Taxonomic variation in the *Mycobacterium fortuitum* third biovariant complex: description of *Mycobacterium boenickei* sp. nov., *Mycobacterium houstonense* sp. nov., *Mycobacterium neworleansense* sp. nov. and *Mycobacterium brisbanense* sp. nov. and recognition of *Mycobacterium porcinum* from human clinical isolates

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The *Mycobacterium fortuitum* third biovariant complex (sorbitol-negative and sorbitol-positive) contains unnamed taxa first characterized in 1991. These organisms can cause respiratory infections, a spectrum of soft tissue and skeletal infections, bacteraemia and disseminated disease. To evaluate this group of organisms, clinical reference isolates and the type strains of *M. fortuitum* third biovariant complex sorbitol-negative ($n=21$), *M. fortuitum* third biovariant complex sorbitol-positive ($n=3$), *M. fortuitum* ($n=3$), *Mycobacterium peregrinum* (pipemidic acid-susceptible) ($n=1$), *Mycobacterium porcinum* ($n=1$), *Mycobacterium senegalense* ($n=2$) and *Mycobacterium septicum* ($n=1$) were characterized by using conventional phenotypic (morphological, physiological and antimicrobial susceptibilities), chemotaxonomic (HPLC and cellular fatty acids) and genotypic [RFLP of the rRNA gene (ribotyping), PCR-RFLP of a

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Abbreviations: PRA, PCR restriction analysis; RRT, relative retention time; RT, ribotype.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences determined in this work are as follows: *Mycobacterium porcinum*, AY012582; AY012574, AY012580 and AY012581; *Mycobacterium boenickei*, AY012573; *Mycobacterium houstonense*, AY012579; *Mycobacterium neworleansense*, AY012575; *Mycobacterium septicum*, AY012576; *Mycobacterium brisbanense*, AY012577.

Differences in the RT patterns of the type and reference strains of *M. fortuitum*, signature nucleotides of hypervariable region A and DNA relatedness among strains in the species and their relatedness to other strains of the *M. fortuitum* third biovariant complex and related species are available as supplementary material in IJSEM Online.

439 bp segment of the 65 kDa *hsp* gene (PCR restriction analysis) and 16S rRNA gene sequence] analysis, DNA G + C content and DNA–DNA relatedness analyses. The results of these studies indicated that the strains comprised *M. porcinum* ($n = 13$), *M. septicum* ($n = 1$) and four novel closely related genetic groups within the *M. fortuitum* third biovariant complex: *Mycobacterium boenickei* sp. nov. ($n = 6$), *Mycobacterium houstonense* sp. nov. ($n = 2$), *Mycobacterium neworleansense* sp. nov. ($n = 1$) and *Mycobacterium brisbanense* sp. nov. ($n = 1$), with type strains ATCC 49935^T (= W5998^T = DSM 44677^T), ATCC 49403^T (= W5198^T = DSM 44676^T) ATCC 49404^T (= W6705^T = DSM 44679^T) and ATCC 49938^T (= W6743^T = DSM 44680^T), respectively.

INTRODUCTION

The organisms that have been classified as comprising the *Mycobacterium fortuitum* third biovariant complex are rapidly growing ubiquitous environmental organisms that normally inhabit soil, dust and water. These organisms frequently are human pathogens that cause a wide spectrum of clinically significant disease. Reported infections include skin and soft-tissue abscesses with associated osteomyelitis, bacteraemia, endocarditis, keratitis, lymphadenitis, peritonitis, post-surgical infections, pulmonary infections and disseminated disease (Wallace *et al.*, 1983, 1991). Involvement of the central nervous system is rare, but meningitis may develop after trauma or surgery. The immunocompromised patient is at special risk for developing severe diseases, especially catheter-related infection with bacteraemia. Disseminated cutaneous disease with skin abscesses, cellulitis and subcutaneous nodules is common with *Mycobacterium chelonae* and *Mycobacterium abscessus* but rare with members of the *M. fortuitum* third biovariant complex (Ingram *et al.*, 1993). It is important for practitioners to be aware of these organisms as possible aetiological agents, as they are resistant to most first-line anti-tuberculous agents.

Wallace *et al.* (1991) were the first to describe the *M. fortuitum* unnamed third biovariant complex, containing two unnamed taxa (sorbitol-positive and sorbitol-negative). Recent work has shown that a strain previously identified phenotypically as a member of the *M. fortuitum* third biovariant complex (sorbitol-negative) represented a novel species, *Mycobacterium septicum* (Schinsky *et al.*, 2000). In this report, we demonstrate that, despite the common conception that the *M. fortuitum* third biovariant complex comprises two phenotypically homogeneous taxa, significant heterogeneity exists. After detailed analysis of 21 clinical isolates formerly classified as the *M. fortuitum* third biovariant complex (sorbitol-negative), three clinical isolates formerly classified as the *M. fortuitum* third biovariant complex (sorbitol-positive), the type strain and two clinical isolates of *M. fortuitum*, the type strain of *Mycobacterium peregrinum* (pipemidic acid-susceptible), the type strain of *Mycobacterium porcinum*, the type strain and one reference strain of *Mycobacterium senegalense* and the type strain of *M. septicum*, we propose four novel species: *Mycobacterium boenickei* sp. nov., *Mycobacterium houstonense* sp. nov., *Mycobacterium neworleansense* sp. nov. and *Mycobacterium brisbanense* sp. nov. In addition, 13 of the *M. fortuitum* third

biovariant complex (sorbitol-negative) human clinical isolates were recognized as *M. porcinum*, previously identified by Tsukamura *et al.* (1983) from submandibular lymph nodes from swine, and one clinical isolate was recognized as *M. septicum*.

METHODS

Mycobacterial strains. The isolates used in this study, together with their clinical and geographical sources, are listed in Table 1. The following strains were obtained from the American Type Culture Collection (ATCC), Manassas, VA, USA: *M. senegalense* ATCC 35796^T, *M. peregrinum* ATCC 14467^T, and *M. fortuitum* group ATCC 49404. *M. septicum* ATCC 700731^T was obtained from the collection of the Actinomycetes Reference Laboratory, Centers for Disease Control and Prevention, Atlanta, GA, USA. The remaining strains were provided by the Mycobacteria/Nocardia Laboratory, University of Texas Health Center at Tyler, TX, USA. Type and reference strains of *M. fortuitum*, *M. peregrinum* (pipemidic acid-susceptible), *M. porcinum*, *M. senegalense* and *M. septicum* were used as controls for the isolates studied. Strains were stored as suspensions in trypticase soy broth (Becton Dickinson) with 20% glycerol (Sigma) at 70 °C and maintained on Löwenstein–Jensen medium slants (Remel) until analysis.

Phenotypic analysis. Colonies were grown on heart infusion agar with 5% (v/v) rabbit blood (BBL, Becton-Dickinson Microbiology Systems) and incubated for 2 days at 35 °C for macroscopic analysis. The presence or absence of aerial hyphae was determined with the use of a stereomicroscope (10×). Gram and modified Kinyoun acid-fast stains were used for studies of microscopic morphology and acid-fastness, respectively (Berd, 1973). Conventional biochemical tests were performed as used routinely in the Actinomycetes Reference Laboratory of the Centers for Disease Control and Prevention (Berd, 1973), and classical tests for species differentiation of the mycobacteria were performed (Silcox *et al.*, 1981). Carbon-utilization tests were performed (Yassin *et al.*, 1995). Semi-quantitative catalase analysis and assessment of the thermostability of catalase at 68 °C were performed (Kirschner *et al.*, 1992; Vestal, 1975). Additionally, we tested for the utilization of acetamide (BBL) and citrate (BBL) as sole carbon and nitrogen sources, and for growth at 42 °C using the methods described by Wallace *et al.* (1991).

A broth microdilution method using cation-supplemented Mueller–Hinton broth, as described by Wallace *et al.* (1991), was used for antimicrobial-susceptibility tests. Plates were incubated at 30 °C for 72 h. MIC values were determined for amikacin, amoxycillin/clavulanate, ampicillin, cefotaxime, ceftriaxone, ciprofloxacin, doxycycline, erythromycin, imipenem, minocycline, sulfamethoxazole, trimethoprim/sulfamethoxazole and vancomycin. Break points were derived from aerobic dilution interpretative criteria found in National Committee for Clinical Laboratory Standards document M7-A4 (NCCLS, 1997).

Table 1. Laboratory and clinical information on *Mycobacterium* isolates used in this study

CDC, Centers for Disease Control and Prevention; GS, genomospecies; NA, not applicable.

CDC strain designation	Other strain designation(s)	<i>M. fortuitum</i> third biovariant complex designation	Clinical source	Geographical location	Species/GS
W5997	ATCC 49937, Mf-628	Sorbitol-negative	Leg wound	USA	<i>Mycobacterium</i> GS 1
W5998 ^T	ATCC 49935 ^T , Mf-657 ^T	Sorbitol-negative	Leg wound	USA	<i>Mycobacterium</i> GS 1
W6229	Mf-609	Sorbitol-negative	Subcutaneous tissue	Australia	<i>Mycobacterium</i> GS 1
W6231	Mf-613	Sorbitol-negative	Thigh wound	Australia	<i>Mycobacterium</i> GS 1
W6240	Mf-319	Sorbitol-negative	Sputum	USA	<i>Mycobacterium</i> GS 1
W6245	Mf-559	Sorbitol-negative	Arm wound	USA	<i>Mycobacterium</i> GS 1
W5198 ^T	ATCC 49403 ^T , Mf-89 ^T	Sorbitol-positive	Face wound	Houston, TX, USA	<i>Mycobacterium</i> GS 2
W6155	ATCC 49934, Mf-633	Sorbitol-positive	Lung biopsy	USA	<i>Mycobacterium</i> GS 2
W6705 ^T	ATCC 49404 ^T , Mf-465 ^T	Sorbitol-negative	Scalp wound	New Orleans, LA, USA	<i>Mycobacterium</i> GS 3
W6743 ^T	ATCC 49938 ^T , Mf-614 ^T	Sorbitol-positive	Antral sinus	Brisbane, Australia	<i>Mycobacterium</i> GS 4
W5196 ^T	ATCC 6841 ^T	NA	Cold abscess	Brazil	<i>M. fortuitum</i>
W5994	Mf-589	NA	Breast wound	USA	<i>M. fortuitum</i>
W5995	Mf-551	NA	Catheter exit wound	USA	<i>M. fortuitum</i>
W6706 ^T	ATCC 14467 ^T	NA	Bronchial aspiration	Mexico	<i>M. peregrinum</i>
W6228	Mf-607	Sorbitol-negative	Sputum	Australia	<i>M. porcinum</i>
W6230	Mf-611	Sorbitol-negative	Leg wound	Australia	<i>M. porcinum</i>
W6232	ATCC BAA-328, Mf-771	Sorbitol-negative	Inguinal node	USA	<i>M. porcinum</i>
W6233	Mf-809	Sorbitol-negative	Thigh wound	USA	<i>M. porcinum</i>
W6234	ATCC 49939, Mf-661	Sorbitol-negative	Back wound	USA	<i>M. porcinum</i>
W6235	Mf-91	Sorbitol-negative	Sputum	USA	<i>M. porcinum</i>
W6236	Mf-131	Sorbitol-negative	Lung biopsy	USA	<i>M. porcinum</i>
W6237	Mf-147	Sorbitol-negative	Foot wound	USA	<i>M. porcinum</i>
W6239	Mf-239	Sorbitol-negative	Sputum	USA	<i>M. porcinum</i>
W6241	Mf-450	Sorbitol-negative	Leg wound	USA	<i>M. porcinum</i>
W6242	Mf-485	Sorbitol-negative	Foot wound	USA	<i>M. porcinum</i>
W6243	Mf-487	Sorbitol-negative	Sputum	USA	<i>M. porcinum</i>
W6244	Mf-533	Sorbitol-negative	Catheter exit site	USA	<i>M. porcinum</i>
W5997	ATCC 49937, Mf-628	Sorbitol-negative	Leg wound	USA	<i>M. porcinum</i>
W7701 ^T	ATCC 33776 ^T	NA	Swine lymph node	Japan	<i>M. porcinum</i>
W6153	ATCC 13781	NA	Bovine farcy lesion	Senegal	<i>M. senegalense</i>
W6605 ^T	ATCC 35796 ^T	NA	Bovine farcy lesion	Senegal	<i>M. senegalense</i>
W6238	ATCC BAA-329, Mf-155	Sorbitol-negative	Not available	Concord, NH, USA	<i>M. septicum</i>
W4964 ^T	ATCC 700731 ^T , DSM 44393 ^T	NA	Catheter tip	Australia	<i>M. septicum</i>

DNA purification. Strains were subcultured from Löwenstein-Jensen medium slants into 200 ml Mueller–Hinton broth containing 1.0 ml Tween 80 (Sigma) and 3.0 g glycine (Sigma) and then grown for 1–2 days at 35 °C before being harvested by centrifugation at 3795 g for 30 min. DNA was purified from lysed protoplasts as previously described by Lasker *et al.* (1992). Repeat extractions were performed with a 20% (w/v) SDS (Roche) solution to improve the DNA yield, a method adapted from Loeffelholz & Scholl (1989).

DNA relatedness and G+C content determination. The hydroxyapatite method used for DNA labelling and DNA relatedness was described previously by Brenner *et al.* (1982). Using a nick translation kit (Gibco), DNA was labelled *in vitro* with [³²P]dCTP. The optimal hybridization temperature used was 75 °C; the percentage divergence was calculated to the nearest 0.5% (Brenner *et al.*, 1983). Relative binding ratios expressed as percentages were the means of at least two hybridization experiments.

The G + C content of DNA from representative isolates was determined spectrophotometrically by using the thermal denaturation method as described by Mandel *et al.* (1970).

Ribotyping. Genomic DNA (1–3 mg ml⁻¹) was digested for 8 h at 35 °C with 20 U of either of the two restriction enzymes *PvuII* or *SalI* (Roche) in the buffers recommended by the manufacturer. DNA fragments were separated by electrophoresis in 0.85% (w/v) agarose gel (Gibco). The DNA fragments were then transferred to a nylon membrane (Nytran; Schleicher & Schuell) and were probed at 37 °C with digoxigenin-labelled cDNA derived from purified 16S and 23S rRNA of *Escherichia coli* by reverse transcription as described previously (Popovic *et al.*, 1993). The rDNA-containing fragments were visualized using the 'Genius' kit (Roche) protocol.

PCR amplification and restriction enzyme analysis. DNA from bacterial cells harvested from trypticase soy agar (Remel) plates

was prepared for PCR amplification according to methods previously described by Steingrube *et al.* (1995), Telenti *et al.* (1993) and Wilson *et al.* (1998). A 439 bp segment of the *hsp65* gene was amplified from ground-cell supernatants by PCR with 1.0 U *Taq* DNA polymerase (Perkin Elmer) in optimized buffer E (1.5 mM MgCl₂, pH 9.0; Invitrogen) containing 83 µM each dNTP, 9% (v/v) DMSO and 1 µM of each primer, i.e. TB11 (5'-ACCAACGATGGTGTGCCAT-3') and TB12 (5'-CTTGTCGAACCGCATACCCT-3') (Midland Certified Reagent Co.), together with the appropriate positive and negative controls according to a modification of the method of Telenti *et al.* (1993). The amplification parameters were 45 cycles of 1 min each at successive temperatures of 94, 55 and 72 °C, followed by a 10 min extension at 72 °C.

Two commercially available restriction endonucleases, *Bst*EII and *Hae*III (Promega), were used to produce PCR restriction analysis (PRA) band patterns, as described previously by Steingrube *et al.* (1995, 1997). Restriction digests were incubated for the appropriate time periods at the appropriate temperatures, using buffers recommended by the manufacturer.

Restriction fragments were electrophoresed on 3% (w/v) Metaphor 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. PRA band sizes (in bp) were estimated on a computerized Bio Image system (Millipore), with a 100 bp ladder (Life Technologies) and a pGem-bp ladder (Promega) as molecular size standards. Restriction fragment sizes were rounded to the nearest 5 bp, as recommended by Telenti *et al.* (1993).

16S rRNA gene sequencing. Purified genomic DNA was amplified using the Expand High Fidelity PCR System (Roche), which includes a low concentration of proof-reading enzyme *Pfu* (Barnes, 1994) to increase the fidelity of products. The protocol was modified with the addition of 5% (v/v) DMSO to increase the yield of high-G + C templates (Scheidl *et al.*, 1995). Each 50 µl reaction contained approximately 10 ng DNA, 2.5 U polymerase, 1.5 mM MgCl₂, 5% DMSO, 200 µM dNTPs, 100 nM each of primers FD1 5'-AGAGT-TTGATCCTGGCTCAG and RD1 5'-AAGGAGGTGATCCAGCC. Amplification was performed on an ABI 9700 (Applied Biosystems) thermocycler at 94 °C for 5 min, followed by 35 cycles of 94 °C for 15 s, 50 °C for 15 s and 72 °C for 90 s, and finalized by a single extension of 72 °C for 5 min followed by maintenance at 4 °C.

Amplification products (5 µl) were electrophoresed on a 1.2% (w/v) agarose gel with a 500 bp ladder for 30 min at 85 V. Excess dNTPs and primers were removed from products with the QIAquick 8 PCR Purification kit (Qiagen), a vacuum-based, chaotropic, salt-silica binding method. Cycle sequencing was performed using standard protocols with the addition of 5% DMSO (Scheidl *et al.*, 1995), utilizing 9 pM each primer: FD1, 5'-AGAGTTTGATCCTGGCTCAG, positions 7–25; 5'-AGTTGATCCTGGCTCAG, 9–25; 5'-TACGGG-AGGCAGCAG, 342–356; 5'-CAGCAGCCGCGTAATAC, 517–535; 5'-CTTGAGTGACGAAGGAGTGG, 650–671; 5'-ATTAGATACC-CTGGTAG, 786–802; 5'-CCCGCAACGAGCGCAACCC, 1094–1113; 5'-GGGCTTGATACACACCG, 1384–1400; 5'-AAGTCGTAAACAAG-GTAACC, 1491–1509; RD1, 5'-GGCTGGATCACCTCCTT, 1524–1540 (positions correspond to positions on the *Escherichia coli* 16S rRNA gene; Brosius *et al.*, 1978). Excess dyes were removed with magnetic carboxylate beads (Agencourt Bioscience) and reactions were sequenced on an ABI 3100 sequencer (Applied Biosystems).

Phylogenetic analysis. The 16S rRNA gene sequences were assembled in SEQMERGE (Accelrys) and trimmed to a minimum of two confirming reads. The sequences (>1400 bp) generated in the present study were compared with the GenBank database gene sequences derived from related *Mycobacterium* species and submitted by other investigators. When representative isolates [BAA-328, RT-A

(*n*=10); ATCC 49939, RT-D; W6236, RT-G; and W6243, RT-F] of one cluster (*n*=13) of *M. fortuitum* third biovariant sorbitol-negative isolates were aligned in PILEUP, the sequences of the representative isolates corresponded to the sequence of *M. porcinum* (GenBank accession no. AF480588) that was deposited in the GenBank database in March 2002 (Turenne *et al.*, 2001). Phylogenetic trees were created in TREECON using the Jukes–Cantor model (Jukes & Cantor, 1969) with 1000 bootstrapping resamples, topology was inferred by using the neighbour-joining method, and sequences of 21 *Mycobacterium* species were rooted with outgroup *Nocardia oitidiscaviarum* (GenBank accession no. M59056). The trimmed 16S rRNA gene sequences were aligned using CLUSTAL W in MegAlign (DNASTAR) to produce similarity values (Thompson *et al.*, 1994).

The 37 bp hypervariable region A, beginning with and corresponding to position 175 in the *E. coli* numbering system designated by Kirschner *et al.* (1993), was extracted from the 16S rRNA gene sequences and aligned with the BioEdit program (Hall, 1999).

HPLC. Mycolic acid analysis of representative isolates was performed according to the established procedure for mycobacteria (Butler *et al.*, 1991, 1992). The chromatographic patterns generated by these isolates were matched to standard HPLC patterns of control species by using relative retention time (RRT) ratios for peaks, as calculated by the chromatographic software (Beckman Instruments). The ratios were derived by using a high-molecular-weight standard (Ribi ImmunoChem Research) as the reference peak.

Cellular fatty acid analysis. Cells were grown for 3 days, saponified and the liberated fatty acids were methylated and analysed by GLC (Weyant *et al.*, 1996). Identification of fatty acids was performed using a commercially available software package (MIDI) utilized with CDC library created using LGS software (Weyant *et al.*, 1996). The presence of a *trans* isomer of hexadecenoic (16:1 ω 9*t*) acid (reported by MIDI software as summed feature 4, which comprises i-2-OH-15:0, 16:1 ω 9*t* or both) was confirmed by acetylation as described by Weyant *et al.* (1996).

RESULTS

Mycobacterial strains

Except for one, clinical sources were available for the studied isolates of the *M. fortuitum* third biovariant complex and the eight reference strains. Fifteen (45%) were isolated from wounds, the remaining strains having been isolated primarily from respiratory or blood sources (Table 1). Most strains studied were isolated either in the USA (66%) or in Australia (19%) (Table 1).

DNA hybridization and G+C content determination

DNA-relatedness studies divided the 24 *M. fortuitum* third biovariant complex (sorbitol-positive and sorbitol-negative) isolates into six genetic groups according to the phylogenetic definition of a species as 'strains with approximately 70% or greater DNA–DNA relatedness and with 5% or less ΔT_m ' (Wayne *et al.*, 1987), and according to the criteria described by Brenner *et al.* (1993) (Supplementary Table available in IJSEM Online). One cluster (*n*=13) represented by ATCC BAA-328, formerly referred to as strains of the *M. fortuitum* third biovariant (sorbitol-negative), was recognized as *M. porcinum*, while one strain

Table 2. RT groups and PRA of isolates used in this study

Isolate	RT pattern with <i>Sall</i> *	PRA pattern with:	
		<i>Bst</i> II	<i>Hae</i> III
<i>Mycobacterium</i> GS 1 (<i>n</i> = 6)			
ATCC 49937	RT-B	235/210	140/125
ATCC 49935 ^T	RT-B	235/205	140/125
W6229	RT-B	235/210	140/120/50
W6231	RT-B	235/210	140/125†
W6240	RT-B	235/210	140/125
W6245	RT-B	235/210	140/125
<i>Mycobacterium</i> GS 2 (<i>n</i> = 2)			
ATCC 49403 ^T	RT-H	235/115/85	140/125
ATCC 49934	RT-H	235/210	140/125/100
<i>Mycobacterium</i> GS 3 (<i>n</i> = 1)			
ATCC 49404 ^T	RT-C	235/115/85	140/125
<i>Mycobacterium</i> GS 4 (<i>n</i> = 1)			
ATCC 49938 ^T	RT-I	235/115/100	140/125/100
<i>M. fortuitum</i> (<i>n</i> = 3)			
ATCC 6841 ^T	RT-Mf1	235/115/85	145/125
W5994	RT-Mf2	230/115/80†	145/120/50†
W5995	RT-Mf2	230/115/80†	145/120/50†
<i>M. peregrinum</i> (<i>n</i> = 1)			
ATCC 14467 ^T	RT-Mp	235/205	145/140/100
<i>M. porcinum</i> (<i>n</i> = 13)			
W6228	RT-A	235/210	140/125/100
W6230	RT-A	235/210	140/125/100
ATCC BAA-328	RT-A	235/205	140/125/100
W6233	RT-A	235/210	140/125/100
ATCC 49939	RT-D	235/205	140/125/100
W6235	RT-A	235/210	140/125/100
W6236	RT-G	235/205	140/125/100
W6237	RT-A	235/210	140/125/100
W6239	RT-A	235/210	140/125/100
W6241	RT-A	235/210	140/125/100
W6242	RT-A	235/210	140/125/100
W6243	RT-F	235/205	140/125/100
W6244	RT-A	235/210	140/125/100
ATCC 33776 ^T	ND	235/210	140/125/100
<i>M. senegalense</i> (<i>n</i> = 2)			
ATCC 35796 ^T	ND‡	235/105/85	185/140
ATCC 13781	RT-Msn	235/115/85	185/140
<i>M. septicum</i> (<i>n</i> = 1)			
ATCC BAA-329	RT-E	235/205	140/125/100
ATCC 700731 ^T	RT-Msp	235/205	140/125/100

ND, Not determined.

*RT-Mf1, *M. fortuitum* pattern 1; RT-Mf2, *M. fortuitum* pattern 2; RT-Mp, *M. peregrinum* pattern; RT-Msn, *M. senegalense* pattern; RT-Msp, *M. septicum* pattern.

†Data from Steingrube *et al.* (1995).

‡ND, Not determined because not digested by the enzyme *SalI*.

(ATCC BAA-329) was recognized as *M. septicum*. In addition to *Mycobacterium* genomospecies 1, consisting of six strains (including ATCC 49935^T), formerly referred to as strains of the *M. fortuitum* third biovariant

(sorbitol-negative), three other genomospecies are described. These include *Mycobacterium* genomospecies 2, consisting of two strains (including ATCC 49403^T) and formerly referred to as strains of the *M. fortuitum* third

biovariant (sorbitol-positive), *Mycobacterium* genomospecies 3, consisting of one strain (ATCC 49404^T) and formerly referred to as the reference strain of the *M. fortuitum* third biovariant (sorbitol-negative), and *Mycobacterium* genomospecies 4, consisting of one strain (ATCC 49938^T) and formerly referred to as the *M. fortuitum* third biovariant (sorbitol-positive).

The G + C contents for representative isolates are given in the species descriptions. Except for *Mycobacterium* genomospecies 3 (ATCC 49404^T), which had a G + C content of 60 mol%, all values were within the 61–71 mol% range for *Mycobacterium* species (Takeuchi & Hatano, 1998).

Ribotyping

Ribotype (RT) analysis with the enzyme *SalI* revealed seven different RT patterns for the 21 *M. fortuitum* third biovariant sorbitol-negative isolates: RT groups A ($n=10$), B ($n=6$), C, D, E, F and G ($n=1$ isolate each). Two different patterns for the three *M. fortuitum* third biovariant sorbitol-positive isolates were revealed, RT groups H ($n=2$) and I ($n=1$), which differed from those of the type strains of *M. fortuitum*, *M. peregrinum*, *M. senegalense* and *M. septicum* (Fig. 1a). In addition, different RT patterns were found between the type and the reference strains of *M. fortuitum* (RT-Mf1 and RT-Mf2) with the enzyme *SalI* (data not shown; results summarized in supplementary data available in IJSEM Online, Table 2). Since the initial RT groups were determined with *SalI* and these RT groups were used as the basis for DNA–DNA relatedness, chemotaxonomic, 16S rRNA gene sequence and PRA analyses, the patterns are retained in Table 2 as references. As *SalI* did not digest DNA from the type strain of *M. senegalense*, further RT analysis with the enzyme *PvuII* was performed to compare representative isolates of the *SalI* RT groups and the studied type strains, including the type strain of *M. senegalense*. As before, nine different RT patterns that differed from those of the type strains of *M. fortuitum*, *M. peregrinum*, *M. senegalense* and *M. septicum* were found among these 24 isolates of the *M. fortuitum* third biovariant complex (Fig. 1a).

PRA

The combined patterns of *HaeIII*- and *BstEII*-derived digests of the PCR amplicons from representative isolates of the nine RT groups, RT-A to RT-I (Fig. 1b, c, lanes 6–14), differed from the PRA patterns of control strains *M. senegalense* ATCC 35796^T, *M. peregrinum* ATCC 14467^T and *M. fortuitum* ATCC 6841^T (Fig. 1b, c, lanes 2–4) but not from *M. septicum* ATCC 700731^T (Fig. 1b, c, lanes 5). Patterns of *HaeIII*- and *BstEII*-derived digests of the PCR amplicons of *M. septicum* ATCC 700731^T, the four isolates of *M. porcinum*, i.e. ATCC BAA-328 (RT-A), ATCC 49939 (RT-D), W6243 (RT-F) and W6236 (RT-G), and *M. septicum* BAA-329 (RT-E) were identical to each other, as shown in Fig. 1(b, c, lanes 5, 6 and 9–12, respectively), and summarized in Table 2, but differed from all other

Fig. 1. (a) RT patterns with *PvuII*-digested genomic DNA, (b) PCR amplification and restriction enzyme analysis with *HaeIII* and (c) PCR amplification and restriction enzyme analysis with *BstEII* of the type strains of *M. senegalense*, *M. peregrinum*, *M. fortuitum*, *M. septicum*, *Mycobacterium* genomospecies 1, *Mycobacterium* genomospecies 2, *Mycobacterium* genomospecies 3, *Mycobacterium* genomospecies 4 and representative studied isolates of *M. porcinum* and *M. septicum* as follows: lanes 1 and 15 (a), λ -DNA molecular size marker digested with *EcoRI* and *HindIII*; lanes 1 and 15 (b, c), 100 bp and pGem markers, respectively; lane 2, *M. senegalense* ATCC 35796^T; 3, *M. peregrinum* ATCC 14467^T; 4, *M. fortuitum* ATCC 6841^T; 5, *M. septicum* ATCC 700731^T; 6, *M. porcinum* ATCC BAA-328 (RT-A); 7, *Mycobacterium* genomospecies 1 (ATCC 49935^T) (RT-B); 8, genomospecies 3 (ATCC 49404^T) (RT-C); 9, *M. porcinum* ATCC 49939 (RT-D); 10, *M. septicum* ATCC BAA-329 (RT-E); 11, *M. porcinum* W6243 (RT-F); 12, *M. porcinum* W6236 (RT-G); 13, *Mycobacterium* genomospecies 2 (ATCC 69403^T) (RT-H); and 14, *Mycobacterium* genomospecies 4 (ATCC 49938^T) (RT-I). Molecular sizes for ribotyping are given in kb. Molecular sizes for PCR-RFLP analysis are given in bp.

patterns. RFLP with PRA of *Mycobacterium* genomospecies 3 (ATCC 49404^T) (Fig. 1b, lane 8) with the enzyme *HaeIII* was similar to the RFLP patterns of *Mycobacterium* genomospecies 1 (ATCC 49935^T) (RT-B) (Fig. 1b, lane 7) and *Mycobacterium* genomospecies 2 (ATCC 49403^T) (RT-H) (Fig. 1b, lane 13). Although the RFLP pattern of *Mycobacterium* genomospecies 3 (ATCC 49404^T) with enzyme *BstEII* (Fig. 1c, lane 8) differed from the pattern of *Mycobacterium* genomospecies 1 (ATCC 49935^T) (RT-B) (Fig. 1c, lane 7), the PRA pattern of *Mycobacterium* genomospecies 3 (ATCC 49404^T) (Fig. 1c, lane 8) with

this enzyme was identical to that of *Mycobacterium* genomospecies 2 (ATCC 49403^T) (RT-H) (Fig. 1c, lane 13). The combined patterns of *Hae*III- and *Bst*EII-derived digests of *Mycobacterium* genomospecies 4 (ATCC 49938^T) (RT-I) (Fig. 1b, c, lanes 14) were unique (Table 2). The PRA pattern of the type strain of *M. porcinum* ATCC 33776^T is given in Table 2. Although the PRA pattern of the type strain is not shown in Fig. 1(b, c), the PRA pattern was identical to those of *M. porcinum* isolates ATCC BAA-328 (RT-A), ATCC 49939 (RT-D), W6243 (RT-F) and W6236 (RT-G).

16S rRNA gene analysis

The 1401 bp region of a 16S rRNA gene, corresponding to *E. coli* positions 84–1494 (GenBank accession no. J01859), was determined and analysed for the *Mycobacterium* isolates shown in Fig. 2. The multiple alignment was used as the input to a phylogeny inference software package (TREECON) employing the neighbour-joining method of Saitou & Nei (1987) to generate a phylogenetic tree (Fig. 2). The GenBank accession numbers and strain designations for the sequences used in this alignment are given in Fig. 2.

M. porcinum ATCC 33776^T was found to be 100 % similar to *M. porcinum* ATCC BAA-328, *M. porcinum* W6243, *M. porcinum* ATCC 49939 and *M. porcinum* W6236 by pairwise comparisons of 16S rRNA gene sequence.

By 16S rRNA gene sequence analysis, *M. septicum* ATCC BAA-329, formerly referred to as the *M. fortuitum* third biovariant (sorbitol-negative), had a similarity of 100 % over the 1401 bp region, to *M. septicum* ATCC 700731^T. These two strains had the same PRA pattern, but differed

phenotypically and genetically when compared using ribotyping.

Mycobacterium genomospecies 1 (ATCC 49935^T) was 99.9 % similar, by pairwise comparisons of 16S rRNA gene sequences, to *M. porcinum* ATCC 33776^T and to the *M. porcinum* isolates (ATCC BAA-328, W6243, ATCC 49939 and W6236) and was 100 % related to *Mycobacterium* genomospecies 3 (ATCC 49404^T).

Mycobacterium genomospecies 2 (ATCC 49403^T), formerly referred to as the *M. fortuitum* third biovariant (sorbitol-positive), was 99.7 % similar, by pairwise comparisons of 16S rRNA gene sequences, to *M. senegalense* ATCC 35796^T and was 99.1 % related to *M. fortuitum* ATCC 6841^T.

The close sequence similarity between *Mycobacterium* genomospecies 3 (ATCC 49404^T) and *Mycobacterium* genomospecies 1 (ATCC 49935^T) was not supported phenotypically or genetically by DNA relatedness studies, by ribotyping or by PRA.

Mycobacterium genomospecies 4 (ATCC 49938^T) was not phylogenetically related by 16S rRNA gene sequence analysis to any of our studied group of isolates of the *M. fortuitum* third biovariant complex (Fig. 2).

The signature nucleotides of hypervariable region A are available as supplementary material in IJSEM Online. As Kirschner *et al.* (1992) have previously pointed out, a highly variable region between positions 175 and 212 (*E. coli* numbering) contains possible species-specific regions. Unique sequence stretches were found for *Mycobacterium* genomospecies 4 (ATCC 49938^T). However, there were

Fig. 2. Phylogenetic tree, based on 16S rRNA gene sequences, showing the positions of *M. septicum* ATCC BAA-329, *M. boenickei* ATCC 49935^T, *M. neworleansense* ATCC 49404^T, *M. porcinum* ATCC 33776^T, *M. porcinum* ATCC BAA-328, *M. porcinum* W6243, *M. porcinum* ATCC 49939, *M. porcinum* W6236, *M. houstonense* ATCC 49403^T and *M. brisbanense* ATCC 49938^T. The tree was rooted by using *N. otitidiscaviarum* as the outgroup. GenBank accession numbers are given in parentheses. Numbers at nodes indicate levels of bootstrap support (%) based on a neighbour-joining analysis of 1000 resampled datasets. Strains sequenced in the present study are shown in bold. Scale bar, 2 % difference in sequence.

no nucleotide differences among the type strains of *M. peregrinum* and *M. septicum*, none among *M. porcinum* ATCC 33776^T, *M. porcinum* isolates, *M. septicum* (ATCC BAA-329), *Mycobacterium* genomospecies 1 (ATCC 49935^T) and *Mycobacterium* genomospecies 3 (ATCC 49404^T), and none between *Mycobacterium* genomospecies 2 (ATCC 49403^T) and *M. senegalense* ATCC 35796^T.

Phenotypic analysis

Microscopic morphological studies showed that all isolates studied were Gram-positive, pleomorphic bacilli lacking spores and capsules, but frequently appearing as long filamentous forms with rudimentary branches. No right-angled branched forms were observed. Each isolate, under low-power stereomicroscopic examination, showed white to slightly beige, small-diameter (approx. 1 mm) colonies after incubation on heart infusion agar with 5% (v/v) rabbit blood for 2 days at 35 °C. No aerial hyphae were seen. Colonial variations in surface texture and edges were seen: all *M. porcinum* strains were mucoid, convex and entire-edged, except isolate W6236, which demonstrated slightly irregular translucent edges; *M. septicum* (ATCC BAA-329) showed both rough, wrinkled, irregularly edged colonies and mucoid, convex, round, entire-edged colonies; all *Mycobacterium* genomospecies 1 strains were matt, domed and scalloped-edged; both *Mycobacterium* genomospecies 2 strains were mucoid, convex, round and entire-edged; *Mycobacterium* genomospecies 3 was rough, wrinkled and irregularly edged; and *Mycobacterium* genomospecies 4 was mucoid, convex, round and entire-edged. All strains were acid-fast when tested using the modified Kinyoun method. All *M. fortuitum* third biovariant complex (sorbitol-positive and sorbitol-negative) isolates under study grew on Löwenstein–Jensen medium at 35 °C in less than 7 days and were negative for both growth in lysozyme and utilization of citrate. All were positive for utilization of acetamide and were positive for 3 day arylsulfatase production, except for two isolates (W6241 and W6229) that were positive in 14 days for arylsulfatase production. All produced acid from *i*-myo-inositol, D-mannitol, mannose and trehalose, were resistant to ampicillin, cefotaxime and ceftriaxone and were susceptible to ciprofloxacin, imipenem and trimethoprim/sulfamethoxazole. Antimicrobial susceptibility and biochemical differences among these isolates and type strains are summarized in Table 3.

HPLC and cellular fatty acid analysis

RRT ratios (± 0.01 min) were used to compare corresponding peaks among chromatograms (Butler *et al.*, 1992). The mycolates for the isolates under study appeared within the expected range for the rapidly growing mycobacteria, which elute after 5 min when compared by the HPLC method. Comparison of HPLC chromatograms and RRT ratios for representative isolates with those of strains of *M. fortuitum*, *M. peregrinum*, *M. senegalense* and *M. septicum* revealed that separation of these species was problematic when the

current HPLC method was used (results not shown). The RRT ratios for initial eluting peaks for each of the species studied were the same, but *M. senegalense* exhibited some differences in the RRT ratios for the late-eluting peaks, as reported previously (Schinsky *et al.*, 2000). The late-eluting peaks for the studied isolates, *M. fortuitum*, *M. peregrinum* and *M. septicum* exhibited the same RRT, with slight differences in relative heights. Visual recognition when the current HPLC method was used clearly distinguished these isolates from *M. abscessus* and *M. chelonae*, which were previously included in the *M. fortuitum* complex.

The cellular fatty acid compositions of saponified whole cells of the type strains of *M. fortuitum*, *M. peregrinum*, *M. septicum* and representative studied isolates from *M. porcinum*, *M. septicum* (ATCC BAA-329), *Mycobacterium* genomospecies 1, *Mycobacterium* genomospecies 2, *Mycobacterium* genomospecies 3 and *Mycobacterium* genomospecies 4 are characterized by large amounts (18–36%) of 16:0 and 18:1 ω 9 c and the presence of 14:0, 16:1 ω 9 c , 16:1 ω 7 c , 16:1 ω 9 t , 18:2, 18:1 ω 7 c , 18:0, 10-methyl-18:0 (tuberculostearic acid) and 20:4. The overall cellular fatty acid profile of *M. senegalense* is similar to those of the other isolates tested, except for the presence of small amounts of 19:0 cyc (2%), the absence of 18:2 and 20:4, and larger amounts of 18:1 ω 7 c (7% vs T-2%) and 10-methyl-18:0 (18% versus 4–10%). The studied isolates of the *M. fortuitum* third biovariant complex all have virtually the same cellular fatty acid content and thus must be identified by additional methods such as DNA relatedness studies.

DISCUSSION

The purpose of the present study was to assess the heterogeneity of previously identified mycobacterial strains, which have been known previously as the *M. fortuitum* third biovariant complex (sorbitol-negative and sorbitol-positive). Since previous studies have shown that ribotyping may be used to provide taxonomic data in addition to strain typing information (Grimont & Grimont, 1986; Kiehlbauch *et al.*, 1991; Popovic *et al.*, 1993), we initially ribotyped 24 phenotypically identified isolates of the *M. fortuitum* third biovariant complex (sorbitol-negative and sorbitol-positive) and compared the RT patterns with type and reference strains of *M. fortuitum*, *M. peregrinum*, *M. senegalense* and *M. septicum*. RT patterns of *Sa*II- and *Pvu*II-derived digests divided the 24 isolates studied into nine groups: seven RT groups of the *M. fortuitum* third biovariant complex (sorbitol-negative) [RT groups A ($n=10$), B ($n=6$) and C, D, E, F and G ($n=1$ isolate each)] and two RT groups of the *M. fortuitum* third biovariant complex (sorbitol-positive) [RT groups H ($n=2$) and I ($n=1$)]. These results suggested that this group of isolates of the *M. fortuitum* third biovariant complex is more heterogeneous than has previously been appreciated, and provided a genetic framework for other taxonomic studies. For this purpose, a polyphasic approach was used, assessing an expanded battery of phenotypic characteristics,

Table 3. Comparison of phenotypic differences among the studied isolates and the type strains of *M. fortuitum*, *M. peregrinum*, *M. porcinum*, *M. senegalense* and *M. septicum*

Strain/species/genomospecies: 1, *Mycobacterium* genomospecies 1 ($n=6$); 2, *Mycobacterium* genomospecies 2 ($n=2$); 3, *Mycobacterium* genomospecies 3 ($n=1$); 4, *Mycobacterium* genomospecies 4 ($n=1$); 5, *M. fortuitum* ATCC 6841^T; 6, *M. peregrinum* ATCC 14467^T; 7, *M. porcinum* ($n=13$); 8, *M. porcinum* ATCC 33776^T; 9, *M. senegalense* ATCC 35796^T; 10, *M. septicum* ($n=1$); 11, *M. septicum* ATCC 700731^T. Abbreviations: –, negative; +, positive; Amox./clav., amoxycillin/clavulanate; NT, not tested. Entries given as numerals represent percentages of isolates that test positive.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Growth at 42 °C	0	100	–	+	+	–	8	–	+	–	–
Nitrate reduction	100	100	+	+	+	+	100	–	+	+	+
Enzymic activity of:											
Arylsulfatase at 3 days*	83	100	+	+	+	+	92	+	+	+	–
Catalase (68 °C)	100	100	+	–	+	+	100	+	+	+	+
Utilization, as sole carbon and nitrogen source, of:											
Acetamide	100	100	+	+	+	–	100	+	+	+	–
Citrate	0	0	–	–	–	–	0	–	+	–	–
Carbon utilization and oxidative acid production from:											
Adonitol	0	0	–	+	–	–	0	–	–	–	–
L-Arabinose	0	0	–	+	+	–	0	–	–	–	–
i-Erythritol	0	0	+	+	+	–	0	–	–	–	–
D-Galactose	0	0	–	–	–	–	0	–	–	+	–
Glycerol	0	0	–	–	+	–	0	–	–	–	+
i-myo-Inositol	100	100	+	+	–	–	100	+	–	+	+
D-Mannitol	100	100	+	+	–	+	100	+	+	+	+
L-Rhamnose	0	0	–	+	–	–	0	–	–	–	–
Salicin	100	0	+	+	–	–	100	+	+	+	+
D-Sorbitol	0	100	–	+	–	–	0	–	–	–	–
Sucrose	0	0	–	–	–	–	0	–	–	+	–
D-Xylose	0	0	+	–	–	–†	0	–	–	–	+
Resistance to:‡											
Amikacin (MIC > 32 µg ml ^{–1})	0	0	–	–	–	–	8	–	–	–	–
Amox./clav. (MIC ≥ 32–16 µg ml ^{–1})	0	0	–	–	–	–	8	–	–	+	–
Doxycycline (MIC ≥ 16 µg ml ^{–1})	83	100	–	+	–	+\$	85	NT	–	–	–
Erythromycin (MIC ≥ 8 µg ml ^{–1})	100	100	+	+	+	+	100	NT	+	+	–
Minocycline (MIC ≥ 16 µg ml ^{–1})	83	100	–	+	–	+\$	23	–	–	–	–
Sulfamethoxazole (MIC ≥ 64 µg ml ^{–1})	0	0	–	–	–	–	8	–	+	–	–
Vancomycin (MIC ≥ 32 µg ml ^{–1})	83	100	+	+	+	+	92	+	–	–	–

*All isolates positive for arylsulfatase activity at 14 days. Alternative methodologies may show varying results at 3 days.

†Results differ from those reported by Kusunoki & Ezaki (1992).

‡The MIC resistance break points that were used are those of the NCCLS (1997).

\$Intermediately resistant; MIC, 8 µg ml^{–1}.

chemotaxonomic profiles and genotypic analysis that included a PCR-based identification method analysing patterns obtained from a 439 bp fragment of the *hsp*-65 kDa gene (PRA), 16S rRNA gene sequencing and DNA–DNA hybridization studies. Decision priority was given to the results of DNA–DNA hybridization in conjunction with 16S rRNA gene sequence similarities, provided that phenotypic properties were in agreement with genetic characteristics (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994).

Relatedness values obtained using the hydroxyapatite

method reflect degrees of overall similarity between genomic DNAs and thus define bacterial species in phylogenetic terms (Wayne *et al.*, 1987). There were two exceptions to the definition for a species designation among the *M. porcinum* strains. Labelled *M. porcinum* ATCC BAA-328 was 69 % related, with 1.0 % divergence, to W6236 (RT-G); however, labelled W6236 (RT-G) was 83 % related, with 0.0 % divergence, to ATCC BAA-328. In addition, *M. porcinum* labelled ATCC BAA-328 was 62 % related, with 0.5 % divergence, to W6243 (RT-F). Strain W6243 (RT-F) was not labelled; however, another labelled *M. porcinum* strain, ATCC 49939, was 73 % related, with 1.5 % divergence, to

W6243 (RT-F). Furthermore, *M. porcinum* ATCC BAA-328 was found to be 100 % similar, in pairwise comparisons of 16S rRNA gene sequence, to ATCC 49939, W6236 and W6243 (Fig. 2). Thus, these three isolates were included in the taxon *M. porcinum*. The labelled type strain of *M. porcinum*, ATCC 33776^T, was 91 % related to ATCC BAA-328, with 1.5 % divergence, and was 58 % related to *Mycobacterium* genomospecies 1 (ATCC 49935^T), with 5.0 % divergence. This close DNA relationship between *M. porcinum* ATCC 33776^T and ATCC BAA-328 confirmed the identity of *M. porcinum* from human clinical sources. *M. porcinum* ATCC 33776^T showed relatively low DNA–DNA relatedness to *Mycobacterium* genomospecies 2 (ATCC 49403^T) (39 %, 10 % divergence), *Mycobacterium* genomospecies 3 (ATCC 49404^T) (43 %, 7.0 % divergence), *Mycobacterium* genomospecies 4 (ATCC 49938^T) (16 %, 11.5 % divergence), *M. septicum* ATCC 700731^T (40 %, 7.5 % divergence) and *M. septicum* ATCC BAA-329 (57 %, 8.0 % divergence).

By 16S rRNA gene sequence analysis, ATCC BAA-329 (RT-E), formerly referred to as a *M. fortuitum* third biovariant (sorbitol-negative), was found to have a similarity of 100 %, over the 1401 bp region, to *M. septicum* ATCC 700731^T and had a PRA profile that was identical to that of *M. septicum* ATCC 700731^T. Labelled genomic DNA of ATCC BAA-329 was 61 % related to *M. septicum* ATCC 700731^T, with 2.0 % divergence, in DNA–DNA hybridization studies. Labelled *M. septicum* ATCC 700731^T was 78 % related to ATCC BAA-329, with 4.5 % divergence, meeting the criteria defined by Wayne *et al.* (1987) for the same species. Although ATCC BAA-329 differed phenotypically from all the other isolates studied (Table 3), genetically, this strain, except for a unique RT pattern, was most similar to *M. septicum* ATCC 700731^T.

The range of relatedness between labelled *M. porcinum* ATCC BAA-328 and the six *Mycobacterium* genomospecies 1 strains was 48–59 %, with divergences ranging from 2.5 to 3.5 %. In addition, the range of relatedness between labelled *Mycobacterium* genomospecies 1 (ATCC 49935^T) and representative *M. porcinum* strains was 47–65 %, with 3.0–4.0 % divergence. Both of these DNA relatedness groups met the criteria for unique taxa, but the strains comprising *Mycobacterium* genomospecies 1 were not sufficiently distinct phenotypically from *M. porcinum* to meet these criteria. The only significant difference was the resistance of 83 % of *Mycobacterium* genomospecies 1 strains to minocycline. *Mycobacterium* genomospecies 1 showed low relatedness values (41 and 42 %) to *Mycobacterium* genomospecies 2 and *Mycobacterium* genomospecies 3. The relatedness of *Mycobacterium* genomospecies 1 to *Mycobacterium* genomospecies 4 was not determined, but the reciprocal level of relatedness was low (3 and 7 %) with *Mycobacterium* genomospecies 1 (ATCC 49935^T and ATCC 49937). Low levels of relatedness (20–47 %) to the type strains of *M. fortuitum*, *M. peregrinum*, *M. senegalense* and *M. septicum* were found.

The labelled genomic DNA of *Mycobacterium* genomospecies 2 (ATCC 49403^T) (RT-H), previously referred to as a reference isolate for the *M. fortuitum* third biovariant complex (sorbitol-positive), was found to be 96 % related, with 0 % divergence, to *Mycobacterium* genomospecies 2 (ATCC 49934) (RT-H), another reference isolate for the *M. fortuitum* third biovariant complex (sorbitol-positive). *Mycobacterium* genomospecies 2 (ATCC 49403^T) showed relatively low DNA–DNA relatedness to *Mycobacterium* genomospecies 1 (ATCC 49935^T) (41 %, 7.5 % divergence) and W6229 (21 %, 8.5 % divergence), *Mycobacterium* genomospecies 3 (ATCC 49404^T) (38 %, 7.5 % divergence) and *Mycobacterium* genomospecies 4 (ATCC 49938^T) (15 %, 9.0 % divergence). Low values of relatedness (35–65 %) to the type or reference strains of *M. fortuitum*, *M. peregrinum*, *M. porcinum*, *M. senegalense* and *M. septicum* were found.

One strain, ATCC 49404^T (RT-C), formerly the reference strain for the *M. fortuitum* third biovariant (sorbitol-negative) and now referred to as *Mycobacterium* genomospecies 3, was 100 % similar in a pairwise comparison of 1401 bp of the 16S rRNA gene sequence with that of *Mycobacterium* genomospecies 1 (ATCC 49935^T) (RT-B). This similarity was not supported phenotypically, by DNA relatedness studies, by ribotyping or by PRA analysis. Phenotypically, *Mycobacterium* genomospecies 3 (ATCC 49404^T) could be distinguished from *Mycobacterium* genomospecies 1 by its production of acid from *D*-erythritol and *D*-xylose, and by its susceptibility to doxycycline and minocycline. Genetically, labelled genomic DNA of *Mycobacterium* genomospecies 3 (ATCC 49404^T) was 46 % related to that of *Mycobacterium* genomospecies 1 (ATCC 49935^T) (RT-B); conversely, labelled genomic DNA of *Mycobacterium* genomospecies 1 (ATCC 49935^T) was 42 % related to that of *Mycobacterium* genomospecies 3 (ATCC 49404^T), with 6.0 % divergence. These values for relatedness do not meet the criteria recommended by Wayne *et al.* (1987) for strains to be considered members of the same species. *Mycobacterium* genomospecies 3 had relatively low values for DNA relatedness to *Mycobacterium* genomospecies 2 (36 %) and with the reciprocal value of *Mycobacterium* genomospecies 4 (1 %, 8 % divergence). Low values of relatedness (31–44 %) to the type or reference strains of *M. fortuitum*, *M. peregrinum*, *M. porcinum*, *M. senegalense* and *M. septicum* were found. RT patterns of *Mycobacterium* genomospecies 3 (ATCC 49404^T) with both *SalI* and *PvuII* differed from the RT pattern of *Mycobacterium* genomospecies 1 (ATCC 49935^T) and the RT patterns of each of the other isolates studied (Fig. 1a). The PRA pattern of *Mycobacterium* genomospecies 3 (ATCC 49404^T) with the enzyme *HaeIII* was similar to the PRA patterns of *Mycobacterium* genomospecies 1 (ATCC 49935^T) (RT-B) and *Mycobacterium* genomospecies 2 (ATCC 49403^T) (RT-H) (Fig. 1b). Although the PRA pattern of *Mycobacterium* genomospecies 3 (ATCC 49404^T) with the enzyme *BstEII* differed from that of *Mycobacterium* genomospecies 1 (ATCC 49935^T) (RT-B) (Fig. 1c), its

PRA pattern was similar to that of *Mycobacterium* genomospecies 2 (ATCC 49403^T) (RT-H) (Fig. 1c).

The labelled genomic DNA of *Mycobacterium* genomospecies 4 (ATCC 49938^T) (RT-I) was related to the other isolates of the *M. fortuitum* third biovariant complex that were studied by DNA–DNA hybridization analysis, with values ranging from 1 to 13 % and divergences ranging from 7.0 to 9.5 %. Low values of relatedness (1–13 %) to type or reference strains of *M. fortuitum*, *M. peregrinum*, *M. porcinum*, *M. senegalense* and *M. septicum* were found. Phenotypically, this isolate differed from all of the other isolates studied (Table 3). Furthermore, the 16S rRNA gene sequence of *Mycobacterium* genomospecies 4 (ATCC 49938^T) was not phylogenetically related to any of our group of studied isolates of the *M. fortuitum* third biovariant complex (Fig. 2). Thus, *Mycobacterium* genomospecies 4 (ATCC 49938^T) met the criteria of Wayne *et al.* (1987) for a novel species.

Application of a PCR-based identification method (PRA) to our group of organisms showed that it had a lower discriminatory ability than ribotyping with either *Pvu*II (Fig. 1a) or *Sal*I (Table 2). This discrepancy could be due to the low degree of heterogeneity existing in a DNA fragment of 439 bp. All *M. porcinum* isolates tested [W6228 (RT A), W6230 (RT A), ATCC 33776^T, ATCC BAA-328 (RT-A), W6233 (RT A), W6235 (RT A), W6237 (RT A), W6239 (RT A), W6241 (RT A), W6242 (RT A), W6244 (RT A), ATCC 49939 (RT-D), W6243 (RT-F) and W6236 (RT-G)] yielded the same PRA restriction pattern: two fragments of 235 and 205–210 bp with *Bst*EII and three fragments of 140/125/100 bp with *Hae*III. In addition to the similar phenotypic characteristics (Table 3), DNA reassociation and 16S rRNA gene sequences (Fig. 2), this PRA grouping further supported the status of these strains as *M. porcinum* according to taxonomic norms. The species *M. porcinum* was studied by Tsukamura *et al.* (1983) and recently recognized by Turenne *et al.* (2001).

Except for their identical *Hae*III and *Bst*EII PRA patterns, both *M. septicum* ATCC 700731^T and ATCC BAA-329 (RT-E) differed from the *M. porcinum* isolates in terms of other phenotypic and genetic characteristics (Tables 2 and 3, Fig. 2).

When the PRA pattern of the type strain of *Mycobacterium* genomospecies 1 was compared with the PRA patterns of other isolates of *Mycobacterium* genomospecies 1 (ATCC 49937, W6229, W6231, W6240 and W6245), both the type strain and the five other isolates of *Mycobacterium* genomospecies 1 gave the same PRA patterns as *M. porcinum* with the enzymes *Bst*EII and *Hae*III, except that they lacked the 100 bp fragment with *Hae*III. These two restriction patterns for *Mycobacterium* genomospecies 1 and *M. porcinum*, *Bst*EII-derived 235/205 and *Hae*III-derived 140/125 and *Bst*EII-derived 235/205 and *Hae*III-derived 140/125/100, respectively, were the recognized patterns of

M. fortuitum third biovariant complex sorbitol-negative isolates previously observed by Steingrube *et al.* (1995).

Although the PRA patterns of the *Hae*III- and *Bst*EII-derived digests of the PCR amplicon of *Mycobacterium* genomospecies 3 (ATCC 49404^T) (RT-C) (Fig. 1b, c, lanes 8) were identical to those of *Mycobacterium* genomospecies 2 (ATCC 49403^T) (RT-H) (Fig. 1b, c, lanes 13), these two strains were not similar phenotypically or genetically (Table 3, Fig. 2). This restriction pattern, 235/115/85 bp with *Bst*EII and 140/125 bp with *Hae*III, was the commonly recognized pattern of the *M. fortuitum* third biovariant complex sorbitol-positive isolates (Steingrube *et al.*, 1995). The other sorbitol-positive strain tested, *Mycobacterium* genomospecies 4 (ATCC 49938^T), displayed a restriction pattern different from that of all other isolates tested.

Further studies on this PCR-based identification method with more isolates of the third biovariant complex and additional enzymes are needed to establish the frequency of the individual taxa among clinical isolates and to establish whether each of these new genetic groups will yield specific, unique PRA patterns.

Phenotypically, all of the *M. fortuitum* third biovariant complex sorbitol-negative and sorbitol-positive isolates under study differed from the type strains of *M. fortuitum* ATCC 6841^T, *M. peregrinum* ATCC 14467^T (pipemidic acid-susceptible), *M. senegalense* ATCC 35796^T and *M. septicum* ATCC 700731^T. They differed from *M. fortuitum* ATCC 6841^T in their ability to produce acid from both D-mannitol and i-myo-inositol, they differed from *M. peregrinum* ATCC 14467^T (pipemidic acid-susceptible) in their ability to produce acid from i-myo-inositol, they differed from *M. peregrinum* ATCC 14467^T and *M. septicum* ATCC 700731^T in their ability to utilize acetamide and they differed from *M. senegalense* ATCC 35796^T in both their ability to produce acid from i-myo-inositol and their inability to utilize citrate. Phenotypic differences among the strains studied can be found in Table 3.

Chemotaxonomically, each of these new taxonomic groups exhibited typical HPLC profiles consistent with rapidly growing *Mycobacterium* species. However, in our present study no distinction could be made either among *M. porcinum*, *M. septicum*, *Mycobacterium* genomospecies 1, *Mycobacterium* genomospecies 2, *Mycobacterium* genomospecies 3 and *Mycobacterium* genomospecies 4 or between these groups and the type strains of *M. fortuitum* ATCC 6841^T, *M. peregrinum* ATCC 14467^T and *M. septicum* ATCC 700731^T, except for *M. senegalense* ATCC 35796^T, as Schinsky *et al.* (2000) also found in their earlier study.

Consistent with a report by Yassin *et al.* (1993), these mycobacterial taxonomic groups showed similar cellular fatty acid profiles that could not be differentiated from each other, from the type strains of *M. fortuitum* ATCC 6841^T, *M. peregrinum* ATCC 14467^T, *M. senegalense* ATCC 35796^T and *M. septicum* ATCC 700731^T or from related

genera, such as *Corynebacterium*, *Nocardia*, *Rhodococcus* or *Tsukamurella*. These new taxonomic groups had a DNA G + C content that ranged from 60 to 64 mol%, within the limits of the genus *Mycobacterium* (Takeuchi & Hatano, 1998).

On the basis of genotypic data, we propose that *Mycobacterium* genomospecies 1 be classified as a novel species, *Mycobacterium bonikei* sp. nov. *Mycobacterium* genomospecies 1 is very similar phenotypically to *M. porcinum*, except in terms of the nitrate-reducing ability of the type strain of *M. porcinum*; however, since this group is very different from all *M. porcinum* isolates in genetic studies, including DNA–DNA reassociation, 16S rRNA gene sequence analysis, ribotyping and PRA, a decision was made to give this group nomenstatus status represented by ATCC 49935^T.

On the basis of phenotypic and genotypic data, we propose that *Mycobacterium* genomospecies 2 be classified as a novel species, *Mycobacterium houstonense* sp. nov.

We also propose that *Mycobacterium* genomospecies 3 and 4, each containing one strain, be formally classified as *Mycobacterium neworleansense* sp. nov. and *Mycobacterium brisbanense* sp. nov., respectively. The decision to give nomenstatus status rather than genomospecies status was based on the determination that each of these proposed novel species exhibited a clear-cut difference from every other species on the basis of at least two phenotypic properties, not including antimicrobial susceptibility differences.

Description of *Mycobacterium boenickei* sp. nov.

Mycobacterium boenickei (boe.nick'e.i. N.L. gen. masc. n. *boenickei* of Bönicke, in honour of the contributions of Rudolf Bönicke, a German mycobacteriologist, who first recognized the heterogeneity within the *M. fortuitum* complex).

The organisms are acid-fast, Gram-positive, pleomorphic bacilli. Long filamentous forms are often observed, but spores and capsules are absent. Each isolate, under low-power stereomicroscopic examination, shows white to slightly beige, small-diameter (approx. 1 mm) colonies after incubation on heart infusion agar with 5% (v/v) rabbit blood for 2 days at 35 °C. Colonies are matt, domed, scalloped-edged and do not demonstrate aerial hyphae. Growth occurs on Löwenstein–Jensen medium at 35 °C in less than 7 days, but no growth occurs at 42 °C; growth occurs on 5% NaCl and on MacConkey's agar without crystal violet at 28 °C. None of the isolates grow in lysozyme or utilize citrate, and five of six (83%) isolates produce arylsulfatase in 3 days. The semi-quantitative catalase activity of all isolates is reactive (>45 mm). All isolates utilize acetamide, reduce nitrate, exhibit iron uptake and produce urease and thermostable catalase. D-Fructose, D-glucose, i-myo-inositol, D-mannitol, D-mannose, salicin and D-trehalose are utilized as sole carbon sources, but adonitol,

L-arabinose, cellobiose, dulcitol, i-erythritol, D-galactose, glycerol, lactose, maltose, melibiose, raffinose, L-rhamnose, D-sorbitol, starch, sucrose and D-xylose are not utilized. Acid is produced oxidatively from D-fructose, D-glucose, i-myo-inositol, D-mannitol, D-mannose, salicin and D-trehalose, but not from adonitol, L-arabinose, cellobiose, dulcitol, i-erythritol, D-galactose, glycerol, lactose, maltose, melibiose, raffinose, L-rhamnose, D-sorbitol, starch, sucrose or D-xylose. All of the isolates are resistant to ampicillin, cefotaxime, ceftriaxone and erythromycin, five of six (83%) are resistant to doxycycline, minocycline and vancomycin and all are susceptible to amikacin, amoxycillin/clavulanate, ciprofloxacin, gentamicin, imipenem, sulfamethoxazole and trimethoprim/sulfamethoxazole. The type strain has a DNA G + C content of 64 mol%. The nearest phylogenetic neighbours, according to 16S rRNA gene sequence similarity, are *M. neworleansense* and all *M. porcinum* isolates studied (all 99.9%).

The type strain, which was isolated from a leg wound, is W5998^T (=ATCC 49935^T=DSM 44677^T). DNA relatedness values among the strains in the species and their relatedness to other strains of the *M. fortuitum* third biovariant complex and related species are available in IJSEM Online.

Description of *Mycobacterium houstonense* sp. nov.

Mycobacterium houstonense [hous'ton.en.se. N.L. neut. adj. *houstonense* pertaining to Houston, TX, USA, where the first isolate of the *M. fortuitum* third biovariant (sorbitol-positive) was identified].

The organisms are acid-fast, Gram-positive, pleomorphic bacilli. Long filamentous forms are often observed, but the organisms have no spores or capsules. Both isolates, under low-power stereomicroscopic examination, show white to slightly beige, small-diameter (approx. 1 mm) colonies after incubation on heart infusion agar with 5% (v/v) rabbit blood for 2 days at 35 °C. Colonies are mucoid, convex, round and entire-edged and do not demonstrate aerial hyphae. Both grow on Löwenstein–Jensen medium at 35 and 42 °C in less than 7 days and grow on 5% NaCl and on MacConkey's agar without crystal violet at 28 °C. Semi-quantitative catalase of both isolates is weakly reactive (<45 mm). Both have arylsulfatase activity by 3 days, utilize acetamide, reduce nitrate, exhibit iron uptake and produce urease and thermostable catalase. Both are unable to utilize citrate or grow in lysozyme. D-Fructose, D-glucose, i-myo-inositol, D-mannitol, D-mannose, D-sorbitol and D-trehalose are utilized as sole carbon sources, but adonitol, L-arabinose, cellobiose, dulcitol, i-erythritol, D-galactose, glycerol, lactose, maltose, melibiose, raffinose, L-rhamnose, salicin, starch, sucrose and D-xylose are not utilized. Acid is produced oxidatively from D-fructose, D-glucose, i-myo-inositol, D-mannitol, D-mannose, D-sorbitol and D-trehalose, but not from adonitol, L-arabinose, cellobiose, dulcitol, i-erythritol, D-galactose, glycerol, lactose, maltose,

melibiose, raffinose, L-rhamnose, salicin, starch, sucrose or D-xylose. Both isolates are resistant to ampicillin, cefotaxime, ceftriaxone, doxycycline, erythromycin, minocycline and vancomycin, and both isolates are susceptible to amikacin, amoxycillin/clavulanate, ciprofloxacin, imipenem, sulfamethoxazole and trimethoprim/sulfamethoxazole. The type strain has a DNA G+C content of 64 mol%. The nearest phylogenetic neighbours, according to 16S rRNA gene sequence similarity, are *M. fortuitum* ATCC 6841^T and *M. senegalense* ATCC 35796^T.

The type strain, which was isolated from a face wound, is W5198^T (=ATCC 49403^T=DSM 44676^T). DNA relatedness among the strains in the species and their relatedness to other strains of *M. fortuitum* third biovariant complex and related species are available in IJSEM Online.

Description of *Mycobacterium neworleansense* sp. nov.

Mycobacterium neworleansense (new.or.le.an.sen'se. N.L. neut. adj. *neworleansense* pertaining to New Orleans, LA, USA, the source of the type strain).

The organisms are acid-fast, Gram-positive, pleomorphic bacilli. Long filamentous forms are often observed, but spores and capsules are absent. Colonies are white to slightly beige and small in diameter (approx. 1 mm) after incubation on heart infusion agar with 5 % (v/v) rabbit blood for 2 days at 35 °C. Colonies are rough, wrinkled, irregular-edged and do not demonstrate aerial hyphae. Growth occurs on Löwenstein–Jensen medium at 35 °C in less than 7 days, but no growth occurs at 42 °C. Growth occurs on 5 % NaCl and on MacConkey's agar without crystal violet at 28 °C. Semi-quantitative catalase activity is positive (>45 mm). The isolate has arylsulfatase activity by 3 days, utilizes acetamide, reduces nitrate, exhibits iron uptake and produces urease and thermostable catalase. It does not utilize citrate or grow in lysozyme. Acid is produced oxidatively from i-erythritol, D-fructose, D-glucose, i-*myo*-inositol, D-mannitol, D-mannose, salicin, D-trehalose and D-xylose but not from adonitol, L-arabinose, cellobiose, dulcitol, D-galactose, glycerol, lactose, maltose, melibiose, raffinose, L-rhamnose, D-sorbitol, starch or sucrose. Resistant to ampicillin, cefotaxime, ceftriaxone, erythromycin and vancomycin, but susceptible to amikacin, amoxycillin/clavulanate, ciprofloxacin, doxycycline, imipenem, minocycline, sulfamethoxazole, trimethoprim/sulfamethoxazole and vancomycin. The type strain has a DNA G+C content of 60 mol%. The nearest phylogenetic neighbours, according to 16S rRNA gene sequence similarity, are *Mycobacterium boenickei* ATCC 49935^T and all *M. porcinum* isolates studied.

The type strain, which was recovered from a scalp wound, is W6705^T (=ATCC 49404^T=DSM 44679^T). DNA relatedness among the strains in the species and their relatedness to other strains of the *M. fortuitum* third biovariant complex and related species are available in IJSEM Online.

Description of *Mycobacterium brisbanense* sp. nov.

Mycobacterium brisbanense (bris.ban.en'se. N.L. neut. adj. *brisbanense* pertaining to Brisbane, Queensland, Australia, the source of the type strain).

The organisms are acid-fast, Gram-positive, pleomorphic bacilli. Long filamentous forms are often observed, but spores and capsules are absent. Colonies are white to slightly beige and small in diameter (approx. 1 mm) after incubation on heart infusion agar with 5 % (v/v) rabbit blood for 2 days at 35 °C. Colonies are mucoid, convex, round, entire-edged and do not demonstrate aerial hyphae. Growth occurs on Löwenstein–Jensen medium at 35 °C in less than 7 days, but no growth occurs at 42 °C. Growth occurs on 5 % NaCl and on MacConkey's agar without crystal violet at 28 °C. The isolate has arylsulfatase activity by 3 days, utilizes acetamide, reduces nitrate, produces urease and exhibits iron uptake. It does not utilize citrate or grow in lysozyme. Semi-quantitative catalase activity is weakly positive (<45 mm). It does not utilize citrate, grow in lysozyme or produce thermostable catalase. Adonitol, L-arabinose, i-erythritol, D-fructose, D-glucose, i-*myo*-inositol, D-mannitol, D-mannose, L-rhamnose, salicin, D-sorbitol and D-trehalose are utilized as sole carbon sources, but cellobiose, dulcitol, D-galactose, glycerol, lactose, maltose, melibiose, raffinose, starch, sucrose and D-xylose are not utilized. Produces acid oxidatively from adonitol, L-arabinose, i-erythritol, D-fructose, D-glucose, i-*myo*-inositol, D-mannitol, D-mannose, L-rhamnose, salicin, D-sorbitol and D-trehalose, but not from cellobiose, dulcitol, D-galactose, glycerol, lactose, maltose, melibiose, raffinose, starch, sucrose or D-xylose. Resistant to ampicillin, cefotaxime, ceftriaxone, doxycycline, erythromycin, minocycline and vancomycin, but susceptible to amikacin, amoxycillin/clavulanate, ciprofloxacin, imipenem, sulfamethoxazole and trimethoprim/sulfamethoxazole. The type strain has a DNA G+C content of 62 mol%. The nearest phylogenetic neighbour, according to 16S rRNA gene sequence similarity, is *Mycobacterium diernhoferi* ATCC 19340^T.

The type strain, which was isolated from an antral sinus, is W6743^T (=ATCC 49938^T=DSM 44680^T). DNA relatedness to other strains of the *M. fortuitum* third biovariant complex and related species are available in IJSEM Online.

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