A new rapidly growing mycobacterial species, *Mycobacterium murale* sp. nov., isolated from the indoor walls of a children's day care centre

R. Vuorio,¹ M. A. Andersson,¹ F. A. Rainey,² R. M. Kroppenstedt,³ P. Kämpfer,⁴ H.-J. Busse,^{1†} M. Viljanen⁵ and M. Salkinoja-Salonen¹

Author for correspondence: R. Vuorio. Tel: +358 9 708 59333. Fax: +358 9 708 59322.
e-mail: Riitta.Vuorio@Helsinki.fi

¹ Department of Applied Chemistry and Microbiology, PO Box 56 (Biocentre), 00014 University of Helsinki, Finland

² Department of Microbiology, 508 Life Sciences Building, Louisiana State University, Baton Rouge, LA 70803, USA

³ DSMZ—Deutsche Sammlung von Mikroorganismen und Zellkulturen, D-38124 Braunschweig, Germany

⁴ Institut fuer Angewandte Mikrobiologie, Justus-Liebig Universität, Senckenbergstr. 3, D-35390 Giessen, Germany

⁵ National Public Health Institute, Department in Turku, Kiinamyllynkatu 13, FIN-20520 Turku, Finland

Scotochromogenic mycobacterial isolates from water-damaged parts of indoor building materials of a children's day care centre represented a phenetically and genetically distinct group of strains. A 16S rDNA dendrogram (1243 bp) showed that the closest species to the new strain MA112/96^T was *Mycobacterium abscessus*. Phylogenetic and phenetic analyses (100 characteristics) grouped the new isolates with *M. abscessus*, *Mycobacterium vaccae*, *Mycobacterium aurum* and *Mycobacterium austroafricanum*. Ribotyping with *PvuII* restriction distinguished the 5 isolates from the other 12 most closely related species by the major bands at 6.5–7 kb and 13–15 kb. The cell morphology of the new isolates was typical of mycobacteria, electron microscopy revealed a triple-layered cell wall with an irregular electron-dense outer layer. They grew at 10–37 °C, with no growth at 45 °C in 5 d. The gene encoding the secreted 32 kDa protein, specific to mycobacteria, was detected by PCR. The main whole-cell fatty acids were characterized by high tuberculostearic acid 10Me-C_{18:0} (17% at 28 °C), which increased with increasing growth temperature (22% at 37 °C). The other main fatty acids were C_{18:1} *cis*9 and C_{16:0} (21–20% each), followed by, C_{17:1} *cis*9 (14%), C_{16:1} *cis*10 (8%) and also a high amount of C₂₀ alcohol (9%). α -Mycolic acids, keto-mycolates and wax esters were present (C₆₀–C₉₀), MK-9(H₂) (90%) and MK-8(H₂) were the main menaquinones. The cellular phospholipids were phosphatidylethanolamine, phosphatidylinositol, phosphatidyl inositolmannosides and diphosphatidylglycerol. Polyamine content was low. G+C content was 72.9 mol%. The new isolates are proposed as a new species, *Mycobacterium murale* sp. nov. The type strain is MA112/96^T (= DSM 44340^T).

Keywords: indoor contaminant, ribotyping, 16S rDNA, *Mycobacterium abscessus*, *Mycobacterium komossense*

INTRODUCTION

Non-tuberculous mycobacteria are widespread in the environment, ranging from harmless inhabitants of water and soil to species pathogenic to man and

animals (Guay, 1996). We recently reported rapidly growing mycobacteria as a major bacterial colonizer of water-damaged sites in the indoor walls of a children's day care centre (Andersson *et al.*, 1997). During the renovation of this moisture-damaged building, 10⁶ c.f.u. of mycobacteria, 10⁷ c.f.u. of Gram-negative bacteria and toxin-producing fungi besides other microbes were found g⁻¹ of the water-damaged gypsum board liner. There are few reports of mycobacteria in dwellings. Dustborne mycobacteria have been reported in tuberculous patients' rooms (Tsukamura *et al.*, 1974), in the indoor air of houses (Reznikov *et al.*, 1971; Flannigan, 1995) and from the tap water in

† Present address: Institut fuer Bakteriologie und Tierhygiene, Veterinärmedizinische Universität, Josef Baumann Gasse 1, A-1210 Wien, Austria.

Abbreviations: DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIM, phosphatidyl inositolmannosides.

The GenBank accession number for the 16S rDNA sequence of strain MA112/96^T (= DSM 44340^T) is Y08857.

hospitals and homes (Peters *et al.*, 1995). Mycobacteria are excellent survivors, living cells were found more than 2 months after inoculation to dry conditions (Hirai, 1991).

The present study is a polyphasic characterization of scotochromogenic mycobacterial isolates from water-damaged building material. Comparison of the almost full 16S rDNA sequences clustered the new isolates closest to *Mycobacterium abscessus* NCTC 13031^T. Chemotaxonomic characters, phylogenetic analysis and ribotyping as a new tool for characterizing mycobacteria, as well as morphological and biochemical tests showed that these strains represent a new mycobacterial species, to which the name *Mycobacterium murale* sp. nov. is proposed.

METHODS

Bacterial strains. The bacterial strains used in this study are described in Table 1. Five mycobacterial isolates were isolated from water-damaged building material of a children's day care centre as described previously (Andersson *et al.*, 1995, 1997). The isolates MA112/96^T, MA113/96, MA142/96 and MA168/96 were isolated at 22 °C and the MA166/96 at 16 °C on Tryptone soy agar plates (Difco).

Characterization of strains. Temperature tolerance of growth was tested on PYE agar plates (0.3% peptone from casein, 0.3% yeast extract, 1.5% agar, pH = 7.2) at 6, 10, 28, 45 and 52 °C, $\Delta T \pm 0.2$ °C. For the measurement of the optimal growth temperature TSB liquid medium (tryptone soy broth) in microtitre plates and a shaking incubator

(Bioscreen) (22, 30 and 37 °C; $\Delta T \pm 0.5$ °C) were used, with inoculum of 1:10, 1:100 or 1:1000 of a Klett = 120 suspension (Klett photometer, red filter), incubated for 4 d. Pigment production, photoreactivity and colony morphology were observed by light microscopy.

Microscopy. Acid-alcohol-fastness and presence of spores were tested as described previously (Murray *et al.*, 1994). For electron microscopy the cells were grown on tryptic soy agar for 7 d at 28 °C. Thin sections were prepared as described previously (Andersson *et al.*, 1995).

Identification by PCR. PCR amplification of a segment of the gene encoding the 32 kDa mycobacterial protein was performed as described previously (Soini *et al.*, 1992).

Antimicrobial sensitivity. Sensitivities of the new isolates to amikacin, azithromycin, chloramphenicol, ciprofloxacin, clarithromycin, doxycycline, penicillin G, ceftazidime and vancomycin were tested with E-test strips (AB Biodisk). Sensitivities to isoniazid (1 mg l⁻¹), rifampin (5 mg l⁻¹), streptomycin (10 mg l⁻¹) and ethambutol (2 mg l⁻¹) were tested by the disk-elutriation method described by Wayne & Krasnow (1966).

Analysis of fatty acids, polar lipids, cell wall, quinones, mycolic acids and G + C content. For whole-cell fatty acids and the other chemotaxonomic analyses, the strains were grown on Middlebrook 7H10 agar (Difco) enriched with Middlebrook OADC or on PYE agar plates. Fatty acid methyl esters were prepared after 4 d incubation at 28 °C and analysed as previously (Briglia *et al.*, 1996). The chemotaxonomic analyses of polar lipids by using authentic phosphatidylethanolamine (PE), phosphatidylinositol (PI) and diphosphatidylglycerol (DPG) (Sigma) as references; sugars and amino acids of whole-cell hydrolysates, isoprenoid quinones, mycolic acids and determination of the

Table 1. Mycobacterial isolates and reference strains used in this study

NCTC, National Collection of Type Cultures, London, UK; DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany.

Name	Number	Obtained from:
New isolate	MA112/96 ^T	This study
New isolate	MA113/96	This study
New isolate	MA142/96	This study
New isolate	MA166/96	This study
New isolate	MA168/96	This study
<i>M. abscessus</i>	NCTC 13031 ^{T*}	NCTC
<i>M. abscessus</i>	NCTC 10882	NCTC
<i>M. aichiense</i>	DSM 44147 ^T	DSMZ
<i>M. aurum</i>	NCTC 10437 ^T	NCTC
<i>M. austroafricanum</i>	DSM 44191 ^T	DSMZ
<i>M. diernhoferi</i>	DSM 43524 ^T	DSMZ
<i>M. fortuitum</i>	DSM 46621 ^T	DSMZ
<i>M. gadium</i>	NCTC 10942 ^T	NCTC
<i>M. hodleri</i>	DSM 44183 ^T	DSMZ
<i>M. komossense</i>	ATCC 33013 ^T	M.-L. Katila, Central Hospital of Kuopio University
<i>M. neoaurum</i>	NCTC 10818 ^T	NCTC
<i>M. obuense</i>	NCTC 10778 ^T	NCTC
<i>M. vaccae</i>	NCTC 10916 ^T	NCTC

* Equivalent to ATCC 19977^T which was used in sequence comparison.

G + C content by HPLC were done as described previously (Minnikin *et al.*, 1980; Luquin *et al.*, 1991; Häggblom *et al.*, 1994; Briglia *et al.*, 1996).

Polyamines. For polyamine analyses the strains were grown on PYE agar plates. Extraction and analysis of polyamines (1,3-diaminopropane, putrescine, cadaverine, sym-norspermidine, spermine and tyramine) was done as described previously (Altenburger *et al.*, 1997).

Physiological tests. Physiological tests in microtitre plates were done as described earlier (Kämpfer *et al.*, 1997). Tests were read after 7 d at 30 °C. Numerical analysis was done using the simple-matching coefficient and the phenogram was generated with the unweighted pair group method with averages (UPGMA) algorithm (Sneath & Sokal, 1973). For the other physiological determinations methods described in (Levy-Frebault & Portaels, 1992; Lutz, 1992) were used. The number of strains studied in the numerical taxonomy is the number of OTUs in the phenogram, i.e. 11 strains, the number of characters is 65 (all tests in Table 5). The test error was not estimated.

Ribotyping. For ribotyping cells were collected from an tryptone soy agar plate, suspended in saline in an Eppendorf tube, centrifuged and the pellet vortexed into 1 ml acetone (1 min). After 15 min at 37 °C and centrifugation, the supernatant was removed and the cell pellet was dried for 5 min at 37 °C. The pellet was vortexed into 1 ml chloroform methanol (2:1, v/v), incubated for 1 h at 37 °C, centrifuged, and the washing protocol was repeated once with chloroform/methanol (2:1, v/v) and then twice with acetone. The dried pellet was suspended in RiboPrinter sample buffer and frozen and thawed a few times; 30 µl of this suspension was analysed using a robotized RiboPrinter Microbial Characterization System (Qualicon) operating as described by Bruce (1996). In every batch of eight samples five molecular mass marker lanes were run also.

Phylogenetic analyses. The ae2 editor (Maidak *et al.*, 1994) was used to align the 16S rDNA sequence of the isolate MA112/96^T against the 16S rDNA sequences of *Mycobacterium* species available from the public databases. The method of Jukes & Cantor (1969) was used to calculate evolutionary distances. Phylogenetic dendrograms were generated using the various treeing algorithms contained in the PHYLIP package (Felsenstein, 1993). The phylogenetic dendrogram was reconstructed from evolutionary distances using the neighbour-joining method (Saitou & Nei, 1987).

Nucleotide sequence accession numbers. The strain designations and 16S rDNA sequence accession numbers of the *Mycobacterium* reference strains used in the phylogenetic analyses are as follows: *Mycobacterium abscessus* ATCC 19977^T, M29559; *Mycobacterium aichiense* ATCC 27280^T, X55598; *Mycobacterium aurum* ATCC 23366^T, X55595; *Mycobacterium austroafricanum* ATCC 33464^T, X93182; *Mycobacterium chitae* ATCC 19627^T, X55603; *Mycobacterium chlorophenolicum* DSM 43826^T, X79292; *Mycobacterium chubuense* ATCC 27278^T, X55596; *Mycobacterium diernhoferi* ATCC 19340^T, X55593; *Mycobacterium fallax* ATCC 35219^T, M29562; *Mycobacterium farcinogenes* DSM 43294, X55592; *Mycobacterium flavescens* ATCC 14474^T, X52932; *Mycobacterium fortuitum* ATCC 6841^T, X52933; *Mycobacterium gadium* ATCC 27726^T, X55594; *Mycobacterium gilvum* ATCC 43909^T, X55599; *Mycobacterium hodleri* EMI2^T, X93184; *Mycobacterium komossense* ATCC 33013^T, X55591; *Mycobacterium madagascariense* ATCC 49865^T, X55600; *Mycobacterium neoaurum* ATCC 25795^T, M29564; *Mycobacterium obuense* ATCC 27023^T, X55597; *Mycobacterium*

parafortuitum DSM 43528^T, X93183; *Mycobacterium senegalense* ATCC 35796^T, M29567; *Mycobacterium smegmatis* ATCC 14468, X52922; *Mycobacterium sphagni* ATCC 33026^T, X55590; *Mycobacterium vaccae* ATCC 15483^T, X55601; strain MA112/96^T DSM 44340^T, Y08857.

RESULTS

Morphological characteristics and growth requirements

The five mycobacterial isolates (MA112/96^T, MA113/96, MA142/96, MA166/96 and MA168/96) grew on TSBA agar as smooth, scotochromogenic colonies of saffron yellow colour. The cells were non-motile, rod-shaped without branching, non-spore-forming and without capsules. Electron microscopic examination (Fig. 1) showed that the cells were short rods or coccoid (varying between 0.4 and 0.5 µm in width, 0.6 and 1.4 µm in length) and exhibited a triple-layered structure of the cell wall that is characteristic of mycobacteria with an irregular electron-dense outer layer. Cells were frequently attached to one another.

Fig. 1. Thin section of cells of strain MA112/96^T. The perpendicular fashion of cell division of a rod-shaped cell is visible and the triple-layered structure of the cell wall: an irregular electron-dense outer layer, an electron-translucent layer and peptidoglycan layer. Cells were frequently attached to one another by the irregular outer layer. Cells were grown for 7 d on tryptic soy agar at 28 °C. Bar, 200 nm.

Table 2. Comparison of selected rapidly growing *Mycobacterium* species with five new isolates

Strains: 1, New isolates (MA112/96^T, MA113/96, MA142/96, MA166/96 and MA168/96); 2, *M. abscessus* NCTC 10882; 3, *M. aurum* NCTC 10437^T; 4, *M. austroafricanum* DSM 44191^T; 5, *M. diernhoferi* DSM 43524^T; 6, *M. gadium* NCTC 10942^T; 7, *M. hodleri* DSM 44183^T; 8, *M. komossense* ATCC 33013^T; 9, *M. neoaurum* NCTC 10818^T; 10, *M. vaccae* NCTC 10916^T. Pigmentation: N, non-chromogenic; P, photochromogenic; S, scotochromogenic.

Character	Strain									
	1	2	3	4	5	6	7	8	9	10
Colonies pigmented	S	N	S	S	N	S	S	S	S	P
Growth at:										
45 °C (5 d)	–	–	–	–	–	–	–	–	–	–
10 °C (10 d)	±	–	±	±	–	–	+	+	±	±
Growth on MacConkey agar without crystal violet	–	+	–	–	–	–	–	–	–	–
Enzymic activities:										
α-Glucosidase*	+	–	±	±	–	–	–	±	–	+
Arylsulfatase	+	+	–	–	–	–	+	–	–	–
Catalase at 22 °C†	+	+	+	+	–	–	+	–	–	+
Catalase at 68 °C†	±	–	+	+	+	+	+	±	+	+
Nitrate reductase	–	–	–	+	+	+	–	–	+	+
Tween 80 hydrolysis (10 d)	+	–	+	+	–	+	+	+	+	+
Urease	+	+	+	+	+	+	–	+	+	+
Resistance to:‡										
Isoniazid (1 µg ml ⁻¹)	–	+	–	–	–	–	+	–	+	–
Isoniazid (10 µg ml ⁻¹)	–	+	–	–	–	–	+	–	–	–
Hydroxylamine (500 µg ml ⁻¹)	–	–	–	–	–	–	–	–	–	–
NaCl (5 %)	–	+	–	–	–	–	–	–	+	–
Picrate (0.2 %)	+	+	–	+	+	–	+	–	+	–

* Activity of α-glucosidase [mmol h⁻¹ (OD₆₀₀ unit)⁻¹]; ±, 0.4; +, 0.7–1.4.

† Semiquantitative catalase: high catalase foam, > 45 mm; low catalase foam, < 45 mm.

‡ Tested in the presence of 0.4 % Tween 80 on Middlebrook 7H10 plates or medium described by Levy-Frebault & Portaels (1992) for the picrate-resistance test.

The optimum growth temperature for the isolates MA112/96^T and MA166/96 was 30 °C in TSB medium. On TSBA plates growth occurred within 2–4 d. The five mycobacterial isolates grew at 10 °C within 10 d. Of all the mycobacterial reference strains tested (listed in Table 1), only *M. komossense* ATCC 33013^T grew at 6 °C. The maximum growth temperature for the five new strains was 37 °C. The temperature requirements of the new strains were similar to those of the type strains of *M. aurum*, *M. austroafricanum*, *M. neoaurum* and *M. vaccae* (Table 2). All five isolates stained Gram-positive when young and were acid-alcohol-fast.

Identification with PCR

The PCR amplification of the gene of the secreted 32 kDa protein specific to mycobacteria gave positive results with both tested isolates, MA112/96^T and MA166/96.

Antimicrobial susceptibility

The susceptibility of the new isolates to antimicrobial agents used against clinically important mycobacteria are shown in Tables 2 and 3. As compared to the opportunistically pathogenic rapidly growing species *M. fortuitum*, MA112/96^T and MA166/96 were susceptible to all antimicrobials tested with E-test strips (Table 3), excepting the β-lactam antibiotics and also chloramphenicol for strain MA112/96^T. The new isolates were also sensitive to rifampin, streptomycin and ethambutol. When tested on plates (with 0.4 % Tween 80) the strains were also sensitive to isoniazid and hydroxylamine but resistant to picrate (Table 2).

Cell wall, fatty acids, respiratory quinones and G + C content

The diagnostic cell wall sugars were arabinose and galactose in isolate MA112/96^T. The cell wall contained *meso*-diaminopimelic acid as the only diamino

Table 3. MICs of selected antimicrobial agents for MA112/96^T, MA166/96 and reference strain *M. fortuitum* DSM 46621^T, as determined at 35 °C

Antimicrobial agent	MIC (µg ml ⁻¹) for:		
	MA112/96 ^T	MA166/96	<i>M. fortuitum</i>
Amikacin	0.38	0.19	1.0
Azithromycin	0.50	0.25	ND
Penicillin G	> 32	> 32	> 32
Ceftazidime	> 256	> 256	> 256
Chloramphenicol	12	0.75	> 256
Ciprofloxacin	< 0.002	< 0.002	0.023
Clarithromycin	0.064	< 0.016	12
Doxycycline	0.047	< 0.016	0.19
Vancomycin	0.38	0.19	32

ND, Not determined.

Table 4. Whole-cell fatty acid compositions of the strain MA112/96^T and selected type strains of genus *Mycobacterium* grown on Middlebrook 7H10 agar

Fatty acids: 1, C_{14:0}; 2, C_{15:0}; 3, C_{16:1} *cis*7; 4, C_{16:1} *cis*9; 5, C_{16:1} *cis*10; 6, C_{16:0}; 7, C_{17:1} *cis*9; 8, C_{18:2}; 9, C_{18:1} *cis*9; 10, C_{18:1} *cis*11; 11, C_{18:0}; 12, 10Me-C_{18:0}; 13, C_{20:0} alcohol; 14, C_{20:0}. The strains are listed in the order of the fatty acid similarity dendrogram as compared to strain MA112/96^T. Minor quantities (< 1%) of the following fatty acids were also found: C_{10:0}, C_{12:0}, 8Me-C_{16:0}, 10Me-C_{16:0}, C_{17:1} *cis*9 and C_{17:0} in some of the following strains MA112/96^T, *M. abscessus*, *M. komossense* and *M. vaccae*.

Strain	Fatty acid (percentage of total):													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
MA112/96 ^T	3.4	< 0.4	2.5	2.4	7.9	19.6	13.6	0.8	21.4	0.8	1.3	16.6	9.1	< 0.4
<i>M. aurum</i> NCTC 10437 ^T	3.8	< 0.4	1.5	1.1	7.6	22.4	15.4	< 0.4	20.0	< 0.4	1.1	13.6	13.5	< 0.4
<i>M. hodleri</i> DSM 44183 ^T	5.4	1.1	2.3	2.8	8.3	19.9	18.3	0.7	23.5	0.8	0.9	13.3	2.7	< 0.4
<i>M. gadium</i> NCTC 10942 ^T	3.7	< 0.4	3.6	3.6	3.7	21.9	11.2	1.1	29.4	0.9	2.7	10.6	6.8	0.7
<i>M. diernhoferi</i> DSM 43524 ^T	7.7	0.5	5.4	0.6	6.6	18.2	33.8	< 0.4	18.1	< 0.4	0.6	4.7	3.3	0.6
<i>M. vaccae</i> NCTC 10916 ^T	5.0	0.5	4.7	1.7	13.0	19.1	18.2	1.9	23.2	1.1	3.0	3.5	2.7	0.5
<i>M. komossense</i> ATCC 33013 ^T	7.8	0.2	2.1	2.6	8.9	23.7	17.4	0.6	22.2	0.4	2.1	8.4	2.4	0.3
<i>M. austroafricanum</i> DSM 44191 ^T	3.2	0.3	2.7	< 0.4	9.5	21.7	25.3	0.4	17.3	0.4	0.9	7.5	10.7	< 0.4
<i>M. neoaurum</i> NCTC 10818 ^T	4.8	< 0.4	3.6	2.6	8.6	23.6	18.4	< 0.4	32.4	< 0.4	1.6	2.0	1.2	1.3
<i>M. abscessus</i> NCTC 10882	6.0	0.3	1.8	4.7	10.4	34.1	< 0.4	0.6	35.5	0.6	1.0	3.9	< 0.4	< 0.4

acid. The whole-cell fatty acid patterns of strain MA112/96^T and selected reference strains are shown in Table 4. The main fatty acids of MA112/96^T were C_{18:1} *cis*9 (21%), C_{16:0} (20%), tuberculostearic acid 10Me-C_{18:0} (17%), C_{17:1} *cis*9 (14%) and C_{16:1} *cis*10 (8%). Also an alcohol of 20 carbon atom chain-length was a major component (9%). The quantity of tuberculostearic acid in strain MA112/96^T was high (17%) when grown at 28 °C and even higher (22%) when grown at 37 °C. The five new mycobacterial isolates had similar fatty acid profiles (data not shown), distinct from those of the other rapidly growing mycobacterial species (Table 4). The fatty acid composition of the new isolates was different from both *M. abscessus* and from *M. komossense* (Table 4). Of the

tested species (Fig. 2), *M. aurum* and *M. hodleri* had fatty acid profiles most similar to those of the new isolates, excepting for the lower amounts of tuberculostearic acid and C₂₀ alcohol which was low in *M. hodleri*. Of the total amount of menaquinones, strain MA112/96^T had 90% of MK-9(H₂) and 10% of MK-8(H₂). The G+C content of strain MA168/96 was 72.9 mol%.

Polar lipids

Two-dimensional TLC analysis showed that the isolates MA112/96^T, MA142/96 and MA166/96 had identical polar lipid patterns. Fig. 3 shows the polar lipids of MA112/96^T and those of *M. komossense*

Fig. 2. Euclidian distance dendrogram based on the whole-cell fatty acid compositions of the new mycobacterial isolate MA112/96^T and the type strains of the nine most closely related mycobacterial species.

Fig. 3. Two-dimensional thin-layer chromatographs of total lipids of (a) MA112/96^T and (b) *M. komossense* ATCC 33013^T. Abbreviations: DPG, diphosphatidylglycerol; GL, glycolipid; L, lipid; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIM, phosphatidylinositolmannoside; PL, unknown polar lipid.

Fig. 4. 16S rDNA sequence based phylogenetic dendrogram reconstructed from evolutionary distances using the neighbour-joining method (Jukes & Cantor, 1969). Scale bar, 1 inferred nucleotide substitution per 100 nucleotides.

ATCC 33013^T. Both displayed PE, PI, PIM and DPG as the major phospholipids. The presence of one unknown lipid (L3) in MA112/96^T (Fig. 3a) and three glycolipids (GL2, GL3 and GL4) in *M. komossense* (Fig. 3b) distinguished the two species.

Mycolic acids

The isolate MA112/96^T contained α -mycolic acids, keto-mycolates and wax esters (ω carboxy mycolates and eicosanol). The overall number of carbon atoms in mycolic acids was 60–90. The chain length of mycolic acids was *Mycobacterium* specific and the composition differentiated tested strains from *M. komossense*, which had also methoxy mycolic acids (Minnikin *et al.*, 1985). The same distribution of mycolic acids has been found in *M. aurum*, *M. austroafricanum*, *M. diernhoferi*, *M. gadium*, *M. hodleri* and *M. neoaurum* (Minnikin *et al.*, 1985; Kleespies *et al.*, 1996).

Polyamines

The analysis of the polyamine patterns of the reference species *M. komossense*, *M. aichiense* and *M. fortuitum* as well as the new isolates demonstrated that in mycobacteria the overall polyamine content is rather low [$\leq 0.05 \mu\text{mol (g dry wt)}^{-1}$]. Putrescine and spermidine were found in all strains and usually spermidine was a predominant compound in the polyamine patterns.

Table 5. Ability of the five new mycobacterial isolates and six type strains of closely related *Mycobacterium* species to degrade various substrates

Strains: 1, New isolates (MA112/96^T, MA113/96, MA142/96, MA166/96 and MA168/96); 2, *M. komossense* ATCC 33013^T; 3, *M. fortuitum* DSM 44621^T; 4, *M. austroafricanum* DSM 44191^T; 5, *M. vaccae* NCTC 10916^T; 6, *M. abscessus* NCTC 13031^T; 7, *M. aurum* NCTC 10437^T. +, All strains positive; —, all strains negative; numbers are percentages of positive isolates. pNP, *para*-nitrophenyl; pNA, *para*-nitroanilide. Acidification of sugars was in some cases weak and difficult to interpret. For this reason no detailed results are given here. All strains were able to utilize gluconate, D-glucose, propionate and fumarate as sole source of carbon and all strains hydrolysed L-alanine-pNA. None of the strains was able to utilize D-melibiose, citrate (in some cases a weak response was visible after 10 d incubation), mesaconate, L-aspartate, L-histidine, L-tryptophan, 3-hydroxybenzoate as sole source of carbon and none hydrolysed pNP- β -D-galactopyranoside, pNP- β -D-glucuronide, 2-deoxythymidine-5'-pNP-phosphate and L-glutamate- γ -3-carboxy-pNA.

Metabolic property	Strain						
	1	2	3	4	5	6	7
Assimilation of:							
N-Acetyl-D-glucosamine	40	+	—	—	—	+	—
L-Arabinose	+	—	—	+	+	—	+
p-Arbutin	20	—	—	—	—	—	—
D-Cellobiose	—	—	+	—	—	—	—
D-Fructose	40	—	—	+	+	—	+
D-Galactose	—	—	—	—	+	—	—
D-Mannose	+	—	—	+	+	+	+
D-Maltose	+	—	—	—	—	—	—
L-Rhamnose	+	—	+	—	+	—	—
D-Ribose	+	—	—	—	—	—	—
D-Sucrose	—	—	—	+	+	—	+
Salicin	—	—	—	—	—	+	—
D-Trehalose	+	—	+	+	+	+	+
D-Xylose	+	—	+	+	+	—	+
Adonitol	+	—	—	—	+	—	+
i-Inositol	+	—	+	+	+	—	+
Maltitol	+	—	—	—	—	—	—
D-Mannitol	+	—	+	+	+	—	+
D-Sorbitol	+	—	+	—	+	—	—
Putrescine	+	+	+	+	—	+	+
Acetate	40	+	+	+	+	+	+
cis-Aconitate	+	—	—	+	—	—	—
Adipate	20	—	—	—	+	—	—
4-Aminobutyrate	20	+	+	—	—	+	+
Azelate	—	—	—	+	—	—	+
Glutarate	+	—	—	+	+	—	+
DL-3-Hydroxybutyrate	+	+	—	+	+	+	+
Itaconate	+	—	—	—	—	—	—
DL-Lactate	+	—	—	+	+	—	+
L-Malate	80	+	+	—	+	—	—
Oxoglutarate	80	—	—	—	—	—	—
Pyruvate	+	+	+	+	+	—	+
Suberate	—	—	—	+	+	—	—
L-Alanine	+	—	—	—	+	—	—
β -Alanine	60	—	—	—	—	+	—
L-Leucine	+	+	+	—	—	+	+
L-Ornithine	80	—	+	+	+	+	—
L-Phenylalanine	20	—	—	—	—	—	—
L-Proline	+	—	—	—	+	+	—
L-Serine	—	—	—	—	—	+	—
4-Hydroxybenzoate	—	—	—	+	—	—	—
Phenylacetate	—	—	+	—	—	+	—
Hydrolysis of:							
Aesculin	—	—	—	—	—	+	—
pNP- α -D-glucopyranoside	20	—	—	+	+	+	+
pNP- β -D-glucopyranoside	80	+	+	—	+	+	—
bis-pNP-phosphate	+	—	—	+	+	—	+
pNP-phenyl-phosphonate	80	—	—	—	+	—	+
pNP-phosphoryl-choline	40	—	+	—	+	—	+
L-Proline-pNA	+	+	+	—	+	—	—

Fig. 5. Phenogram showing the relationships of the five new mycobacterial isolates as compared to the type strains of mycobacterial species most closely related by 16S rDNA.

16S rDNA sequence analysis

The almost complete 16S rDNA sequence of the isolate MA112/96^T (Y08857) comprising 1478 nucleotides [$> 95\%$ of the *Escherichia coli* sequence (Brosius *et al.*, 1978)] was used in this study. The phylogenetic dendrogram shown in Fig. 4 was reconstructed from evolutionary distances (Jukes & Cantor, 1969) by the neighbour-joining method (Saitou & Nei, 1987). A total of 1243 nucleotides present in all strains between positions 39 and 1376 (*E. coli* positions; Brosius *et al.*, 1978) were used for this analysis. The phylogenetic dendrogram (Fig. 4) shows isolate MA112/96^T to fall within the radiation of the genus *Mycobacterium*. Sequence of the 16S rDNA in the *Mycobacterium* specific hypervariable region (*E. coli* positions 129–260) of isolate MA166/96 was identical to that of MA112/96^T. Isolate MA112/96^T showed 16S rDNA

sequence similarity in the range 95.8–98.1% (*M. komossense*) to the species of the genus *Mycobacterium* shown in Fig. 4. Even though the overall highest sequence similarity of MA112/96^T was with *M. komossense* ATCC 33013^T; the closest species in the 16S rDNA dendrogram was *M. abscessus* ATCC 19977^T due to the treeing algorithm and how it calculates the alignment. In the 16S rDNA dendrogram the next closest species were *M. aurum*, *M. vaccae* and *M. austroafricanum*.

Physiological properties

The substrate utilization of the new isolates were compared to that of the type strains of the phylogenetically closest six species in Table 5. The new isolates used a broader spectrum of carbon sources for growth than the reference species. The ability to assimilate D-maltose, D-ribose, maltitol and itaconate differentiated the new isolates from type strains of *M. komossense*, *M. fortuitum*, *M. austroafricanum*, *M. vaccae*, *M. abscessus* and *M. aurum*. The physiological tests showed (Table 5, Fig. 5) that the new isolates form a homogeneous group distinct from the mycobacterial type strains listed above.

Scotochromogenic pigment formation separates the new isolates from *M. abscessus*, *M. diernhoferi* and *M. vaccae* (Table 2). The new isolates did not grow on MacConkey agar without crystal violet like *M. abscessus* did. Classical mycobacterial tests (Table 2) showed the new isolates to be most similar to *M. aurum* and *M. austroafricanum*, the next closest species were *M. komossense* and *M. vaccae* while *M. abscessus* differed in most tests.

Fig. 6. Ribotypes obtained after digestion with *EcoRI* or *PvuII* and hybridization of the *E. coli* *rrnB* operon of strains MA112/96^T, MA113/96, MA142/96, MA166/96, MA168/96 and of type strains of selected *Mycobacterium* species.

Ribotyping

The five new isolates and 12 most closely related mycobacterial species were ribotyped with two restriction enzymes and the results are shown in Fig. 6. *EcoRI* yielded ribotyping patterns with 4–7 bands in the size range 1–50 kb. *PvuII* yielded 2–5 bands of 3–40 kb from the different mycobacterial species. The new isolates showed four bands (2, 3, 15 and 40–50 kb) with *EcoRI* and two bands (7 and 12 kb) with *PvuII*. These patterns allow for a species recognition of the new isolates from the 12 closest species of mycobacteria (cf. Fig. 6 to Fig. 4). The 12–15 kb band but not the 7 kb *PvuII* band, typical of the new isolates, was also present in *M. abscessus*. 7 kb band, but not the *PvuII* 12–15 kb band, was present in *M. aurum* and *M. vaccae*, which were in the same 16S rDNA cluster with the new isolates (Fig. 4). *M. hodleri* differed in ribotype from all the other species. Based on these results, the new strains are concluded to represent a new species of *Mycobacterium*, to which the name *M. murale* is proposed.

DISCUSSION

In this paper we describe a new mycobacterial species, *M. murale*, with strain MA112/96^T proposed as the type strain. Phylogenetical and chemotaxonomic characterization, together with PCR amplification of a DNA fragment specific for a mycobacterial antigen (Soini *et al.*, 1992) showed that the five isolates, representing actinomycetes that colonize water-damaged building materials were mycobacteria. The characteristics of the new isolates which formed a homogeneous group fit the description of mesophilic, aerobic, rapidly growing, pigmented mycobacteria which stain weakly Gram-positive and are genus specifically acid–alcohol-fast. Mycobacteria were not found in non-water damaged parts of the same building materials (Andersson *et al.*, 1997).

The 16S rDNA sequence of strain MA112/96^T contains all the signature nucleotides defined for the family *Mycobacteriaceae* (Stackebrandt *et al.*, 1997). 16S rDNA sequence comparisons demonstrated the distinct lineage of strain MA112/96^T within the radiation of the genus *Mycobacterium*. This was confirmed by phylogenetic dendrogram and also in the range of 16S rDNA sequence similarity values to the closest relatives within this genus. The physiological and chemotaxonomic properties of strain MA112/96^T were compared to the nine most closely related mycobacterial species selected by 16S rDNA sequence data, up to 98.1% similarity to MA112/96^T. The 16S rDNA sequence data along with the morphological, physiological and chemotaxonomic data presented confirm the novel species status of this strain.

The results of the carbon substrate utilization tests were confirmed by the system used by (Tsukamura *et al.*, 1983; Tsukamura & Ichiyama, 1986) with some of the tested strains shared in both systems. *M. murale*

could use a much broader set of compounds as carbon sources for growth than *M. komossense* ATCC 33013^T or *M. abscessus* NCTC 13031^T.

MA112/96^T could be easily identified as a member of the genus *Mycobacterium* and differentiated from all other genera of the suborder *Corynebacterineae* by the occurrence of a multi spot mycolic acid pattern detected on TLC. MA112/96^T synthesized α -mycolates, keto-mycolates and the wax esters. This pattern is also present in the closely related species *M. aurum*, *M. vaccae* and *M. austroafricanum*, but here α' -mycolates are found in addition to the other three mycolates. Another member of this taxon, *M. abscessus*, is easily differentiated from the others by its simple mycolate pattern containing α -mycolates and α' -mycolates.

Previously, it has been shown that the polyamine content of the mycolic-acid-containing genera *Corynebacterium* and *Tsukamurella* is rather low and suitable for distinguishing members of these two genera from other coryneform genera (Altenburger *et al.*, 1997). The finding of extremely low polyamine concentrations in mycobacteria indicates that this feature is a characteristic of mycolic acid containing bacteria and might be useful for distinction from other groups. However, the polyamine content of the mycobacterial reference strains were found to be significantly lower than in *Corynebacterium*, *Tsukamurella* (Altenburger *et al.*, 1997) and *Gordonia* species (H.-J. Busse, unpublished data). The polyamine contents of the new mycobacterial isolates were in the same range as the reference strains of the genus *Mycobacterium*, indicating that these isolates were mycobacteria.

Some rapidly growing mycobacteria may cause infections that are difficult to treat due to the widespread antimicrobial resistance, including the specific antituberculosis drugs. The new *Mycobacterium* species described in this paper represents an antimicrobial susceptibility profile commonly encountered among apathogenic environmental mycobacteria. However, the presence of mycobacteria at high density (10^6 c.f.u. g⁻¹) in indoor building material may expose the occupants to mycobacterial antigens.

Because the number of environmental mycobacteria that may be opportunistic pathogens in certain circumstances is growing, there is a need for simple identification methods. Our results show that ribotyping together with whole-cell fatty acid analysis provides an option for two-step differentiation of closely related mycobacterial species. The whole-cell fatty acid composition of MA112/96^T is typical of mycobacteria, but contains larger amounts of tuberculostearic acid (17–22%) than its closest relatives. MA112/96^T contained large quantities of C_{17:1} *cis*9 and C_{20:0} alcohol unlike *M. abscessus* and also C_{16:0} and C_{18:1} *cis*9. This report on the use of ribotyping to distinguish related mycobacterial species, is to our knowledge the first one of its kind. Amplified rDNA restriction analysis (ARDRA) has

been used to differentiate several mycobacterial species by using two or three different restriction enzymes (Vanechoutte *et al.*, 1993). Although the total number of strains ribotyped is yet low, the results indicate that *EcoRI* and *PvuII* can be used to differentiate the tested 13 species. The slightly different ribotype patterns for MA142/96 and MA166/96 with *PvuII* and that of MA168/96 with *EcoRI* could be explained by a difference in restriction sites. *M. hodleri* DSM 44183^T displayed an *EcoRI* multiband ribotype with no similarity to any of the other mycobacterial species. Due to the unusual ribotype, the analysis of *M. hodleri* was repeated with a new culture retrieved from the DSMZ (German Collection of Microorganisms and Cell Cultures) with identical results. Although species with multiple different rRNA operons have been described (Mylvaganam & Dennis, 1992; Ninet *et al.*, 1996; Rainey *et al.*, 1996), in the sequencing of *M. hodleri* there was no evidence of operons with different sequence (Kleespies *et al.*, 1996). Fast-growing mycobacteria have two ribosomal operons while slow-growing species usually have one (Domenech *et al.*, 1994; Ji *et al.*, 1994). The single-band ribotype by *PvuII* for *M. obuense* (Fig. 5) indicates that this species may possess only one *rrn* operon as is the case for *M. abscessus* (Ninet *et al.*, 1996).

Description of *Mycobacterium murale* sp. nov.

Mycobacterium murale [mu.ra'le. L. adj. *muralis*, -le pertaining or belonging to wall(s)].

Gram-positive, aerobic, acid-alcohol-fast, non-spore forming, non-motile rods or coccoid cells without branching (0.4–0.5 µm in width, 0.6–1.4 µm in length). Smooth, scotochromogenic colonies of saffron yellow colour on TSBA agar. Cells have a triple-layered structure that is typical of mycobacteria visible by electron microscope. The temperature range for growth is 10–37 °C, optimum at 30 °C. The cell wall contains arabinose, galactose and *meso*-diaminopimelic acid. Contains α -mycolic acids, keto-mycolates and wax esters (ω carboxy mycolates and eicosanol). Mainly straight-chain saturated (C_{16:0}) and mono-unsaturated (C_{16:1} *cis*10, C_{17:1} *cis*9, C_{18:1} *cis*9) fatty acids, C_{20:0} alcohol and tuberculostearic acid (17% at 28 °C). The G+C content is 72.9 mol%. The main menaquinone is MK-9(H₂). Contains phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylinositol, phosphatidylinositolmannosides. Polyamine content is low. Yields two major bands, 7 and 12–15 kb, upon cleavage with *PvuII* and hybridization to the full ribosomal operon probe of *E. coli*. α -Glucosidase, arylsulfatase, catalase- and urease-positive. Hydrolyses Tween 80; nitrate-reductase-negative. Does not tolerate 5% NaCl or grow on MacConkey agar without crystal violet. Assimilates D-maltose, D-ribose, adonitol, i-inositol, maltitol, D-mannitol, D-sorbitol and itaconate but not D-sucrose, salicin, aesculin, D-melibiose, citrate, mesaconate, L-aspartate, L-histidine, L-tryptophan or 3-hydroxybenzoate. No

hydrolysis of pNP- β -D-galactopyranoside, pNP- β -D-glucuronide, 2-deoxythymidine-5'-pNP-phosphate and L-glutamate- γ -3-carboxy-pNA. Sensitive to amikacin, azithromycin, ciprofloxacin, clarithromycin, doxycycline, vancomycin, rifampin, streptomycin and ethambutol but resistant to isoniazid, penicillin G, ceftazidime and chloramphenicol. Isolated from water-damaged indoor building material, Finland. MA112/96^T is the type strain of the species and has been deposited in the DSMZ as DSM 44340^T.

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