Mycobacterium parmense sp. nov.

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The isolation and identification of a novel, slow-growing, scotochromogenic, mycobacterial species is reported. A strain, designated MUP 1182^T, was isolated from a cervical lymph node of a 3-year-old child. MUP 1182^T is alcohol- and acid-fast, with a lipid pattern that is consistent with those of species that belong to the genus *Mycobacterium*. It grows slowly at 25–37 °C, but does not grow at 42 °C. The isolate was revealed to be biochemically distinct from previously described mycobacterial species: it has urease and Tween hydrolysis activities and lacks nitrate reductase, 3-day arylsulfatase and β-glucosidase activities. Comparative 16S rDNA sequencing showed that isolate MUP 1182^T represents a novel, slow-growing species that is related closely to *Mycobacterium lentiflavum* and *Mycobacterium simiae*. On the basis of these findings, the name *Mycobacterium parmense* sp. nov. is proposed, with MUP 1182^T (=CIP 107385^T=DSM 44553^T) as the type strain.

In recent years, the role of *Mycobacterium scrofulaceum*, one of the non-tuberculous mycobacteria, as an aetiological agent of mycobacterial cervical lymphadenitis (scrofula) seems to have declined. An increasing number of cases caused by *Mycobacterium avium* has been reported, and other cases due to newly identified, scotochromogenic mycobacteria, such as *Mycobacterium celatum*, *Mycobacterium heidelbergense*, *Mycobacterium interjectum* and *Mycobacterium tusciae*, have been described (Springer *et al.*, 1993; Haase *et al.*, 1994; Wolinsky, 1995; Haas *et al.*, 1997; Howell *et al.*, 1997; Tortoli *et al.*, 1999).

In this study, the isolation of a slow-growing, scotochromogenic mycobacterium from material that was obtained surgically from a cervical lymph node from a 3-year-old girl is reported. The micro-organism proved to be unidentifiable by conventional biochemical tests that are specific for this group. Chromatographic and genetic analyses indicated that the isolate represents a novel species, which is named *Mycobacterium parmense* sp. nov. because of the place of its first isolation.

Isolate MUP 1182^T was recovered from a 3-year-old girl who presented in May 1999 with a local swelling of the left submandibular region that was ~4 cm in diameter and mildly painful to the touch. While under standard antibiotic therapy, other lymph nodes enlarged and the patient was hospitalized because of her bilateral cervical lymphadenitis. In August 1999, surgical exploration allowed the collection of colliquated material. The specimen was processed in accordance with standard procedures. Microscopic examination revealed the presence of acid-fast bacilli. Tests for *Mycobacterium tuberculosis* DNA (LCx MTB; Abbott) gave negative results. A slow-growing, scotochromogenic mycobacterium (MUP 1182^T) was recovered by using both conventional and radiometric culture procedures (Kent & Kubica, 1985; Siddiqi, 1996).

Therapy with a triple regimen that included isoniazid, rifampicin and pyrazinamide, the latter being changed

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The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of strain MUP 1182^T is AF466821.

Supplementary material showing the sequence alignment, fatty acid content and mycolic acid pattern of strain MUP 1182^T is available in IJSEM Online.

subsequently to clarithromycin, was unsuccessful. The mandibular lymph nodes were eventually removed by surgery. No relapses occurred thereafter.

Colony morphology, pigment production in the dark and after photoinduction and ability to grow at 25–45 °C were determined during 6 weeks incubation on Löwenstein–Jensen medium. Acid- and alcohol-fastness was determined by Ziehl–Neelsen staining.

Previously described methods were used to determine the standard biochemical reactions of the isolate (Kent & Kubica, 1985). The following biochemical features were investigated: niacin accumulation, nitrate reductase, arylsulfatase on days 3 and 14, drop-method catalase, semi-quantitative catalase, heat-stable catalase (pH 7, 68 °C), tellurite reductase, Tween 80 hydrolysis, β-glucosidase activity and urease activity. Inhibition tests included tolerance to isoniazid, hydroxylamine, *p*-nitro-α-acetylamino-β-hydroxypropiophenone, *p*-nitrobenzoate, oleate, 5 % (w/v) NaCl, thiacetazone, thiophene-2-carboxylic acid hydrazide and growth on MacConkey's agar without crystal violet.

The macrodilution method in radiometric broth, recommended for *M. avium* (Siddiqi *et al.*, 1993), was used to test the susceptibility of the isolate to ciprofloxacin, clofazimine, ethambutol, rifabutin, rifampicin and streptomycin.

The mycolic acids of whole-organism methanolysates were investigated by TLC as described by Minnikin *et al.* (1984), as well as by HPLC of bromo-phenacyl esters by using a C_{18} Ultrasphere XL cartridge column (Beckman) on a System Gold instrument (Beckman), according to standard procedures (Butler *et al.*, 1992; Tortoli & Bartoloni, 1996). Low- and high-molecular-mass internal standards (Ribi; ImmunoChem) were added for peak identification.

Fatty acid methyl esters, alcohols and mycolic acid cleavage products were obtained from 40 mg wet biomass of the isolate, which was saponified, methylated and extracted as described by Miller (1982). They were subsequently separated by GLC using a model 5898A gas chromatograph (Hewlett Packard). Microbial Identification system software (Microbial ID) was used to identify the fatty acids.

DNA was extracted from cells of the isolate by the alkaline-wash and heat-lysis method. Briefly, several small colonies were placed into a tube that contained 0·5 ml alkaline wash solution (0·5 M NaOH and 0·05 M sodium citrate). Tubes were vortexed and allowed to stand for 5 min at room temperature. The tubes were then centrifuged at 20 800 ***g*** for 5 min and the supernatant was discarded; 0·5 ml 0·5 M Tris/HCl, pH 8·0, was added and the tubes were vortexed again, then centrifuged at 20 800 ***g*** for 5 min. The supernatant was discarded, the pellet of cells was suspended in 100 µl RNase-free water and the tubes were held at 95 °C for 15 min in a heat block.

Amplification and sequencing of the first 500 bp of the 16S rRNA gene were carried out with the MicroSeq 500 16S rDNA Bacterial Sequencing kit (Applied Biosystems). The rest of the gene was sequenced by using the MicroSeq Full Gene 16S rDNA Bacterial Sequencing kit (Applied Biosystems). Sequencing analysis of the 16S rRNA gene was performed by using a MicroSeq sequencing module. Sequencing was performed in an ABI 3100 16 capillary genetic analyser (Applied Biosystems).

All sequence sample files were assembled and the final sequence was compared with those in a bacterial database containing 1434 entries, including 83 species of mycobacteria (version 1.4.2, February 2002). The Mayo Clinic library of nucleic acid sequences was composed of an additional 24 isolates, including different genotypes of common mycobacterial species (Hall *et al.*, 2003).

The newly determined sequence was aligned with reference 16S rDNA sequences from closely related mycobacterial species by using the computer program CLUSTAL W, version 1.81 (Thompson *et al.*, 1994). A phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987). The method was applied to distances corrected for multiple hits and for unequal transition and transversion rates, according to Kimura's two-parameter model (Kimura, 1980), omitting regions of uncertain alignment at both ends of the gene; tree topology was confirmed by parsimony analysis.

Microscopically, cells of strain MUP 1182^T were small, rod-shaped, acid- and alcohol-fast and non-motile; no spores or capsules were observed.

The isolate grew slowly (>2 weeks) on Löwenstein–Jensen slants at temperatures ranging from 25 to 37 °C, whereas no growth was seen at 42 or 45 °C. Colonies were small ($\leqslant 1$ mm), smooth, raised with round or lobate regular margins and scotochromogenic. Growth was not inhibited by isoniazid, *p*-nitrobenzoate, *p*-nitro-α-acetylamino-β-hydroxypropiophenone or thiophene-2-carboxylic acid hydrazide, but the micro-organism was sensitive to hydroxylamine, oleate, sodium chloride and thiacetazone. No growth was observed on MacConkey's agar without crystal violet.

Strain MUP 1182^T possessed urease activity and was able to hydrolyse Tween 80 in <5 days. Tests for niacin production, nitrate reduction, tellurite reduction and β-glucosidase activity were negative. Arylsulfatase activity was negative in the 3-day test and weakly positive after 14 days, catalase activity was rapid by the drop method and was not inactivated by heating to 68 °C, and foam production was <45 mm in the semi-quantitative test. Patterns of enzymic activities and metabolic properties demonstrated that the isolate differed from previously described species (Table 1). In particular, isolate MUP 1182^T was distinguishable from *M. tusciae* by a negative nitrate reductase test and from *Mycobacterium bohemicum* by a positive Tween 80 hydrolysis test. MUP 1182^T was characterized by a positive Tween 80 hydrolysis test, unlike

Table 1. Selected distinguishing characteristics of strain MUP 1182^T with respect to other slow-growing, scotochromogenic mycobacteria

Species: 1, *M. parmense* MUP 1182^T; 2, *M. bohemicum*; 3, *M. flavescens*; 4, *M. gordonae*; 5, *M. interjectum*; 6, *M. lentiflavum*; 7, *M. scrofulaceum*; 8, *M. szulgai*; 9, *M. tusciae*; 10, *M. xenopi*. Reactions are scored as follows: −, <15 % of isolates positive; +, >85 % of isolates positive; V, 16–85 % of isolates positive; +/−, usually absent, or a weak reaction was possible. Blank spaces indicate that the information is not currently available or that the property is unimportant. Data from other taxa were taken from Kent & Kubica (1985), Reischl *et al.* (1998), Springer *et al.* (1993, 1996) and Tortoli *et al.* (1999).

Characteristic	1	2	3	4	5	6	7	8	9	10
Nitrate reduction	−	−	+	−	−	−	−	+	+	−
Semi-quantitative catalase activity	−	−	+	+	+	V	+	+	−	−
Catalase activity (68 °C)	+	+	+	+	+	+	+	+	+	+
Tween 80 hydrolysis	+	−	+	+	−	−	−	V	+	−
Arylsulfatase activity (3 days)	−	−	−	−	−	−	−	−	−	V
Urease activity	+	+/−	V	−	+	−	+	+	+	−
NaCl tolerance	−	−	+	−			−	−	−	−
Growth at:										
25 °C	+	+	+	+	−	+	+	+	+	−
45 °C	−	−	−	−	−	−	−	−	−	+

M. lentiflavum, *M. scrofulaceum* and *Mycobacterium xenopi*, and by urease activity, unlike *Mycobacterium flavescens*, *Mycobacterium gordonae* and *M. xenopi*.

Strain MUP 1182^T proved to be susceptible to all drugs tested, with the exception of isoniazid. MICs (μg ml^{-1}) were as follows: ciprofloxacin, ⩽1; clofazimine, ⩽0·12; ethambutol, ⩽2; rifabutin, ⩽0·12; rifampicin, ⩽0·5; streptomycin, ⩽2.

Mycolic acid analysis by TLC showed a pattern that was reminiscent of those of other slow-growing mycobacteria, such as *M. bohemicum*, *M. interjectum*, *M. scrofulaceum*, *M. tusciae* and *M. xenopi*. Strain MUP 1182^T contained α-mycolates, ketomycolates and wax esters. Fatty acid analysis revealed a typical mycobacterial pattern that was characterized by the presence of tuberculostearic acid (10-methyl $C_{18:0}$) and saturated and unsaturated elements without side chains. The presence of eicosanol, derived from wax cleavage, was also detected.

Analysis of mycolic acids by HPLC showed a profile that was characterized by two groups of peaks and was not similar in any significant way to that of any mycobacterial species described to date (Fig. 1).

Sequencing of the PCR-amplified 16S rDNA of strain MUP 1182^T (1533 bp) yielded a unique, as yet undescribed sequence for a mycobacterial species. In contrast to growth behaviour in culture, the sequence pattern was characterized by the occurrence of a short helix 18 within the hypervariable B region of the 16S rRNA gene, which is a molecular signature of fast-growing mycobacteria. These features are shared by a group of micro-organisms that includes *M. simiae* and other emerging mycobacterial species (Böttger *et al.*, 1993; Kirschner *et al.*, 1993; Meier *et al.*, 1993; Springer *et al.*, 1993, 1996; Floyd *et al.*, 1996, 2000; Haas *et al.*, 1997; Tortoli *et al.*, 1997; Herbst *et al.*, 2001).

Phylogenetic analysis grouped the novel species close to *Mycobacterium genavense*, *M. heidelbergense*, *M. interjectum*, *Mycobacterium kubicae*, *M. lentiflavum*, *M. simiae*, *Mycobacterium triplex* and *Mycobacterium montefiorense* (GenBank accession no. AF330038). In particular, isolate MUP 1182^T clusters with *M. lentiflavum* and *M. simiae*, with which it shares high 16S rDNA sequence similarity (about 98 %). A phylogenetic tree showing the position of the novel species with respect to closely related mycobacterial species is shown in Fig. 2.

Cervical lymphadenitis (scrofula) represents the most

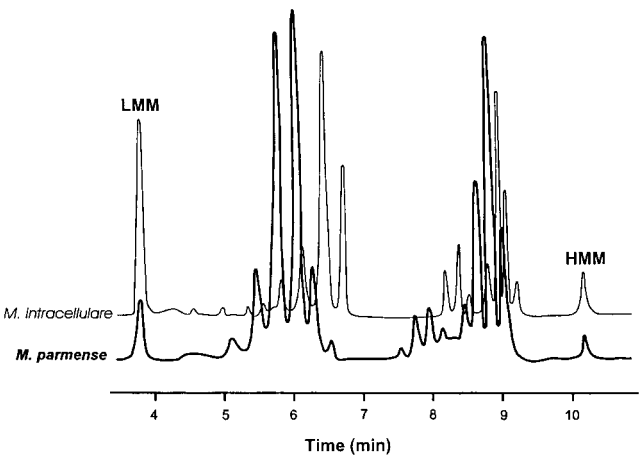

Fig. 1. Mycolic acid pattern of strain MUP 1182^T, obtained by HPLC analysis, compared with that of *Mycobacterium intracellulare*. LMM, Low-molecular-mass internal standard; HMM, high-molecular-mass internal standard.

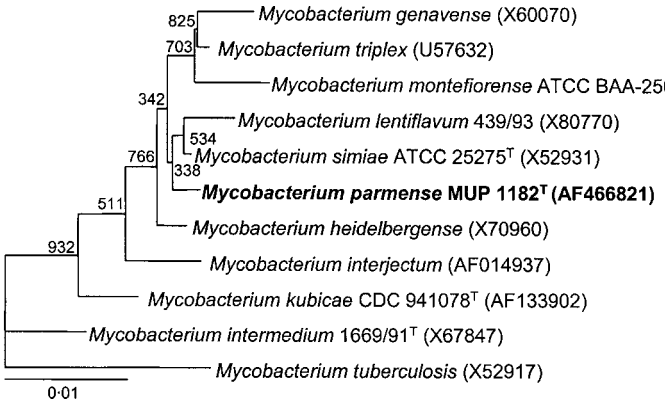

Fig. 2. Phylogenetic tree constructed by using the neighbour-joining method, illustrating the position of *M. parmense*. The sequence of *M. tuberculosis* was used as the outgroup. Bootstrap values for each node are reported. GenBank accession numbers for sequences used to construct the tree are shown in parentheses.

common manifestation of infection with non-tuberculous mycobacteria in childhood. Isolation of a mycobacterial strain from surgically obtained material from a sterile body site strongly suggests a pathogenic role for such micro-organisms (Wallace *et al.*, 1990). Isolate MUP 1182^T, described in this study, perfectly meets the above-mentioned conditions, particularly in view of the fact that microscopy of the clinical specimen revealed acid-fast bacilli.

Strain MUP 1182^T was presumptively identified as a *Mycobacterium* species by acid-fastness and morphological and growth characteristics, and its identification was confirmed by lipid analysis and comparative 16S rRNA gene sequencing.

Overall, the combined results of nucleic acid analyses, biochemical tests and lipid analysis indicate that the strain described in this study is representative of a novel species in the genus *Mycobacterium*, for which the name *Mycobacterium parmense* sp. nov. is proposed.

Description of *Mycobacterium parmense* sp. nov.

Mycobacterium parmense (par.men′se. L. neut. adj. *parmense* pertaining to the Italian city of Parma, where the strain was isolated).

Rod-shaped cells; acid- and alcohol-fast. Growth requires >2 weeks at 25–37 °C. No growth occurs at 42 °C. Colonies on Löwenstein–Jensen medium are smooth, raised with round or lobate regular margins and scotochromogenic. The organism produces <45 mm foam in the semi-quantitative catalase test. Positive for heat-stable catalase, Tween hydrolysis and urease activity. Reactions for nitrate and tellurite reduction, arylsulfatase (3-day test) and β-glucosidase activities are negative. Growth is inhibited on MacConkey's agar without crystal violet and by addition to the culture medium of 5 % (w/v) NaCl, hydroxylamine, thiacetazone or oleate. No inhibition is observed in the presence of *p*-nitrobenzoate, thiophene-2-carboxylic acid hydrazide or *p*-nitro-α-acetylamino-β-hydroxypropiophenone. Susceptible to ciprofloxacin, clofazimine, ethambutol, rifabutin, rifampicin and streptomycin. Resistant to isoniazid. TLC of mycolic acid methanolysates indicates the presence of α-mycolates, ketomycolates and wax esters. The unique HPLC profile is supportive of a novel species. The fatty acid pattern is characterized by hexadecanoic acid (25 %), hexadecenoic acids (13 %), octadecanoic acid (3 %), octadecenoic acids (24 %) and tuberculostearic acid (7 %). Two alcohols, $C_{18:0}$ (8 %) and $C_{20:0}$ (10 %), are also present. Phylogenetic analysis based on the 16S rRNA gene sequence reveals a unique profile; the species was placed in an intermediate position between slow- and fast-growing mycobacteria.

The type strain of *M. parmense* is MUP 1182^T (=CIP 107385^T=DSM 44553^T).

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