members of the *M. fortuitum* complex. They are most commonly seen in post-traumatic wound infections, especially those following open fractures and with associated osteomyelitis. They differ from members of the *M. fortuitum* in the type of chronic lung disease they produce, with

essentially all cases occurring in the setting of chronic lipoid pneumonia, either secondary to chronic oil ingestion or chronic aspiration (usually achalasia).

The IWGMT has had one major polyphasic study of rapidly growing mycobacteria which was published in 1972 (Kubica *et al.*, 1972). This included nine strains which clustered with *M. smegmatis* ATCC 14468. Seven of the isolates were reference strains, and only one strain was of human origin. The current study expands this study to include a large number of human clinical *M. smegmatis* isolates and defines that they actually consist of three different species. The current study utilized more advanced genetic and chromatographic methods than were available in the 1972 study.

ACKNOWLEDGEMENTS

The authors wish to thank Michio Tsukamura, formerly of the National Chubu Hospital, Obu, Aichi, Japan; Robbie Dunlap, Denise Dunbar and Arthi Kalkura of the Texas Department of Health; Sylvia Chomyc of the Provincial Laboratory of Public Health, Edmonton, Alberta, Canada; and Vella A. Silcox from the Centers for Disease Control and Prevention, Atlanta, GA, USA, for kindly performing biochemical testing on selected isolates reported in this paper. We also thank Hans Trüper for help with the nomenclature of the new species. We also wish to thank Joanne Woodring for preparation of the manuscript.

This manuscript is dedicated to the memory of Vincent A. Steingrube, a co-author, colleague, and friend of many years, who passed away before completion of this study.

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