Mycobacterium palustre sp. nov., a potentially pathogenic, slowly growing mycobacterium isolated from clinical and veterinary specimens and from Finnish stream waters

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Taxonomic studies were performed on a phenotypically homogeneous group of 13 mycobacteria isolated from clinical, veterinary and stream-water samples. The methods applied included chromatographic analyses of bacterial lipids, biochemical tests and sequencing of the 16S rDNA and the internal transcribed spacer 1 (ITS1) region. Positive results in urease, Tween 80 hydrolysis and pyrazinamidase tests and a negative result in a semi-quantitative catalase test, combined with the ability to grow at 42 °C, distinguished this group among the yellow-pigmented, slowly growing mycobacteria. Unique fatty acid and mycolic acid profiles in chromatographic analyses and the results of gene sequencing indicated that the novel isolates represent a previously undescribed species, for which the name *Mycobacterium palustre* sp. nov. is proposed. The fatty acid profile obtained by GLC was characterized by the presence of several methyl-branched fatty acid markers. The most prominent markers were 2-methyleicosanoic, tetracosanoic and hexacosanoic acids. According to 16S rDNA sequencing, *M. palustre* is phylogenetically closest to *Mycobacterium kubicae*, a recently described species. *M. palustre* gives a false-positive result in a hybridization test with the AccuProbe *Mycobacterium avium* complex. One of the strains was isolated from a lymph-node biopsy from a child with cervical lymphadenitis. Thus, *M. palustre* should be listed among potential inducers of paediatric lymphadenitis. The veterinary isolates originated from the lymph nodes of slaughter pigs. The majority of the strains were recovered from natural waters, which highlights the role of the environment as a source of potentially pathogenic mycobacteria. The type strain of *M. palustre* is strain E846[T] (= DSM 44572[T] = ATCC BAA-377[T]).

Keywords: *Mycobacterium palustre* sp. nov., identification, taxonomy, GLC, HPLC

INTRODUCTION

In an environmental study on mycobacteria in Finnish stream waters (Iivanainen *et al*., 1993), we recovered eight isolates with a unique combination of phenotypic

properties, including identical GLC profiles of fatty acids and mycolic acid cleavage products (MACPs). Similar GLC profiles were also recognized among our collections of clinical and veterinary isolates that had remained without definite species identification. These comprised four isolates from Finland and one from Italy (Tortoli *et al*., 1997). The polyphasic characterization of these isolates is reported in this paper. The isolates formed a distinct group most closely related to a recently described species, *Mycobacterium kubicae* (Floyd *et al*., 2000). Several genetic and

chemotaxonomic differences distinguished this group from all known mycobacterial species, including *M. kubicae*. On the basis of the results described, this group of 13 isolates is considered to represent a novel species of the genus *Mycobacterium*, for which we propose the name *Mycobacterium palustre* sp. nov.

METHODS

Bacterial strains. Thirteen isolates were included in the study. Eight isolates (E58, E168, E171, E340, E520C, E712, E832 and E846[T]) were cultured from water samples from four Finnish streams, as described previously in detail (Iivanainen *et al.*, 1993). They were recovered after decontamination with 2 M H_2SO_4 + cycloheximide. No similar colonies were detected after decontamination with 4% NaOH and 5% oxalic acid (Iivanainen *et al.*, 1993). Six of the eight isolates were recovered in egg-based media at pH 5·5. Two Finnish clinical isolates (HO 036/91 and 3739-1/99) were grown from sputum specimens, one of them exclusively in the MGIT 960 system (BD Biosciences) in the primary isolation (Katila *et al.*, 2000). An Italian clinical strain (Fi-16795) was recovered from a lymph-node biopsy from a 4-year-old girl with swelling in the right submandibular region. She presented with a slightly aching lymph node. The infection progressed rapidly, manifesting as colliquation of the lymph node within a week, and the appearance of another one. Her tuberculin skin test (Mantoux) was positive but, otherwise, haematological and other clinical laboratory findings were within the normal range. Microscopy of the biopsy specimen of the lymph node revealed acid-fast bacilli, and the culture grew mycobacteria in both solid and liquid media (Bactec 12B; Becton Dickinson) at 4 and 2 weeks, respectively. Despite treatment with various antibiotics, also including isoniazid and rifampicin at a later stage, the lesion fistulated, secreting a yellowish, purulent discharge. The lymph node conglomerate was removed surgically and the child recovered without relapse. Two Finnish veterinary isolates (629 and 4942) were grown from specimens of submandibular lymph nodes from finishing pigs sampled at a normal meat inspection in a slaughterhouse in 1996. The reference strains used in this study were *Mycobacterium interjectum* ATCC 51457[T] and *Mycobacterium intermedium* DSM 44049[T] for GLC analyses and *Mycobacterium intracellulare* ATCC 13950[T] and *M. kubicae* ATCC 700732[T] for HPLC analyses.

Lipid analyses. For the present study, all isolates were grown on Middlebrook 7H11 agar (Difco). GLC of fatty acids, fatty alcohols and MACPs was performed as described previously in detail (Torkko *et al.*, 1998). For identification of fatty acid methyl esters and MACPs, GLC-MS analyses were performed as described previously (Torkko *et al.*, 1998). Trimethylsilyl derivatives of alcohols were prepared as described elsewhere (Torkko *et al.*, 1998) and double bonds were hydrated using a method published previously (Suutari & Laakso, 1993).

Mycolic acids were saponified and extracted from whole cells and derivatized to bromophenacyl esters as reported previously (Butler *et al.*, 1992; Tortoli & Bartoloni, 1996). HPLC was performed using a reverse-phase C_{18} Ultrasphere-XL cartridge column (Beckman) on an HPLC instrument (System Gold model; Beckman).

Conventional tests. The isolates were investigated for the following properties, as described previously (Torkko *et al.*, 1998, 2000): nitrate reduction, production of arylsulfatase (10 days), pyrazinamidase, semi-quantitative catalase and urease and hydrolysis of Tween 80. The production of alkaline phosphatase, acid phosphatase, α-galactosidase and β-galactosidase was determined using the API ZYM system (bioMérieux) as recommended by the manufacturer, but using overnight incubation at 36 °C as reported by Tortoli *et al.* (1996). Testing for the temperature range for growth was performed on egg-based media (Vincent Lévy-Frébault & Portaels, 1992).

All isolates were tested with a commercial DNA probe (AccuProbe; GenProbe) specific for the *Mycobacterium avium* complex and for the species *M. avium* and *M. intracellulare*. Furthermore, five isolates were also tested with another commercial probe system (INNO LiPA Myco-bacterium; Innogenetics). In both cases, the instructions of the manufacturers were followed.

16S rDNA sequencing and phylogenetic analysis. Amplification of the complete 16S rRNA gene and sequencing of the amplified DNA fragments of isolates E171, E520C, E712 and E846[T] were performed as described previously (Koukila-Kähkölä *et al.*, 1995). The other isolates were sequenced, the hypervariable regions A and B of the 16S rRNA gene being partially covered. A phylogenetic tree was constructed by using methods described in detail previously (Torkko *et al.*, 2000).

Internal transcribed spacer (ITS) region sequencing. All isolates were amplified and the amplification products were sequenced for the ITS1 region between the 16S and 23S rDNAs as described in detail previously (Torkko *et al.*, 2000).

Environmental analyses. Characteristics of the drainage areas and the chemical, physical and microbiological quality of the natural water reservoirs of the environmental isolates in the target group were analysed as described in detail previously (Iivanainen *et al.*, 1993).

RESULTS AND DISCUSSION

Lipid analyses

GLC fatty acid analysis of the novel isolates revealed a unique pattern. In addition to fatty acids regarded as typical of mycobacteria (Jantzen *et al.*, 1989), four different methyl-branched fatty acids were detected. These included 2-methyleicosanoic acid (2-Me-20:0), which was the most prominent, 2-methyldecanoic acid (2-Me-10:0), 2-methylnonadecanoic acid (2-Me-19:0) and 2,9-dimethyleicosanoic acid (2,9-diMe-20:0) (Fig. 1). Additional minor, but constant, markers, which could be used in separation of this species, included 14-phenyltetradecanoate (14-phe-14:0) and 2-methyl-16-phenylhexadecanoate (2-Me-16-phe-16:0), respectively with mass ions at *m/z* 318 and 360 and relative retention times of 1·81 and 2·04 (tetradecanoate = 1·0). The mass spectra of these previously undescribed compounds among the MACPs contained ions *m/z* 91 and 104, respectively originating from fragments phenyl–CH_2– and phenyl–CH_2–CH–. Phenyl groups have previously been found as structural components of mycobacteria, though unrelated to MACPs (Brennan & Nikaido, 1995; Barry *et al.*, 1998). In addition, the compound 2-methyl-16-phenylhexadecanoate contained fragments *m/z* 88 and 101,
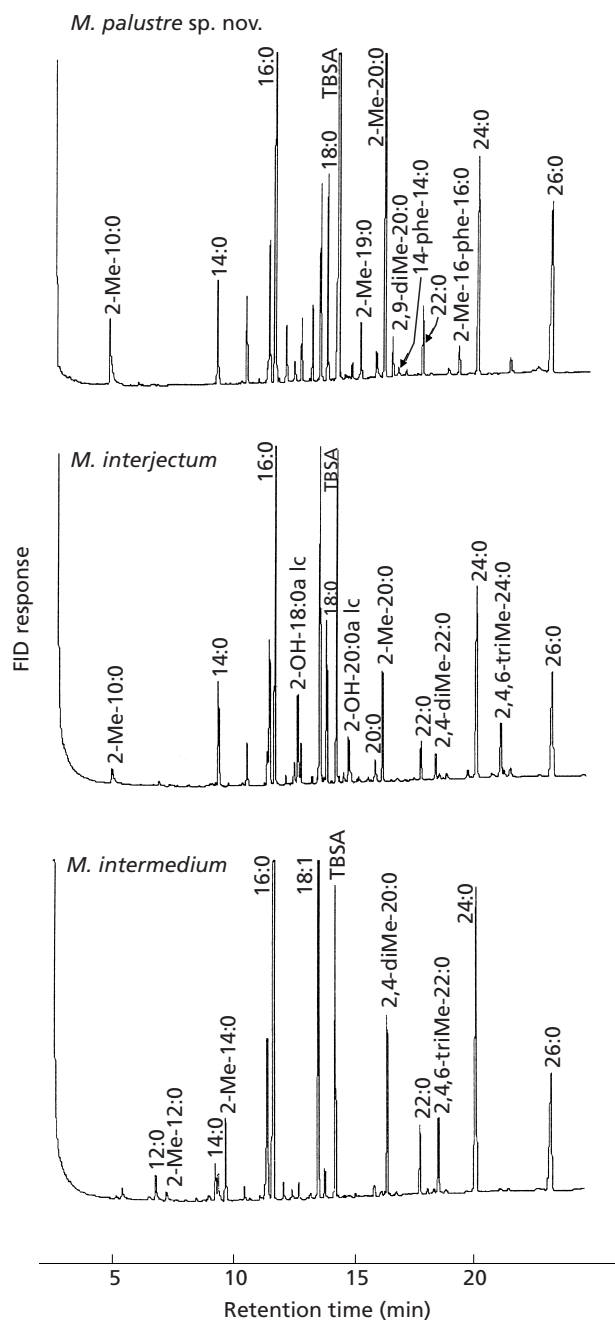
Fig. 1. GLC profiles of *M. palustre* sp. nov., *M. interjectum* and *M. intermedium*. For definitions of marker designations, see Results and Discussion. FID, Flame ionization detector; TBSA, tuberculostearic acid.

Fig. 2. Mycolic acid patterns of *M. palustre* sp. nov., *M. intracellulare* and *M. kubicae* obtained by HPLC analyses. LMMIS, Low-molecular-mass internal standard; HMMIS, high-molecular-mass internal standard.

known to be typical fragments of 2-methyl-branched fatty acid methyl esters (Julák *et al.*, 1980). In addition to these branched-chain fatty acids, both tetracosanoic (24:0) and hexacosanoic acids (26:0) were present.

Fig. 1 presents the GLC profiles of strain E846[T] and two other yellow-pigmented mycobacteria, *M. intermedium* and *M. interjectum*, which biochemically resemble the group of strains studied. In addition to
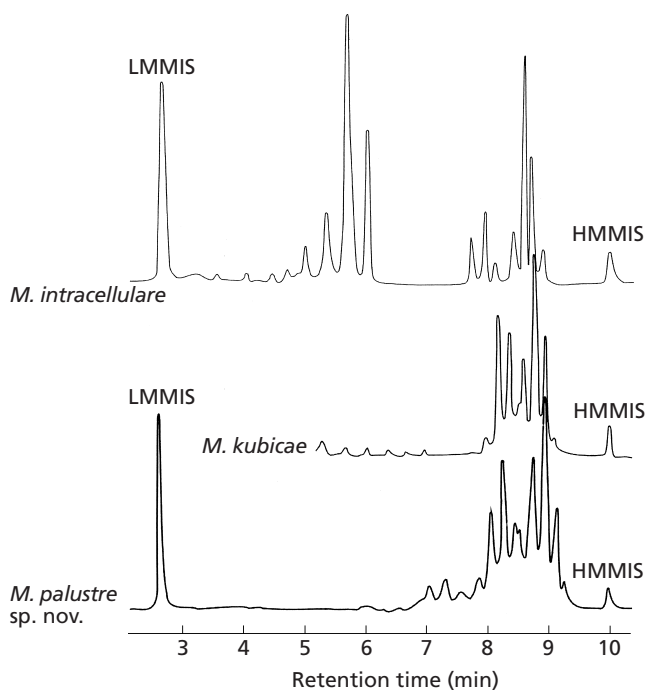
containing MACPs 24:0 and 26:0, these two mycobacterial species are also rich in methyl-branched fatty acids. However, each of these three species has a unique pattern of branched-chain fatty acid markers, which enables their separation on the basis of GLC profiles.

In HPLC analysis of mycolic acids, the profile of the isolates studied showed a single cluster of peaks similar to that present in *Mycobacterium asiaticum* and *M. kubicae* (Floyd *et al.*, 2000) (Fig. 2). However, differences in peak-heights and the presence of an additional late-emerging peak allowed their differentiation.

16S rDNA and ITS region sequencing and phylogenetic analysis

The 13 pigmented isolates comprising the group studied had identical 16S rDNA and ITS1 region sequences (Fig. 3). A similar 16S rDNA sequence, from an unidentified, non-pigmented isolate, was previously submitted to GenBank (*Mycobacterium* sp. IWGMT 90210; Wayne *et al.*, 1996). A difference of two nucleotides was detected between this sequence and the 16S rDNA of our isolates. Strain IWGMT 90210 differed from the novel isolates in the absence of pigment production and growth at 45 °C (Wayne *et al.*, 1996). This isolate was unavailable for comparative analyses.

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M. tuberculosis taxon 1773   AAGGAGCACC ACGA-AAA-C GCCCCAACTG GTGGGGCGTA GGCCGTGAGG GGTTCTTGTC    58
M. palustre sp.nov. E846ᵀ    .......... ....G.. A......... ......T.C. .......... ....CC.C..    58
M. avium taxon 1764          .......... .......G. A......... ......T.CG A......... ....CC....    59
M. malmoense taxon 1780      .......... .......G. .......... ......T.C. A......... ......C.C.    59
M. simiae taxon 1784         .......... ....G.... A.T....T.. ......T..G A......... ......C...    59
M. triplex taxon 47839       .......... ....G.... A.T....T.. ......T..G A......... ......C...    59


M. tuberculosis taxon 1773   TGTAGTGGGC GAGAGCCGGG TGCATGACAA CA----AAGT TGGCCACCAA CACACTGTTG   114
M. palustre sp.nov. E846ᵀ    .......... .G.G...... ...G----... ..GCA...CG AA....G--. ......A...   112
M. avium taxon 1764          ........A. .G.G...... ...G----... ..GCAA.T.A .T....G--. ......A...   114
M. malmoense taxon 1780      .......... .G.G...... ...G----... ..GCAA.T.A .T....G--. ......A...   114
M. simiae taxon 1784         ........A. ...G...... ....CA.... ..G..GCAA .C....G--. ......A...   114
M. triplex taxon 47839       ........A. ...G...... ....CA.... ..G..GCAA .C....G--. ......A...   114


M. tuberculosis taxon 1773   GGTCCTGAGG CAACACTCGG ---ACTT--- -GTTCCAGGT GTTGTCCCAC CGCCTTGGTG   167
M. palustre sp.nov. E846ᵀ    ..C....... .......... ....TCG... ..CATG..TG .G...T.C. .AT.......   165
M. avium taxon 1764          ..C.....A .......... ...TC.G... .CCGTGT..A .----...T. .AT.......   165
M. malmoense taxon 1780      ..C.....A .......... ...CCAG... ..CCG.GT... .----...C. .AT.......   165
M. simiae taxon 1784         ..C.....A .......... ...CCG....CGG TTGAAGT... .----...T. .AT.......   171
M. triplex taxon 47839       ..C.....A .......... ...CCGG..GAGG TCGATGT... .----...T. .AT.......   171


M. tuberculosis taxon 1773   GTGGGGTGTG GTGTTTGAGA ACTGGATAGT GGTTGCGAGC   207
M. palustre sp.nov. E846ᵀ    .......... .......... .......... ..........   205
M. avium taxon 1764          .......... .......T .T......... ..........   205
M. malmoense taxon 1780      .......... .......T .T......... ..........   205
M. simiae taxon 1784         .......... .......T .T......... ..........   211
M. triplex taxon 47839       .......... .......C .T..A..... ..........   211
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Fig. 3. Alignment of selected ITS1 sequences of mycobacteria, including *M. palustre* sp. nov. The reference sequence is that of *Mycobacterium tuberculosis*. The length of the ITS1 is indicated (in nt) from the beginning of the region. Dots indicate identity; dashes represent alignment gaps.
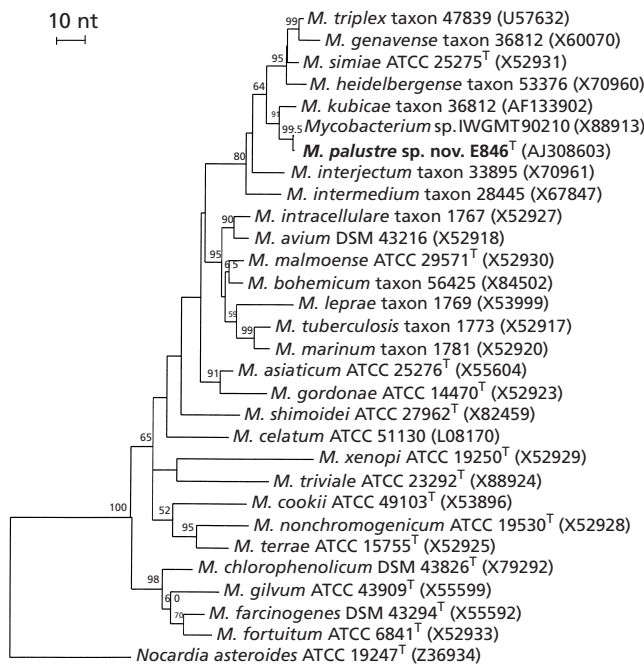

Fig. 4. Phylogenetic tree based on 16S rDNA sequences showing the relationships of *M. palustre* sp. nov. and selected *Mycobacterium* species. This tree was constructed using the neighbour-joining method. The tree was rooted by using *Nocardia asteroides* as the outgroup. Sequence accession numbers are given in parentheses. Bar, 10 nt difference.

In a phylogenetic tree (Fig. 4) based on evaluation of 16S rDNA sequences, the novel group is located among the slowly growing mycobacteria, being most closely related to *M. kubicae*.

Cultural, biochemical and hybridization properties

Colonies were smooth and yellow to pale yellow. Isolates grew as scotochromogenic colonies at 36 °C, but 11 of 13 were photochromogenic at 42 °C. Mature colonies were observed on egg media from dilute inocula after 4–5 weeks incubation at the optimum temperature of 36 °C. Colonies were larger on Middlebrook agar than on egg-based media, and reached a colony size of 1–1·5 mm in diameter in 4 weeks. The results of biochemical testing of the examined group and three other slowly growing species, all of which grow at 42 °C, are presented in Table 1.

All clinical, veterinary and environmental isolates hybridized with *M. avium* complex AccuProbe but not with the species-specific probes for *M. avium* and *M. intracellulare*. In contrast, no hybridization was obtained with LiPA DNA probes, either with the probe for the *M. avium–intracellulare–scrofulaceum* group or with any of the probes specific for single species (Tortoli *et al.*, 2001). The different genomic regions involved in these tests could explain this difference: AccuProbe is based on the 16S rDNA and LiPA on the ITS region.

Environmental data

Natural water, especially in peaty regions, appears to be a rich source of mycobacteria in Finland (Iivanainen *et al.*, 1993). Such water was also found to be a possible reservoir of the newly described potential pathogen, which was isolated from four of the 53 streams analysed in our initial environmental survey. The sampling sites were located within a distance of approx. 350 km. The four water samples were acidic (between pH 5·6 and 5·9). Seven of the eight isolates were recovered from three drainage areas widely covered by peatlands (60–75 % of the area). These water samples were rich in mycobacteria (800–2200 c.f.u. l⁻¹) and all had a high content of organic matter, as indicated by chemical oxygen demands of 80–140 mg KMnO₄ l⁻¹ and colour values of 160–600 mg Pt l⁻¹. In contrast, one isolate was recovered from a drainage area with 20 % peatland coverage. This water also had lower contents of organic matter (chemical oxygen demand of 65 mg KMnO₄ l⁻¹ and colour value of 180 mg Pt l⁻¹) and mycobacteria (320 c.f.u. l⁻¹).

Table 1. Comparison of cultural and biochemical characteristics that help in the differentiation of *M. palustre* sp. nov., *M. avium*, *M. xenopi* and *M. bohemicum*

Values are percentages of strains that tested positive. Data for *M. avium* and *M. xenopi* were taken from Wayne *et al.* (1991). Data for *M. bohemicum* were modified from Torkko *et al.* (2001). Growth of *M. bohemicum* at 22 °C was calculated from the results for the five environmental isolates in that study. ND, Not determined.

Characteristic	*M. palustre*	*M. avium*	*M. xenopi*	*M. bohemicum*
Pigmentation	Yellow	None*	Yellow	Yellow
Scotochromogenic	100†	8	86	100
Photochromogenic	85†	1	1	0
Tween 80 hydrolysis	100	1	1	20
Semi-quantitative catalase (45 mm foam)	0	8	1	100
Urease	100	1	1	93
Arylsulfatase (10 days)	46	29	99	87
Nitrate reduction	54	1	1	60
Pyrazinamidase	100	50	50	27
Acid phosphatase	100	1	1	ND
Alkaline phosphatase	85	ND	ND	ND
α-Galactosidase	0	ND	ND	ND
β-Galactosidase	0	1	1	ND
Growth at:				
22 °C	0	99‡	1‡	60
42 °C	100	92	99	100
45 °C	23§	50	99	0

*Usually non-pigmented, but may develop yellow pigment.

†Scotochromogenic at 36 °C; 85 % of isolates photochromogenic at 42 °C.

‡Results for growth at 25 °C.

§All isolates grew from heavy inocula on Middlebrook agar at 45 °C in 6 weeks.

Clinical significance of human and veterinary isolates

One of the clinical isolates was regarded as an unquestionable inducer of mycobacterial lymphadenitis in an Italian child. Thus, the proposed novel species should be included among potential inducers of paediatric cervical lymphadenitis (Wolinsky, 1995; Springer *et al.*, 1993; Tortoli *et al.*, 1999, 2000). On the basis of the available information, the clinical significance of the other clinical strains could not be verified. One was co-isolated with *Mycobacterium bohemicum* from a single sputum specimen from an elderly patient; the other was isolated from another elderly patient, lost to follow-up.

Known veterinary mycobacterial pathogens, such as the *M. avium* complex and *Mycobacterium xenopi*, prefer higher temperatures for growth than most non-pathogenic species. The broad range of growth temperatures of the isolates studied may indicate enhanced ability to colonize warm-blooded animals such as pigs. In our cases, these veterinary isolates were recovered from abnormal lymph nodes taken at meat inspections of otherwise healthy slaughter pigs. During the short lifetime of slaughter pigs, infections due to slowly growing mycobacteria are not likely to be manifested as more severe disease forms. Thus, the role of this novel species as an inducer of true porcine disease remains to be evaluated.

An interesting co-isolation with *M. bohemicum*, another recently described species (Reischl *et al.*, 1998), occurred not only from one clinical specimen but also from one environmental sample (E846[T]; formerly numbered E170 and E170-1) (Torkko *et al.*, 2001). These two rare, yellow-pigmented species differ in colony appearance, and they can be separated easily by fatty acid, mycolic acid or biochemical analyses.

Differentiation of *M. palustre* sp. nov. from other slowly growing, pigmented mycobacteria

M. palustre sp. nov. is a yellow-pigmented species that hydrolyses Tween 80, splits urea and produces less than 45 mm foam in the semi-quantitative catalase test. It grows at temperatures in the range 30–42 °C. This pattern of test results is unique among pigmented, slowly growing mycobacteria. Other pigmented, slowly growing mycobacteria that grow at 42 °C include the three related species *M. xenopi*, *Mycobacterium botniense* (Torkko *et al.*, 2000) and *Mycobacterium heckeshornense* (Roth *et al.*, 2000) and most

isolates of *M. avium* and *Mycobacterium celatum*. Both *M. avium* and *M. celatum* may vary in pigment production. An important characteristic of *M. palustre* is its confusing hybridization with the *M. avium*-complex AccuProbe. However, if two simple conventional tests, urease and Tween 80 hydrolysis, are included in the species verification, *M. palustre* is easily separated even from yellow-pigmented isolates of the *M. avium* complex, *M. xenopi* and *M. celatum*.

In GLC analyses, *M. palustre* is characterized by the presence of notable amounts of 2-Me-20:0, 24:0 and 26:0 in addition to minor amounts of 2-Me-19:0, 2,9-diMe-20:0, 14-phe-14:0 and 2-Me-16-phe-16:0. *M. avium*, *M. xenopi* and *M. celatum* are devoid of these methyl-branched fatty acids. In all, a combination of 26:0 and methyl-branched fatty acids other than tuberculostearic acid (10-Me-18:0) is uncommon. It is a characteristic of *M. interjectum* and *M. intermedium* (in addition to *M. palustre*) (Fig. 1). Among these two species, *M. interjectum* typically contains the secondary alcohols 2-octadecanol (2-OH-18:0alc) and 2-eicosanol (2-OH-20:0alc) and *M. intermedium* is characterized by a fatty acid pattern containing a combination of 2-methyldodecanoic acid (2-Me-12:0), 2-methyltetradecanoic acid (2-Me-14:0), 2,4-dimethyleicosanoic (2,4-diMe-20:0) and 2,4,6-trimethyldocosanoic acid (2,4,6-triMe-22:0). The HPLC profile of mycolic acid bromophenacyl esters of *M. palustre* is similar, but not identical, to that of *M. kubicae*. These two recently described species can be further separated by biochemical and growth characteristics.

Description of *Mycobacterium palustre* sp. nov.

Mycobacterium palustre (pa′lus.tre. L. adj. *palustre* living in swamps, referring to the isolation of most members of this novel species from peatland run-off water).

Cells are acid- and alcohol-fast bacilli. Visible growth from a dilute inoculum requires 2–3 weeks. Mature colony size is reached in 4 weeks at 36 °C. Growth occurs at temperatures between 30 and 42 °C, 36 °C being regarded as the optimum temperature. Growth at 45 °C is detected from a heavy inoculum on Middlebrook agar after 5–6 weeks. Colonies on egg-based media and Middlebrook agar are smooth, scotochromogenic at 36 °C and photochromogenic or scotochromogenic at 42 °C. The species yields positive results in urease, pyrazinamidase, acid phosphatase and Tween 80 hydrolysis tests and variable results in nitrate reduction and alkaline phosphatase tests. Test results are negative for semi-quantitative catalase (45 mm foam), α-galactosidase and β-galactosidase. In GLC analyses, *M. palustre* is characterized by the presence of notable amounts of 2-Me-20:0, 24:0 and 26:0 in addition to minor amounts of 2-Me-10:0, 2-Me-19:0, 2,9-diMe-20:0, 14-phe-14:0 and 2-Me-16-phe-16:0. Phylogenetic analysis, based on 16S rDNA sequences, places *M. palustre* among the slowly growing mycobacteria, being most closely related to *M. kubicae*.

The type strain is strain E846[T] (= ATCC BAA-377[T] = DSM 44572[T]), isolated from water from a stream in Finland. A veterinary isolate (strain 629) has also been deposited in the DSMZ, as DSM 44571.

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